Investigating Mechanisms of Toxicity and Detoxification of BMAA in *Escherichia coli*

by Carly Jade Italiano

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (Science)

under the supervision of Associate Professor Kenneth Rodgers and Associate Professor Iain Duggin

University of Technology Sydney
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Certificate of Original Authorship

I, Carly Jade Italiano declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy in the School of Life Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by the Australian Government Research Training Program.

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For Spud
2005 -2020

COVID-19 Impact Statement

The latter part of this thesis was conducted during the COVID-19 pandemic and as such, a number of adaptations to the project were necessary.

Travel restrictions during 2020 resulted in disruptions and delays to ongoing experiments. The continuing delays in sourcing required laboratory supplies due to global changes to transport extended experiment timelines beyond anticipation. Reduced access to facilities required for data analysis meant much processing was conducted in a home setting. Resulting analysis using limited internet services in a rural area meant delays in data analysis, particularly for Aim 3.
Despite the various delays and difficulties experienced due to COVID-19, a solid body of work has been produced, culminating in this thesis.

Out of the four Aims covered in this thesis, Aims 3 and 4 were particularly impacted by difficulties that arose due to COVID-19 impacts. As a result, the number of experiments able to be completed was somewhat less than initially planned prior to COVID-19. Despite this, each Aim has still been covered satisfactorily and represents a meaningful contribution to the research field. I ask that the examiners please take this into consideration during their assessment.

Publications

Steele JR, Italiano CJ, Phillips CR, Violi JP, Pu L, Rodgers KJ, and Padula MP, *Misincorporation Proteomics Technologies: A Review.* Proteomes 2021;**9**(1).

Main BJ, Italiano CJ, and Rodgers KJ, *Investigation of the interaction of beta-methylamino-L-alanine with eukaryotic and prokaryotic proteins.* Amino Acids 2018;**50**(3-4):397-407.

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List of Abbreviations

AARS - aminoacyl-tRNA synthetase

ALS - Amyotrophic Lateral Sclerosis

ALS-PDC - Amyotrophic Lateral Sclerosis – parkinsonism/dementia complex

AQC - 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate

BCA - bicinchoninic acid protein quantification

BMAA - β-Methylamino-L-alanine

CFU/mL - colony forming units per millilitre

CGSC - Coli Genetic Stock Centre

Cm - Chloramphenicol

CmR - Chloramphenicol Resistant

DNA - deoxyribonucleic acid

DTT - Dithiothreitol

E. coli - *Escherichia coli*

EDTA - Ethylenediaminetetraacetic acid

FA - Formic acid

FC – fold change

Flp - Flippase recombinase

FRT - Flippase recognition target

GSH - glutathione

GSSG - glutathione disulfide

HCl - hydrochloric acid

IGV - integrative genomics viewer

OD_{600} - Optical density at 600 nanometres

IPTG - Isopropyl β-D-1-thiogalactopyranoside

Km - Kanamycin

KmR - Kanamycin Resistant

L-DOPA - L-3,4-dihydroxyphenylalanine

LB - Luria Bertani medium

LC-MS/MS - Liquid chromatography with tandem mass spectrometry

LOD - limit of detection

MND - Motor Neurone Disease

MRM - Multiple reaction monitoring

LOQ - limit of quantification

NADPH - reduced form of nicotinamide adenine dinucleotide phosphate

NAS - N-Acetyl-L-serine

NCBI - National Center for Biotechnology Information.

NMDA - N-methyl-D-aspartate

NPAA - Non-protein amino acid

OAS - O-acetyl-L-Serine

OASase - O-acetylserine sulfhydrylase

PAGE - polyacrylamide gel electrophoresis

PBS - phosphate buffered saline

PCR - Polymerase Chain Reaction

PLP - pyridoxal-5’ phosphate

RNA - Ribonucleic acid

ROS - reactive oxygen species

Rpm – revolutions per minute

RSD - relative standard deviation

SAT - Serine O-Acetyltransferase

SDS - sodium dodecyl sulfate

Snp - single nucleotide polymorphism

STRING - Search Tool for the Retrieval of Interacting Genes/Proteins

TIC - Total ion chromatogram

TQMS - Triple quadrupole mass spectrometry

UHPLC - ultra high-performance liquid chromatography

UTS - University of Technology Sydney

Vo - Initial velocity

β-PA - β-(Pyrazol-1-yl)-L-alanine

Abstract

Incurable neurodegenerative diseases are becoming an increasingly common problem affecting our population. Research into environmental triggers which contribute to development of these conditions is vital for understanding disease pathogenesis and prevention. Non-protein amino acids are a class of naturally occurring molecules that can interfere with metabolic processes in cells to cause toxicity. Exposure to the neurotoxic non-protein amino acid BMAA has been linked to the development of neurodegenerative disease. BMAA is known to be produced by cyanobacteria, and their wide distribution globally increases the likelihood of human contact with this toxin. Additionally, BMAA can bioaccumulate in ecosystems, compounding the risk of human exposure. While there are a variety of studies on the neurotoxic effects of BMAA in mammalian systems, less work has been conducted in prokaryotic species such as bacteria. Previous work has indicated that the bacterium *Escherichia coli* exhibits tolerance towards BMAA that is in contrast to the toxicity seen in mammalian cells and animal models. However, there has been no studies into the mechanism behind this tolerance.

In this project, screening of *Escherichia coli* mutants with disruptions to various amino acid biosynthesis pathways indicated that cysteine biosynthesis genes (*cysE, cysK,* and *cysM*) are important for BMAA tolerance. Disruption to any of these genes resulted in susceptibility to BMAA and growth inhibition, which manifested as a substantial delay in the onset of logarithmic growth. Severity of delay was dependent on BMAA concentration. However, following a period of BMAA-induced growth delay, a recovery in growth was observed. The possibility that additional genetic mutations spontaneously arise in cultures and suppress sensitivity to BMAA in *Escherichia coli* (*cysE* mutant) was also investigated. Results showed that genetic changes were not responsible for the restoration of growth that occurs following BMAA-induced inhibition. Analysis of gene expression changes in *Escherichia coli* exposed to BMAA showed a pattern indicative of an iron starvation response. There were also changes to sulfur homeostasis and amino acid metabolism (cysteine and threonine).

O-acetylserine sulfhydrylase (*cysM*) from the cysteine biosynthetic pathway was tested for ability to enzymatically degrade BMAA, which would explain the requirement of

cysteine biosynthetic genes for BMAA tolerance. While the enzyme did not degrade BMAA, the cofactor pyridoxal-5'phosphate which the enzyme uses to catalyse cysteine biosynthesis reactions did, resulting in the production of methylamine. Results from this project have explored different possibilities for *Escherichia coli* tolerance towards BMAA and provided insights into BMAA metabolism. This work has opened up many future directions of BMAA research including the importance of cysteine metabolism in BMAA toxicity, enzymatic degradation of BMAA via pyridoxal-5'phosphate dependent enzymes, and exploration of iron chelating properties of BMAA.

Chapter One – Thesis Introduction and Background

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1.1 Neurodegenerative Disease

Progressive decline and death of neuronal cells is common to a collection of disorders referred to as 'neurodegenerative diseases'. Deterioration of the nervous system leads to impairments in normal functioning and decreased quality of life for those affected [1]. The prevalence of these diseases is increasing, and with neurodegenerative disease currently standing as the second leading cause of death in Australia [2]. The three most common neurodegenerative diseases affecting our population are Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS) [3]. Of all people diagnosed with these diseases, roughly 10% of cases are due to a direct genetic link alone. The remaining 90% of cases are termed 'sporadic' with their cause(s) remaining unknown [4]. In the absence of a single genetic link, these sporadic cases are likely to have arisen due to environmental influence [5]. As understanding of neurodegenerative disease has increased, there is the growing suggestion that these diseases are not caused by a single factor but by a multitude of different events, including genetic propensity and exposure to environmental insults [6]. For ALS in particular, analysis of epidemiological data suggest that ALS is a multistage disease with up to six different molecular steps occurring prior to disease onset, and that as age increases so does the chance of progression through more steps [7]. While genetic susceptibility may result in a predisposition to development of neurodegenerative disease, exposure to environmental factors may add to these steps of disease development.

As these diseases are incurable, and treatments are often administered only to delay disease progression and alleviate symptoms, prevention is highly desirable [8]. As such, identification of environmental triggers which contribute to neurodegeneration is an important component of neurodegenerative research. Studies have already shown that exposure to substances such as pesticides and heavy metals can predispose individuals to the development of neurodegenerative disease later in life [9, 10]. Identification of these triggers allows for not only preventative strategies to be employed, but also gives insight into disease pathogenesis. There is increasing interest

in investigating environmental triggers for neurodegenerative disease, one of which is the neurotoxic amino acid β-methylamino-L-alanine (BMAA).

1.2 BMAA and Neurodegenerative Disease

While various environmental triggers for neurodegenerative disease have been studied, BMAA is a trigger of interest which has received relatively less attention. Exposure to cyanobacterial blooms that contain several known toxins including BMAA has been shown to be associated with an increased incidence of neurodegenerative disease [11, 12]. This highlights the importance of investigating the non-protein amino acid BMAA and understanding its impact on disease development.

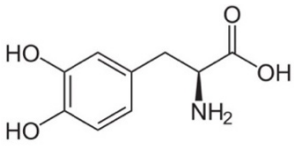
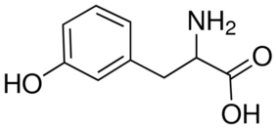
Amino acids which do not belong to the 20 classical amino acids used in protein synthesis are termed non-canonical or non-protein amino acids (NPAAs) [13]. Some NPAAs resemble protein amino acids, this allows them to act as antimetabolites and displace canonical amino acids in physiological systems (reviewed in [14]). Various cellular pathways which involve amino acids may be influenced by the presence of NPAAs, such as protein synthesis and amino acid metabolism [15]. This enables NPAAs to exert different kinds of toxic effects on the organism exposed. In addition, naturally occurring NPAAs are abundant in our environment and are often produced by plants [13] (Table 1).

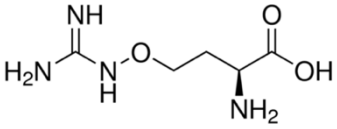
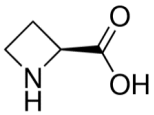
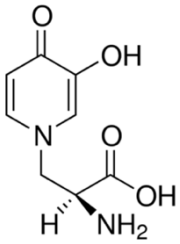
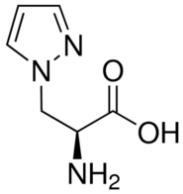
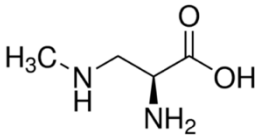
Many NPAAs including the Parkinson's disease drug L-DOPA (L-3,4-dihydroxyphenylalanine) are synthesised by plants, which use them as a form of chemical warfare against predators and to outcompete other plant species [16]. Fescue grasses (*Festuca spp*.) release the NPAA *meta*-tyrosine into the soil where it acts as an allelochemical by inhibiting the growth of nearby plants [17]. NPAAs can also be used as nitrogen storage molecules in species that synthesise them [18], which may explain the plant origin of many NPAAs. However, unlike many plant derived NPAAs, BMAA is known to be produced by cyanobacteria and diatoms [19, 20]. Cyanobacteria inhabit a diverse range of environments, and their photosynthetic ability means they share traits common to both plants and bacteria [21]. Despite knowledge of

cyanobacteria being BMAA synthesisers, the purpose of this toxin within cyanobacteria is still being explored.

Because BMAA is a NPAA and does not belong to the 20 canonical amino acids, its role within cells is complicated and not fully established. In terms of BMAA toxicity, proposed mechanisms of action have been put forward, most notably oxidative stress [22], and excitotoxicity [23, 24] (see section 1.4 BMAA Mechanisms of Toxicity). In addition, misincorporation of BMAA into neuronal proteins leading to their aggregation has also been suggested [25, 26]. However, there is a distinct lack of information regarding the metabolism of BMAA within cells. This includes how BMAA may be modified during cellular processing, and what key pathways and aspects of metabolism it may be interacting with. Understanding the chemical interaction of BMAA with elements of cells is the key to uncovering the different ways in which exposure can potentiate the development of neurodegenerative disease.

Table 1. Examples of Toxic Non-protein Amino Acids (NPAAs).

NPAA	Structure	Mechanism	Produced by
L-DOPA (L-3,4-dihydroxyphenylalanine)		Oxidative stress [27], Protein misincorporation (tyrosine) [28]	Velvet bean plant (*Mucuna pruriens*) [29]
meta-Tyrosine		Protein misincorporation (phenylalanine) [30]	Fescue grass (*Festuca spp.*) [31]

Canavanine		Protein misincorporation (arginine) [32]	Jack bean plant (*Canavalia ensiformis*) [33]
Azetidine-2-carboxylic acid		Protein misincorporation (proline) [34]	Lily of the valley (*Convallaria majalis*); Garden beet (*Beta vulgaris*) [35]
Mimosine		Arrest of cell cycle replication [36], neurotoxic and depilatory effects in animals [37]	Leucaena (*Leucaena spp.*) and some *Mimosa* species [37]
β-PA (β-(Pyrazol-1-yl)-L-alanine)		Brain glutamate receptor interactions [38]	Watermelon (*Citrullus vulgaris*) [39]
BMAA (β-methylamino-L-alanine)		Oxidative stress [22], Protein misincorporation (serine) [25] (alanine) [26]	Cycad palms (*Cycas spp.)* and cyanobacteria [19, 40]

1.3 BMAA Discovery and History

The first report of BMAA isolation was in 1967 by Vega and Bell, who extracted BMAA from the seeds of the cycad palm *Cycas circinalis* [40]. Cycad palms were often used as a food source by the native people of the Western Pacific island of Guam [41]. The event which sparked interest in the contents of the seeds was the suggestion that they may contain toxins that were contributing to the high incidence of neurodegenerative disease on the island [42], which at its peak was 50 to 100 times higher in incidence than that of neighbouring countries [43]. The complex disease experienced by the native Chamorro people of Guam was termed amyotrophic lateral sclerosis – Parkinsonism/dementia complex (ALS-PDC), as the features resembled this collection of diseases [43]. Unusually, it also affected people of different cultural and genetic backgrounds who came to live on Guam and adopted the Chamorro lifestyle [44]. Despite efforts of researchers, no genetic links could be found to explain this disease [43]. This lead to the suggestion that cycad seeds prominent in the diet of the Chamorros may explain the development of ALS-PDC, and resulted in the isolation of BMAA as a suspect toxin [40].

It was later established that cyanobacteria inhabited the roots of cycad palms as symbionts and were responsible for the presence of BMAA in the cycad seeds [45], which were extensively used to provide flour for staple food items [46]. Cyanobacteria are ubiquitous microorganisms and are now known to be producers of BMAA [19]. Additionally, the BMAA content in Guamanian flying foxes which fed on cycad seeds was shown to be high due to bioaccumulation of BMAA through trophic levels of the food web [47]. As flying foxes were frequently eaten as a Chamorro delicacy, this suggested that BMAA was accumulating throughout the food chain, with the Chamorros receiving the highest doses via their traditional diet [47, 48]. Post-mortem investigations revealed that BMAA was present in the brain tissue of those affected by ALS-PDC, but not in control patients who died of non-neurological disease [45]. This offered strong support for BMAA being the causative agent behind the neurodegeneration on Guam and gave rise to further investigations into the mechanisms of disease development.

Due to it links to neurodegenerative disease, a number of animal studies were applied to the study of BMAA. Early research showed that administration of BMAA results in neurotoxicity in chicks [40], rats [49, 50], and mice [51]. However conflicting studies followed, showing no occurrence of neuropathological effects after BMAA exposure [52, 53]. Due to the differences in susceptibility across different species, development of an animal model for BMAA-induced neurological disease has been difficult. Administration of BMAA to macaque monkeys (*Macaca fasicularis*) resulted in the development of symptoms resembling the ALS-PDC seen on Guam [11]. However, uncertainty arose from this early study as the disease experienced by humans on Guam was one of long latency, often with the disease becoming apparent many years after BMAA exposure [54]. In contrast, acute effects experienced by macaques occurred after relatively short-term exposure of up to 13 weeks, with different motor effects apparent earlier and later in the treatment [11]. This discrepancy in disease characteristics made it difficult to apply such animal models to what was occurring in humans. This was also because the model used on the macaques was not representative of accurate levels of BMAA exposure by a person on Guam, with daily doses in the study exceeding 100 mg/kg [11]. More recent studies using prolonged BMAA administration at lower doses have shown widespread neurofibrillary tangles in the brains of Vervet monkeys (*Chlorocebus sabaeus*) that resemble plaques seen in Parkinson's disease and Alzheimer's disease [12]. However, there remains discrepancies between different primate species from the various studies described, including the types and spread of neuronal plaques and the motor symptoms displayed [55]. While it is difficult to say how transferrable the animal models are to humans, this is compelling evidence that BMAA is worth investigating further as a trigger for neurodegenerative disease.

In recent times there has been focus on bioaccumulation of BMAA, particularly in aquatic environments where cyanobacterial blooms are common. BMAA appears to be prominent in aquatic species. For example, a comprehensive study by Jonasson and colleagues demonstrated BMAA accumulation throughout trophic levels in the Baltic Sea, which experiences frequent cyanobacterial blooms [56]. Similar studies have also shown bioaccumulation in other regions and food webs, such as Gonghu Bay, China

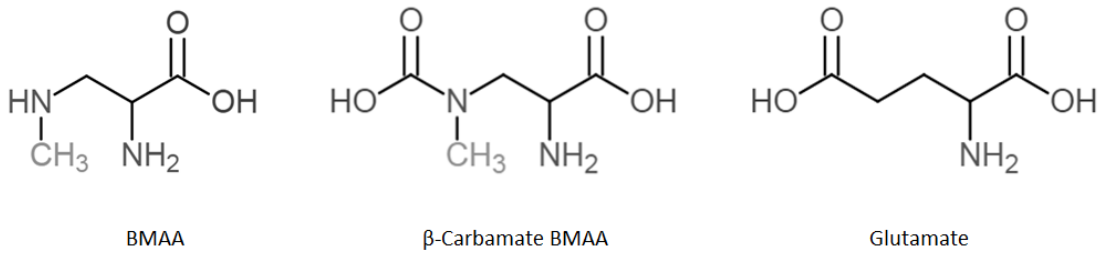
[57] and Thau lagoon in the French Mediterranean Sea [58]. Detection of BMAA has been especially prominent in shellfish such as oysters and mussels [58-62] as well as fish consumed by humans [56, 63, 64]. Fin tissue of sharks, which are known to accumulate toxins in the food web have also tested positive for the presence of this NPAA [65], and concerningly BMAA has also been detected in shark fin supplements sold to the public [66]. This trend of food web bioaccumulation ending with human consumption mirrors the previous situation in Guam. However, despite knowledge of the presence of BMAA in our environment, the exact details of how BMAA contributes to neurodegeneration are not completely understood.

Due to the risk to human health initial BMAA research was heavily focused on animal studies and to date, only two studies in bacteria other than cyanobacteria have been published, one of which arises from our lab [67, 68]. The wealth of animal toxicology studies undertaken has provided lots of information on the consequences of BMAA exposure, but there is a distinct lack of information concerning BMAA metabolism in prokaryotic species such as bacteria and cyanobacteria which are thought to be the predominant producers of this toxin. An understanding of how prokaryotic organisms respond to this toxin may provide valuable insights into BMAA metabolism including mechanisms that could reveal how BMAA is produced or potentially detoxified.

1.4 BMAA Mechanisms of Toxicity

Since its discovery as a suspect in ALS-PDC development, research has focused on unravelling how BMAA exerts its toxic effects on mammalian systems. Studies using mammalian cells *in vitro* have repeatedly demonstrated the cytotoxic effects of BMAA [24, 69, 70]. The mechanisms behind the observed effects in these studies have shown recurring themes of excitotoxicity and oxidative stress (reviewed in [71]). These findings were further bolstered when it was discovered that BMAA can mimic the excitatory neurotransmitter glutamate when in the presence of bicarbonate [72] (Figure 1). This mimicry of glutamate allows BMAA to activate N-methyl-D-aspartate (NMDA) receptors, triggering excitotoxic effects in neuronal cells. Activation of other glutamate receptor types is also implicated in BMAA toxicity, with motor neurons

being particularly susceptible [23]. Apart from the ability to activate glutamate receptors, BMAA has more recently been shown to inhibit cystine uptake in neuronal cells via inhibition of the cystine/glutamate antiporter known as system xc-, leading to reduced levels of glutathione [22]. Glutathione is one of the most important antioxidants used by neuronal cells to combats oxidative stress, and the impacts of BMAA on glutathione adds to the association between BMAA and oxidative stress. Both excitotoxicity and oxidative stress are heavily involved components of neurodegenerative disease [73, 74]. As BMAA has shown involvement in both processes, it is an increasingly important molecule to investigate in relation to the development of neurodegeneration.



BMAA β-Carbamate BMAA Glutamate

Figure 1. Molecular structures of BMAA, β-Carbamate BMAA and the excitatory neurotransmitter glutamate

It has also been suggested that being an amino acid, BMAA may be mistakenly incorporated into growing peptide chains in place of canonical amino acids during protein synthesis [25]. Incorporation of non-protein amino acids into proteins is known to contribute to destabilisation of protein structure, leading to misfolding and aggregation [75]. Additionally, many NPAAs are well known for replacing canonical amino acids during protein synthesis and causing toxicity [14]. Examples of NPAAs which replace canonical amino acids during protein synthesis are shown in Table 1, including canavanine and azetidine-2-carboxylic acid. BMAA has also been shown to promote misfolding and aggregation of proteins within neuronal cells *in vitro* [25]. This protein aggregation was prevented when the amino acid serine was co-administered, suggesting that BMAA competes with serine for incorporation into proteins during

their synthesis or may be connected to serine metabolism [25]. Recent research has also indicated that BMAA may be mistakenly charged onto alanine tRNA, bypassing proofreading mechanisms for protein misincorporation [26]. In addition to influencing amino acids during protein synthesis, many NPAAs are capable of interfering with other aspects of amino acid metabolism including their synthesis via mechanisms such as feedback inhibition [15]. The mimicry of amino acids by NPAAs therefore has the potential to impact all areas of the cell where amino acids are involved. There is particular risk to long lived cells such as neurones, as terminally differentiated cells with an inability to divide, they may be more susceptible to toxic burdens because they cannot dilute down toxic species by passing them on to daughter cells.

Despite the increasing evidence for BMAA involvement in neurodegenerative disease development, detailed knowledge of the mechanisms behind the toxic effects of BMAA is still lacking. As mentioned, oxidative stress, receptor activation, and excitatory mechanisms have been demonstrated, along with protein misincorporation and amino acid interference. Despite this, the metabolism of BMAA and the reactions which it undergoes in living systems requires deeper investigation. Understanding the metabolic reactions of BMAA is valuable information considering that a mechanism of toxicity may rely on metabolism of BMAA into different forms. BMAA can form carbamates in the presence of bicarbonate and can only activate glutamate receptors when in this particular form, illustrating the importance of understanding BMAA metabolism [72]. A study has recently been published showing that BMAA interferes with amino acid metabolism in neuroblastoma cells [76], and this area of BMAA research is likely to reveal more promising insights into BMAA toxicity. The knowledge of BMAA reactions and metabolism within different kinds of cells and species would increase our understanding of the toxic effects of BMAA and could help explain why they might be species dependent. This information may also lead to the uncovering of novel toxicity mechanisms yet to be observed. This is especially relevant for the organisms which produce BMAA, and those which evade its toxic effects.

1.5 BMAA Research in Bacteria

Despite the research into mechanisms by which BMAA causes detrimental effects on mammalian cells, there has been limited research on the effect BMAA has on prokaryotic species and bacteria. This is particularly of interest because BMAA is known to be produced by prokaryotic cyanobacteria, though the production pathway is yet to be definitively shown.

Cyanobacteria are known producers of toxins and secondary metabolites, the production of which is influenced by environmental conditions [77]. Initial studies of BMAA production in a range of cyanobacteria indicated that 97% of strains tested were capable of BMAA production [19]. It has been shown that the concentration of BMAA in cyanobacterial cells is linked to carbon and nitrogen metabolism, which also occurs for microcystin, another cyanotoxin [78, 79]. Further support for BMAA being involved in nitrogen metabolism is that under conditions of nitrogen starvation, BMAA production increases in cyanobacteria [80]. It has been suggested that production of BMAA may help cyanobacteria cope with nutrient-limited environments, as it prevents continuation of photosynthesis under the stressful conditions of nutrient deprivation [81]. In addition, others have suggested that BMAA production acts as a way to limit oxidative damage which would result from the use of photosynthesis in nutrient-starved settings [78]. However, the connection between BMAA and nitrogen metabolism in cyanobacteria is yet to be explored fully and does not explain the importance of BMAA in cyanobacterial physiology completely. While cyanobacteria are BMAA producers, they appear to not be spared from some of its toxic effects. For example, BMAA inhibits the growth of the cyanobacterial strain *Synechocystis* PCC6803 in a concentration dependent manner [82]. Similar growth inhibition also occurred for another cyanobacterial species, *Nostoc sp.* PCC 7120 [81]. A recent proteomic study of the same species also showed negative impacts on nitrogen and carbon metabolism, amino acid metabolism and protein synthesis [83]. With this information and the emerging nature of cyanobacterial metabolism regarding BMAA, a complete understanding of BMAA's role in cyanobacteria is yet to be accomplished.

While cyanobacteria are thought to be the main producers of BMAA, studies have also suggested that other microbial species and plants are capable of BMAA synthesis,

suggesting perhaps a shared mechanism. The most notable example is a study by Marler and colleagues, who grew cycad palms in the absence of the symbiotic cyanobacteria which they usually possess [84]. The palms were shown to contain BMAA despite the lack of BMAA-producing cyanobacteria, suggesting that cyanobacteria are not solely responsible for synthesising BMAA. Production of BMAA has also been shown to occur in other species such as diatoms [20, 85] and dinoflagellates [86]. It also appears that BMAA impedes nitrogen metabolism in diatoms, resulting in reduced chlorophyll levels [87], similar to what has been observed in cyanobacteria. Overall, there is diversity in the microorganisms which can produce BMAA and similarities in how they respond to it. It therefore could be possible that there are universal features across microorganisms which explain their interaction with BMAA.

The first study to test BMAA on bacterial species other than cyanobacteria showed no decrease in growth over a period ranging from 4-16 hours, this was consistent in both nutrient rich and nutrient poor medium [67]. In contrast, exposure to the NPAA canavanine showed an obvious decrease in growth of the bacteria and a decrease in respiration [67]. Due to its ability to interfere with vital processes and inhibit growth in the species which produce it, and its cytotoxic effects in mammalian cells, it is interesting that bacterial species such as *Escherichia coli* (*E. coli*) show no negative growth effects when exposed to BMAA [67]. The authors of this study showed that the lack of BMAA toxicity in bacteria was in contrast to other toxic NPAAs such as canavanine and azetidine-2-carboxylic acid which produced slow growth and death upon exposure [67]. In addition, the main finding of this paper was that unlike mammalian cells, bacteria such as *E. coli* do not incorporate BMAA into their proteins, a mechanism which is thought to explain BMAAs toxic effects and connection to neurogenerative disease development [67]. However, mammalian cells may have a different level of susceptibility to BMAA than *E. coli* cells. Amino acyl tRNA synthetase (AARS) proofreading enzymes can differ slightly between eukaryotes and prokaryotes with some possessing more or less editing capabilities [88]. The serine AARS of *E. coli* for example has been found to only share 37% sequence identity with its mammalian counterpart [89]. These differences may impact on the toxicity of BMAA in these

organisms. It is possible that while other NPAAs can bypass proofreading mechanisms across almost all species, BMAA is only able to exert its effects on eukaryotes. As BMAA has been shown to be taken up by *E. coli* cells [67], the lack of growth inhibition suggests that *E. coli* possess a form of resistance that other mammalian organisms do not have.

A recent study from our laboratory has shown that in addition to growth being unaffected by BMAA, *E. coli* displays a differing pattern of BMAA uptake and association with cellular protein when compared to mammalian neuroblastoma cells [68]. Interestingly, while mammalian cells had a gradual and steady increase in BMAA protein association over time, levels of BMAA within *E. coli* spiked after 1 hour before declining [68]. Such results suggest that *E. coli* may possess a mechanism to metabolise BMAA over time or otherwise negate its toxic effects, and that this does not occur in neuroblastoma cells which are susceptible to BMAA toxicity. To date, no study has been conducted into the mechanism by which *E. coli* are able to tolerate this toxin. An understanding of how BMAA in processed and metabolised by *E. coli* has the potential to reveal further insights into the nature of this toxin and potentially uncover useful protective elements against its toxic effects. Further research into the multifaceted nature of BMAA chemistry and metabolism in cells would undoubtedly reveal many interesting features of this NPAA. As there is potentially a commonality in the way bacteria and microorganisms respond to BMAA, identification of these features in one organism could prove useful for directing the study of others with BMAA. This would potentially lead to more defined explanations for the role of BMAA in prokaryotes such as cyanobacteria, possibly a resulting in ways to limit the presence of BMAA in the environment

1.6 Thesis Aims and Structure

The overall purpose of this project was to investigate how *E. coli* respond to the neurotoxic amino acid BMAA. With the aim of gaining greater understanding of the unique tolerance that *E. coli* display towards BMAA, and possibly identify how BMAA is metabolised and detoxified in these bacteria. In this discovery-based project, as new data and information was discovered over the course of the investigation, a number of subsequent aims were created and are as follows:

Aim 1 – Determine if specific amino acids and their biosynthetic pathways in *E. coli* are affected by BMAA exposure during growth.

Aim 2 – Investigate the connection between cysteine biosynthesis genes of *E. coli* and BMAA induced growth delays.

Aim 3 – Analyse global genomic and transcriptomic changes occurring in *E. coli* exposed to BMAA.

Aim 4 – Investigate the ability of the isolated *E. coli* enzyme O-acetylserine sulfhydrylase to degrade BMAA *in vitro*

The organisation of the thesis following this introductory chapter is outlined below, each aim is addressed in its specified chapter.

Chapter Two – This chapter describes the screening of *E. coli* genetic mutants for growth with BMAA and addresses Aim 1. It is presented in manuscript form under the title "*Tolerance towards β-methylamino-L-alanine in Escherichia coli requires cysteine biosynthesis genes*" and is currently under review in the journal *Research in Microbiology*. Please note that tables and figures within Chapter 2 are self-contained within the manuscript and their numbering is therefore independent of other thesis chapters.

Chapter Three – Aim 2 is described in this chapter and covers the investigation into the impacts of deletion and complementation of various cysteine biosynthesis genes on BMAA tolerance in *E. coli*.

Chapter Four – This chapter explores the genetic and transcriptional changes that occur in *E. coli* exposed to BMAA during growth and covers Aim 3.

Chapter Five – The subject of this chapter is Aim 4 and the investigation into whether the cysteine biosynthesis enzyme of *E. coli,* O-acetylserine sulfhydrylase has the ability to degrade BMAA into methylamine when isolated from *E. coli*.

Chapter Six – General discussion and future directions.

Chapter Two – Manuscript for Publication “Tolerance towards β-methylamino-L-alanine in *Escherichia coli* requires cysteine biosynthesis genes”

Chapter Two – Manuscript for Publication

"Tolerance towards β-methylamino-L-alanine in *Escherichia coli* requires cysteine biosynthesis genes"

*This manuscript was accepted for publication on 29th June 2021, and available online from 8th July 2021, during review the title was updated:

Italiano CJ, Pu L, Violi JP, Duggin IG, and Rodgers KJ, ***Cysteine biosynthesis contributes to β-methylamino-l-alanine tolerance in Escherichia coli***. Research in Microbiology 2021;**172**(6):103852.
DOI: https://doi.org/10.1016/j.resmic.2021.103852

2.1 Statement

Certificate of Authorship and Originality

This chapter is presented in manuscript format as submitted to the Journal *Research in Microbiology* and is currently under review (April 2021). Figures and Tables referenced in-text are presented at the end of the manuscript and are independent of other Chapters. I certify that the work present in Chapter 2 of this thesis has not been previously submitted as part of the requirements for this degree. I also certify that I carried out all the experimental work, analysis and interpretation of the data presented here.

Author contributions are as follows:

Author	Contribution
Carly J. Italiano	conceived, planned, and conducted experiments. Wrote majority of the manuscript (~95%). Signature: Production Note: Signature removed prior to publication. Date: 20/04/2021
Lisa Pu	sample processing and analysis for glutathione experiments, contributed to writing methods section of manuscript
Jake P. Violi	technical support for glutathione experiments, contributed to writing methods section of manuscript.
Iain G. Duggin	feedback on research and data analysis, supervision and proofreading of manuscript
Kenneth J. Rodgers	feedback and supervision of the research, proofreading of manuscript, and conception of research project.

2.2 Abstract

In contrast to mammalian cells, bacteria such as *E. coli* display tolerance towards the neurotoxin β-methylamino-L-alanine (BMAA) suggesting that these prokaryotes possess a way to metabolise BMAA resulting in its export, degradation, or recycling into alternate metabolites. Single gene deletion mutants of *E. coli* K-12 with inactivated amino acid biosynthesis pathways were treated with 500 µg/ml BMAA and the resulting growth was monitored. Wild type *E. coli* and most gene deletion mutants displayed unaltered growth in the presence of BMAA over 12 hours. Conversely, deletion of the gene *cysE*, a key component of the cysteine biosynthesis pathway, was shown to result in susceptibility to BMAA and a lack of growth in a dose-dependent manner. This strain also showed increased susceptibility to oxidative stress in the form of H_2O_2, and disruptions in glutathione levels. The cysteine biosynthesis pathway is linked to the tolerance of BMAA in *E. coli*, and potentially represents a mechanism of BMAA detoxification.

Keywords: β-methylamino-L-alanine; amino acid; neurotoxin; oxidative stress; glutathione; Serine O-acetyltransferase

2.3 Introduction

The non-protein amino acid (NPAA), β-methylamino-L-alanine (BMAA) has been implicated as an environmental neurotoxin. Exposure has been associated with development of neurodegenerative disease such as amyotrophic lateral sclerosis (ALS) [11, 12]. Mechanisms behind the toxic effects of BMAA that could contribute to neuronal damage include oxidative stress [22], excitotoxicity [23, 24], and proteotoxic stress [25]. BMAA is known to be produced by cyanobacteria; ubiquitous photosynthetic microorganisms that inhabit a diverse range of aquatic and terrestrial environments [90, 91]. This presents a growing public health concern, as the global problem of increased cyanobacterial blooms brings with it the risk of increased levels of cyanotoxin production, including BMAA [92]. So far, researchers have been unable to identify a common taxonomic group or geographical characteristic shared by BMAA producing species of cyanobacteria [19]. To further complicate matters, recent studies have found that along with cyanobacteria, other aquatic organisms such as diatoms appear to be capable of BMAA production [85, 93]. Despite ongoing research into how BMAA causes harmful effects to humans, researchers are still unable to answer the question of how BMAA is produced and how it is metabolised by different species; including those that produce it. Due to the many potential routes of exposure and its putative links to fatal neurodegenerative disease, it is imperative that the metabolism of BMAA be explored. This is particularly important in prokaryotic systems, due to the tolerance that bacteria such as *Escherichia coli* (*E. coli*) display towards BMAA, which is not observed in mammalian systems [67, 68].

As many NPAAs structurally resemble protein amino acids, this allows them to act as antimetabolites and displace or mimic canonical amino acids in physiological systems (reviewed in [14]). Various cellular pathways that involve amino acids may be influenced by the presence of NPAAs, including protein synthesis, amino acid synthesis, and amino acid metabolism [15]. This enables NPAAs to exert different kinds of toxic effects on the organism exposed. However, due to its links to human disease BMAA research has understandably focused on mammalian systems, with prokaryotic systems receiving less study. Prokaryotic systems have previously been used to reveal

amino-acid mimicking ability and protein misincorporation as a mechanism of action for other NPAAs including azetidine-2-carboxylic acid (Aze) [94, 95], canavanine [95], and *meta*-tyrosine [96]. The effects that BMAA may have on bacteria such as *E. coli* could reveal important information that can be used to understand how BMAA is produced, processed, or even detoxified in prokaryotic species generally, including cyanobacteria which are known producers of BMAA [19]. It is of particular interest to investigate the effects BMAA has on amino acid metabolism, as evidence has already demonstrated its ability to influence amino acid metabolism in other cell models [76]. The effects that BMAA has on prokaryotes such as *E. coli* is particularly interesting, as it is becoming increasingly likely that BMAA has additional effects on cells that are not typical of many standard-amino-acid mimicking NPAAs.

This study aimed to investigate the effects of BMAA on a typical prokaryotic system using the model organism *E. coli*. Auxotrophic strains of *E. coli* that each have one amino acid biosynthetic pathway inactivated were used to identify any essential interactions between BMAA and the biosynthesis of specific protein amino acids. As toxic NPAAs tend to mimic specific canonical amino acids, toxicity can often be alleviated by supplementation with the competing canonical amino acid [95]. Considering this, it was hypothesised that if BMAA competed with a particular canonical amino acid, the auxotrophic strain that was supplemented with additional amino acid would resist toxic effects of BMAA more effectively than strains supplemented with non-competing canonical amino acids. Identification of interactions between BMAA and amino acid pathways may give wider insight into the complex effects and metabolism of BMAA in prokaryotic cells.

2.4 Materials and methods

2.4.1 Materials

All reagents were analytical grade or cell culture grade and sourced commercially. Amino acids including BMAA and Aze were sourced from Sigma-Aldrich (St. Louis, MO, USA). Solutions of BMAA and Aze were prepared in 10 mM hydrochloric acid (HCl) and MilliQ water respectively and stored at -20°C.

2.4.2 Bacterial strains

E. coli strains from the Keio collection were obtained from the Coli Genetic Stock Centre (CGSC) and derived from parent strain BW25113 [97]. Strains and their relevant phenotypes are listed in Table 1. Strains were stored at -80 °C in 16% glycerol and revived on solid media as needed. An auxotrophic strain for alanine was not available as this deletion is non-viable. In addition, deletions to create auxotrophy for glycine, asparagine, aspartic acid, and glutamic acid rendered *E. coli* unable to grow sufficiently in minimal medium despite external amino acid supplementation, these strains were excluded from the screening study.

2.4.3 Growth of amino acid auxotrophic strains with Aze and BMAA

Strains were streaked onto LB agar and incubated for 18 hours at 37 °C, single colonies were then inoculated into 3 ml of M9 minimal media which was prepared as described by Sambrook, Fritsch, and Maniatis [98] with addition of 10 μg/ml uracil and a trace-elements solution [99]. Auxotrophy of strains was alleviated by the addition of 100 μg/ml of required amino acid to the media. *E. coli* were cultured for 16-18 hours at 37 °C with shaking at 150 rpm. These starter cultures were then used to inoculate 10 ml of fresh media of the same composition to an OD_{600} of 0.05 and growth was continued until exponential phase (OD_{600} 0.5-0.6) before cells were washed in M9 media lacking amino acids. Cells were re-suspended to OD_{600} 0.1 in M9 media with 100 μg/ml of required amino acid for each auxotrophic strain. Wild type *E. coli* was not supplemented with amino acids. Diluted cultures were grown in 96-well plates with

either 500 µg/ml BMAA or 500 µg/ml Aze, controls received a 10 mM HCl blank or water for BMAA and Aze respectively. Washed cultures not supplemented with amino acids served as negative controls and confirmed auxotrophy of strains. *E. coli* were grown at 37 °C with constant shaking in a microplate reader (PowerWave HT Microplate Spectrophotometer, BioTek), with absorbance measurements taken at 30-minute intervals (600 nm) for 12 hours. Final absorbance measurements were appropriately blanked to correct for background absorbance and the data used to generate growth curves for all cultures. For growth of the cysteine auxotrophic strain with varied BMAA concentrations, the same procedure was followed but for 18 hours total growth time and with concentrations of BMAA at 25, 50, 100, 200, and 400 µg/ml.

2.4.4 Hydrogen peroxide (H_2O_2) treatment of wild type and cysteine auxotrophic *E. coli*

Wild type *E. coli* and the cysteine auxotrophic strain JW3582-2 were grown to exponential phase as described in section 2.3. Strain JW3582-2 was supplied with 100 µg/ml cysteine throughout. Exponentially growing cultures were diluted to OD_{600} of 0.1 in additional medium pre-warmed to 37 °C. H_2O_2 was added at a final concentration of either 5 mM or 25 mM, and then incubated at 37 °C for 15 minutes. 1 ml of cells were washed in 30 ml of PBS and pelleted at 4000 xg for 15 minutes. Pellets were re-suspended in 1 ml PBS and serially diluted before spotting onto LB agar plates. Agar plates were then incubated at 37 °C for 18 hours and subsequent colonies counted and used to calculate CFU/ml of original culture.

2.4.5 Glutathione analysis of wild type and cysteine auxotrophic *E. coli*

Wild type *E. coli* and the cysteine auxotrophic strain JW3582-2 were grown to exponential phase as described previously in 30 ml of nutrient poor M9 minimal or nutrient rich LB medium. Cultures were centrifuged at 4000 xg for 15 minutes and washed in 30 ml of PBS before further centrifugation. Pellets were stored at -80 °C prior to glutathione analysis. During sample preparation, pellets were reconstituted in

3 ml of 80% ice cold methanol and probe sonicated twice at 40% power on ice. Samples were then centrifuged at 3900 xg for 10 minutes and the supernatant transferred into new tubes for glutathione analysis while the pellet was kept for protein quantification. 2 ml of 0.1% Triton was added to the pellet and vortexed before performing the bicinchoninic acid assay (BCA) for protein quantification [100].

2.4.6 Glutathione analysis by LC-MS/MS

Glutathione reduced (GSH) and oxidised (GSSG) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and analysed using the Agilent Technologies 6490 triple quadrupole mass spectrometer (TQMS) coupled with an Agilent 1290 Infinity UHPLC. Liquid chromatography was performed using a Waters Cortecs® UPLC C18 column (150 x 2.1 mm, 1.6 µM particle size, 90 Å pore size). Solvent A consisted of ultrapure water + 0.1% (v/v) formic acid (FA) with solvent B consisting of LC-MS grade methanol + 0.1 % FA (v/v). GSH and GSSG were chromatographically separated and eluted via gradient elution (see Fig. 1). Flow rate was set to 0.25 ml/min with the column temperature being maintained at 20 °C. Solvent B conditions were as follows: 0.0 min 0%, 2.0 min 0%, 2.1 min 50%, 5.0 min 50%, 5.1 min 100%, 7.0 min 100%, 7.1 min 0%, 9.5 min 0%.

The TQMS was operated with an electrospray ionisation source in positive mode. The operating settings for the TQMS were as follows: 250 °C drying gas temperature at 14 l/min, 250 °C sheath gas temperature at 11 l/min, 20 psi nebuliser pressure, 3000 V capillary voltage and 1500 V nozzle voltage. Retention time and multiple reactions monitoring (MRM) transitions were used to identify GSH and GSSG (Table 2). Each MRM transiton had a dwell time of 200 ms. Quanitification of GSH was conducted on the most abundant transition (308 m/z > 75.9 m/z). In-source fragmentation was observed for GSSG, resulting in its most abundant ion transiton to be the same as GSH (308 m/z > 75.9 m/z). Thus, quantification was conducted using the 308 m/z > 75.9 m/z transition, this was possible due to the chromatographic seperation between the two compunds. The remaining transitions that utiilised the protonated mass of GSSG (613.2 m/z) were used for its qualification. Limits of detection (LOD) and limits of

quantification (LOQ) were calculated with a signal to noise ratio of 3:1 and 10:1 respectivly. GSH was determined to have a LOD of 0.095 µg/l with a LOQ of 0.28 µg/l while GSSG had a LOD of 0.12 µg/l with an LOQ of 0.37 µg/l. GSH was found to have a linear range of 1 – 1000 µg/l and GSSG had a linear range of 1 – 500 µg/l. Repeatability was determined via calculation of %RSD from 7 repeat injections of one point in the standard curve, with both GSH and GSSG having % RSD values of < 10%. Stability of GSH and GSSG were monitored via back-to-back injections overnight at 4 °C with no notable decrease or increase in levels for either compound. Samples were run alongside a 7-point calibration curve (1 µg/l, 5 µg/l, 10 µg/l, 50 µg/l, 100 µg/l, 500 µg/l, and 1000 µg/l) to allow for quantification, and several blanks to ensure no carry-over was occurring. No carry-over was observed following either samples or standards. Injection volume was set to 2 µl, with all samples and standards being run in triplicate. All data analysis was conducted on Agilent's Masshunter Qualitative Analysis with the quantified values for samples later being normalised to protein.

2.5 Results

To explore potential relationships between amino acid biosynthetic pathways of *E. coli* and BMAA, growth studies of single-gene knockout strains exposed to BMAA were undertaken (Fig. 2). Parallel studies were carried out with Aze, a NPAA known to compete with proline for insertion into proteins. Control cultures of individual strains were grown under the same conditions but received no BMAA or Aze. The optical density (OD_{600}) of NPAA exposed cultures was compared to that of control cultures and a relative percentage of growth was calculated and is displayed in Fig. 2. The NPAAs Aze and BMAA were added to a final concentration of 500 µg/ml, the ratio of NPAA to supplied amino acid being 5:1. Wild type *E. coli* and single-gene knockout mutants all showed decreased final growth yield (OD_{600}) after 12 hours when Aze (500 µg/ml) was present in the growth medium (Fig. 2a). The exception to this Aze-induced growth inhibition is demonstrated by the proline auxotrophic strain. The deletion of the gene *proA* renders this strain unable to synthesize proline and therefore supplementation with proline (100 µg/ml) was necessary for growth. This supplementation with proline was enough to outcompete the proline analogue Aze and reduce its growth inhibitory effects by 90%. Supplementation with glutamine also reduced growth inhibition but only slightly (Fig. 2a).

The effect of BMAA administration (500 µg/ml) on the final density of each *E. coli* gene knockout strain after 12 hours of growth was determined (Fig. 2b). Wild type *E. coli* treated with BMAA attained the same density of growth as BMAA-free controls (Fig. 2b). Additionally, similar to the wild type strain, the gene knockout mutants tested also showed no or minimal growth inhibition in media containing BMAA. The exception to this pattern is the cysteine auxotrophic strain with a deletion in the gene *cysE* which failed to grow in the presence of BMAA (Fig. 2b).

A growth time-course of wild type and cysteine auxotrophic strain shows that growth of wild type *E. coli* with BMAA is near identical to that of the control culture (Fig. 3a) Cultures of the *ΔcysE* mutant which has a non-functional cysteine biosynthesis pathway, failed to produce any noticeable change in optical density during the entire

12-hour time period in BMAA-containing medium, yet grew well in BMAA-free medium (Fig. 3b).

Due to its unique susceptibility to BMAA, the *ΔcysE* mutant was selected for further study. A concentration of 500 µg/ml proved to be inhibitory for the *ΔcysE* mutant after 12 hours of growth, and thus concentrations lower than this were selected (25 µg/ml to 400 µg/ml) to determine the minimal inhibitory concentration. However, for the *ΔcysE* mutant, there was a dose-dependent lag in the growth of cultures exposed to BMAA (Fig. 4), rather than complete inhibition of growth that was apparent at the 12-hour time-point with exposure to 500 µM BMAA (Fig. 3b). At all concentrations of BMAA tested, the *E. coli* cultures reached similar optical densities to control cultures by the 18 hours (Fig. 4). Therefore, instead of indefinite inhibition of growth or death, the pattern was one of a growth lag preceding a return to normal growth rates and final yield.

Due to the apparent BMAA susceptibility of the *ΔcysE* mutant, it was of interest to compare the *ΔcysE* mutant and wild type *E. coli* in terms of tolerance to oxidative stress. Viability was determined after exposure to 5 mM and 25 mM H_2O_2 as an inducer of oxidative stress (Fig. 5). There was a marked increase in susceptibility to H_2O_2 treatment for the *ΔcysE* strain for both concentrations.

Analysis of the glutathione content of both the wild type strain and *cysE* deletion mutant is displayed in Fig. 6. In rich medium, wild type *E. coli* has significantly more total glutathione than the *ΔcysE* mutant (Fig. 6a). In nutrient poor media there was a similar level of glutathione in both strains, that was less than the amounts observed in rich medium. The ratio of reduced to oxidised glutathione is significantly lower in the *ΔcysE* mutant when compared to wild type, and this is consistent for both rich and poor medium conditions (Fig. 6b).

2.6 Discussion

The pattern of response observed across BMAA and Aze treated *E. coli* strains contrasted greatly, suggesting different mechanisms of action and effects on the cells. The addition of Aze to culture media resulted in complete inhibition of growth of *E. coli* strains at the time points measured (Fig. 2a). Only the proline auxotroph which was supplied with 100 μg/ml of exogenous proline showed resistance to the toxic effects of Aze. The growth inhibitory effects of Aze and the protective effects of proline are consistent with other reports [94, 101, 102]. Previous studies have shown that toxicity of Aze in *E. coli* may be prevented by the addition of an equal concentration of proline [94] and may explain why 100 μg/ml of proline used in this study did not completely protect the proline auxotroph from the higher levels of Aze used (500 μg/ml). A similar effect of growth inhibition which is alleviated by the addition of the competing protein amino acid has been reported for other NPAAs, such as the arginine analogue canavanine, a well-studied NPAA [95, 103, 104]. Conversely, with BMAA exposure significant growth inhibition of wild type *E. coli* and most of the auxotrophic strains examined did not occur (Fig. 2b). This is consistent with previous wild type *E. coli* data published by our group [68]. In addition, Van Onselen and colleagues showed that exposure of wild type *E. coli* to BMAA (100 μM) in minimal media resulted in no inhibition in growth over an 8 hour time period [67]. The response of *E. coli* suggests that BMAA does not share the amino acid mimicry mechanism of toxicity observed to occur with other NPAAs such as Aze and canavanine. Typically, inhibition of growth upon exposure to a protein amino acid mimicking NPAA is relieved by addition of the competing 'parent' amino acid [95, 102], as was seen for Aze (Fig. 2a). For strain Δ*cysE*, no recovery was seen with the addition of cysteine to media. Similarly, the addition of serine or alanine for the respective auxotrophic strains failed to allow growth of cysteine auxotrophic *E. coli* in the presence of BMAA, though both have been implicated as BMAA-mimetics [25, 26, 105]. BMAA toxicity is well documented in mammalian cell models [11, 24, 69, 70, 76]. Competition with the amino acid L-serine has been suggested [25], and some reports have provided evidence of interference with amino acid metabolism in mammalian cells [76]. The lack of BMAA toxicity in *E. coli* is at odds with the toxicity seen in mammalian systems. The perceived tolerance of

E. coli towards this toxin raises the possibility of a defence or detoxification mechanism possessed by *E. coli* that could be absent in BMAA-susceptible mammalian cells.

A potential means to explain BMAA tolerance in *E. coli* lies within the unique response observed in the cysteine auxotrophic strain. The complete lack of detectable growth in the cysteine auxotroph over 12 hours upon exposure to 500 µg/ml BMAA contrasted with the relatively uninhibited growth across the other strains (Fig. 2b). In this strain, the pathway for cysteine production is impaired via deletion of the gene *cysE*, which expresses an essential enzyme for cysteine biosynthesis [97]. This suggests that this pathway or its downstream effects on the cell are important for tolerance towards BMAA in bacteria. As the main route of sulfur assimilation in cells, cysteine biosynthesis is central to many downstream activities essential for cell survival [106]. Free cysteine can also contribute to the protection of cells from oxidative damage via oxidation to cystine which can be reduced back to cysteine in a continual cycle, providing a reservoir of antioxidant activity [107-110]. In addition, it is a precursor for many important vitamins and antioxidants, including thiamine, biotin, and glutathione. As such, a lack of cysteine often results in reduced levels of glutathione and subsequently an increased susceptibility to oxidative damage across many species [111-113]. Lowered levels of glutathione are also known to increase the susceptibility of *E. coli* to external stressors and toxins [114]. Total glutathione levels for *E. coli* in minimal medium were similar (Fig. 6a), however the ratio of reduced to oxidized glutathione was lower for the *cysE* lacking strain (Fig. 6b). This would suggest that glutathione reserves are being oxidised in response to oxidative insult within this strain. Oxidative stress has previously been shown to be a component of BMAA toxicity [22, 115], perhaps explaining why a lack of cysteine-producing ability could render this cysteine auxotrophic strain more susceptible to BMAA. This was supported by results showing that the Δ*cysE* strain is more susceptible to oxidative stress in the form of H_2O_2 than wild type *E. coli* (Fig. 5) and has a lowered ratio of reduced to oxidised glutathione (Fig. 6b). This may mean that the normal defences against oxidation in *E. coli* are sufficient to deal with BMAA in wild type *E. coli*. This is supported by the van Onselen study showing cellular respiration was not decreased, nor was there an

increase in reactive oxygen species generation upon 100 µM BMAA exposure [67]. Conversely, the Δ*cysE* mutant may be rendered more susceptible to oxidative stress due to an inability to synthesis cysteine. Impairment of metabolic pathways that influence glutathione homeostasis such as cysteine biosynthesis could result in this strain experiencing the toxic effects of BMAA and severely delayed growth.

It is also apparent that the inhibition of growth BMAA caused for strain Δ*cysE* is not permanent. Fig. 4 shows a BMAA concentration dependence in growth lag time, but not maximum growth rate or final growth yield. A possibility to explain this pattern is that BMAA is metabolised using a process that may take some time for the *E. coli* to initiate. This is supported by the dose-dependent increase in time taken to recover based on the concentration of BMAA present in the media (Fig. 4). While this strain may be initially susceptible, processes within the cell may adapt to the stressor and enable it to metabolise BMAA, such reinforcement of the impaired cysteine biosynthesis pathway with alternate enzymes to restore cell defences.

The observed link between BMAA and the enzymes in the cysteine biosynthesis pathway of *E. coli* also presents interesting possibilities for BMAA detoxification. In *E. coli*, the formation of cysteine is catalysed via two sequential enzymes in the cysteine biosynthesis pathway: serine O-acetyltransferase (SAT; EC 2.3.1.30) encoded by *cysE* and O-acetylserine sulfhydrylase (OASase; EC 4.2.99.8) encoded by *cysK* or *cysM*. In the BMAA-susceptible strain Δ*cysE*, the enzyme SAT cannot be produced by the cell and thus the pathway for cysteine biosynthesis is rendered non-functional. The implications of absent SAT enzymes from the cell being related to BMAA sensitivity suggest that this enzyme either directly acts on BMAA as an alternate substrate instead of serine, or that it indirectly affects another downstream aspect of the *E. coli* metabolism to render it susceptible to BMAA. The pyridoxal 5'-phosphate dependent OASase enzymes encoded by *cysK* and *cysM* have previously been linked to NPAA metabolism, including enzymatic production of the toxic NPAAs mimosine and β-Pyrazol-1-yl-L-alanine [116-118]. As such, these enzymes may also be important to investigate as being involved in BMAA metabolism, perhaps as detoxifiers. And their

impairment in the cysteine auxotrophic mutant may explain the BMAA concentration-dependent delay in return to normal growth in this strain.

This is the first study to examine the effects of BMAA on a range of auxotrophic *E. coli* strains and it presents new evidence from which to design new investigations. Further research into connections between BMAA-induced oxidative stress and cysteine biosynthesis is warranted and could reveal more about how BMAA behaves within cells. Additionally, connections to the cysteine biosynthesis pathway of *E. coli* could point towards potential detoxification mechanisms for BMAA in prokaryotes and the complex relationship between BMAA and amino acid metabolism.

Investigations into cyanobacteria have revealed that they are the likely source of BMAA production, yet how they tolerate its toxic effects is not known. It has been shown that BMAA can inhibit growth of certain cyanobacterial strains [81, 82]. Considering that even producers of BMAA are seemingly not immune to its toxic effects, it is interesting that bacterial species like *E. coli* are so tolerant of BMAA. As cyanobacteria share many similarities to other prokaryotic species such as *E. coli*, further exploration of BMAA metabolism in bacteria may provide insights into cyanobacterial metabolism as well. This could pave the way for the discovery of metabolic processes in cyanobacteria that involve BMAA, including mechanisms of production and protection. Such knowledge would present the opportunity to develop methods for combating BMAA production in these producing species and prevent BMAA release into the environment.

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Conflicts of Interest: The authors declare no conflict of interest.

2.7 Legends to figures

Figure 1. Example chromatogram of GSH and GSSG from one of the standards used for the calibration curve.

Figure 2. Maximum growth yield (%) of wild type *E. coli* BW25113 and single-gene knockout strains exposed to Aze or BMAA. The maximum growth of strains treated with either 500 µg/ml Aze (a) or BMAA (b) is plotted as a percentage of growth relative to untreated control cultures (100%). Strains were grown in M9-minimal medium at 37°C with shaking for 12 hours, auxotrophy of knockout strains was alleviated by supplementation with 100 µg/ml of required amino acid. The growth of cultures was measured via optical density readings at 600 nm. Error bars represent standard deviation from biological triplicate cultures.

Figure 3. Effect of BMAA on the growth of wild type *E. coli* and cysteine deletion mutant. (a) Growth over 12 hours of Wild Type *E. coli* treated with 500 µg/ml BMAA (blue) or untreated control (black) in M9-minimal medium. (b) Growth of *cysE* deletion strain with BMAA (blue) compared to untreated control (black) in M9-minimal medium supplemented with 100 µg/ml L-cysteine. Figures are representative of curves generated from biological triplicate cultures.

Figure 4. Growth time-course of *ΔcysE* mutant exposed to a range of BMAA concentrations. Mean optical density measured at 600 nm of *E. coli* cultures grown in 96-well plates with shaking at 37°C. *ΔcysE* mutant *E. coli* growth over 18 hours in M9 minimal media is shown with indicated BMAA concentrations. Cysteine auxotrophy in this strain was alleviated by supplementation with 100 µg/ml cysteine. Figures are representative of curves generated from biological triplicate cultures.

Figure 5. Viability of *E. coli* strains treated with H_2O_2. Wild Type and *cysE* deletion mutant *E. coli* were treated with 5 mM and 25 mM H2O2 for 15 minutes at 37°C in M9 minimal medium. Serially diluted cultures were plated on LB agar and resulting colonies were used to calculate CFU/ml. Cysteine auxotrophic strain ∆*cysE* was supplemented with 100 µg/ml cysteine. Error bars represent standard deviation from the mean n=3. * Significantly different ($p < 0.05$) using T-test.

Figure 6. Measurement of glutathione in *E. coli* grown in nutrient poor and nutrient rich media. (a) Quantification of total glutathione levels in *E. coli* grown in nutrient poor (M9 minimal media) and nutrient rich (LB) media during exponential growth. Concentration is normalized to protein as detected by BCA assay. (b) Ratio of reduced to oxidised glutathione in *E. coli* strains grown in nutrient poor and nutrient rich medium. Glutathione content was determined using triple quadrupole mass spectrometer (TQMS) coupled with UHPLC, n=4. * Significantly different ($p < 0.05$) using T-test.

2.8 Tables

Table 1. List of E. coli Strains used in this project.

Strain	Genotype	Phenotype
BW25113		Wild Type
JW0003-2	*ΔthrC724::kan*	Threonine Auxotroph
JW0233-2	*ΔproA761::kan*	Proline Auxotroph
JW1254-2	*ΔtrpC770::kan*	Tryptophan Auxotroph
JW2004-1	*ΔhisB720::kan*	Histidine Auxotroph
JW2580-1	*ΔpheA762::kan*	Phenylalanine Auxotroph
JW2581-1	*ΔtyrA763::kan*	Tyrosine Auxotroph
JW2786-1	*ΔargA743::kan*	Arginine Auxotroph
JW2806-1	*ΔlysA763::kan*	Lysine Auxotroph
JW2880-1	*ΔserA764::kan*	Serine Auxotroph
JW3745-2	*ΔilvA723::kan*	Isoleucine Auxotroph
JW3841-1	*ΔglnA732::kan*	Glutamine Auxotroph
JW3973-1	*ΔmetA780::kan*	Methionine Auxotroph
JW5605-1	*ΔilvD722::kan*	Valine, Isoleucine, Leucine Auxotroph
JW5807-2	*ΔleuB780::kan*	Leucine Auxotroph
JW3582-2	*ΔcysE720::kan*	Cysteine Auxotroph

Table 2. Retention times and MRM ion transitions for all targeted compounds. *denotes transition used for quantification.

Analyte	Retention time (min)	Collision energy	MRM transition (m/z)
Glutathione reduced (GSH)	2.7	34	308.0 → 75.9*
		38	→ 84
		74	→ 59
Glutathione oxidised (GSSG)	4.2	34	308.0 → 75.9*
		40	613.2 → 176.9
		38	→ 230.8
		22	→ 355.1

2.9 Figures

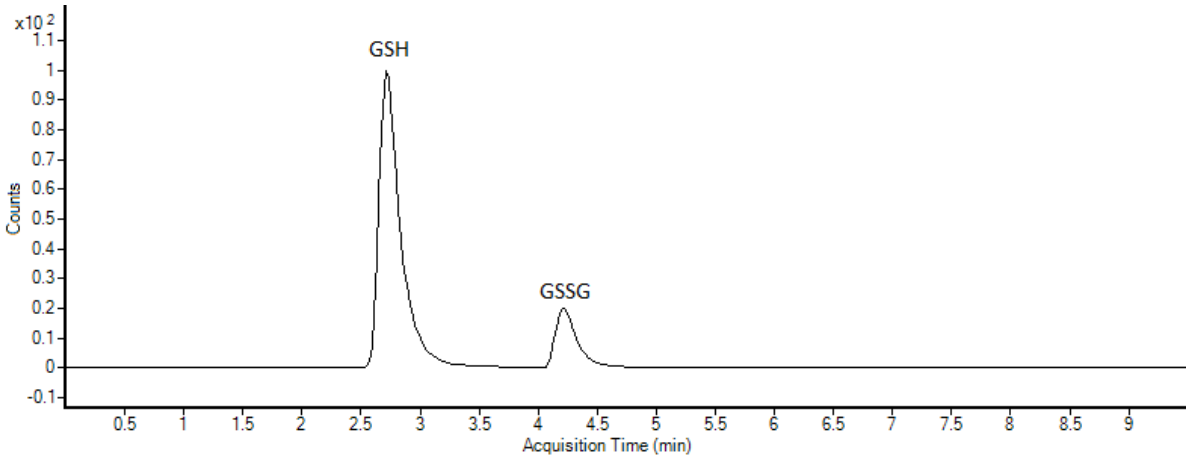


Figure 1. Example chromatogram of GSH and GSSG from one of the standards used for the calibration curve.

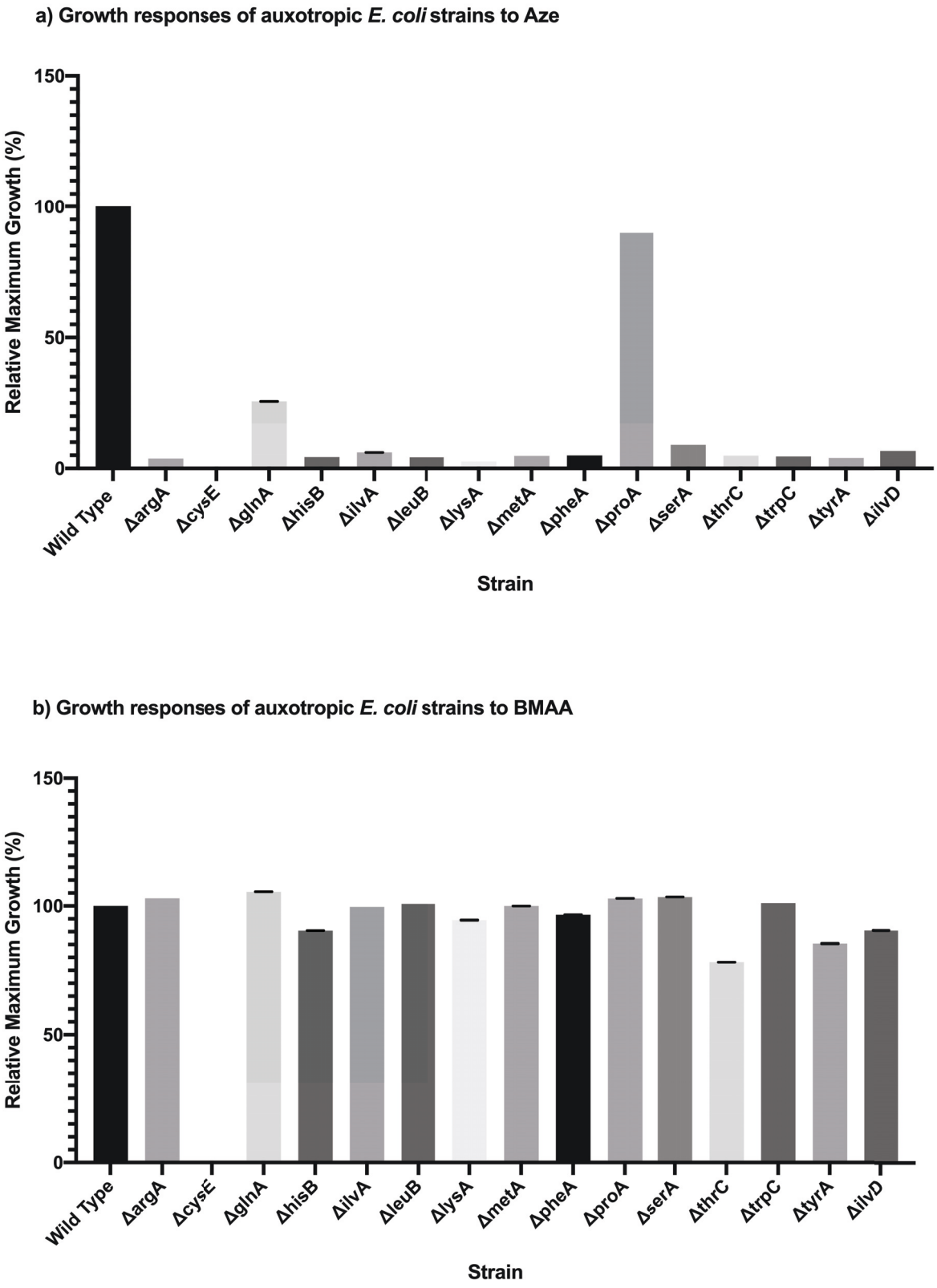


Figure 2. Maximum growth yield (%) of wild type *E. coli* BW25113 and single-gene knockout strains exposed to Aze or BMAA.

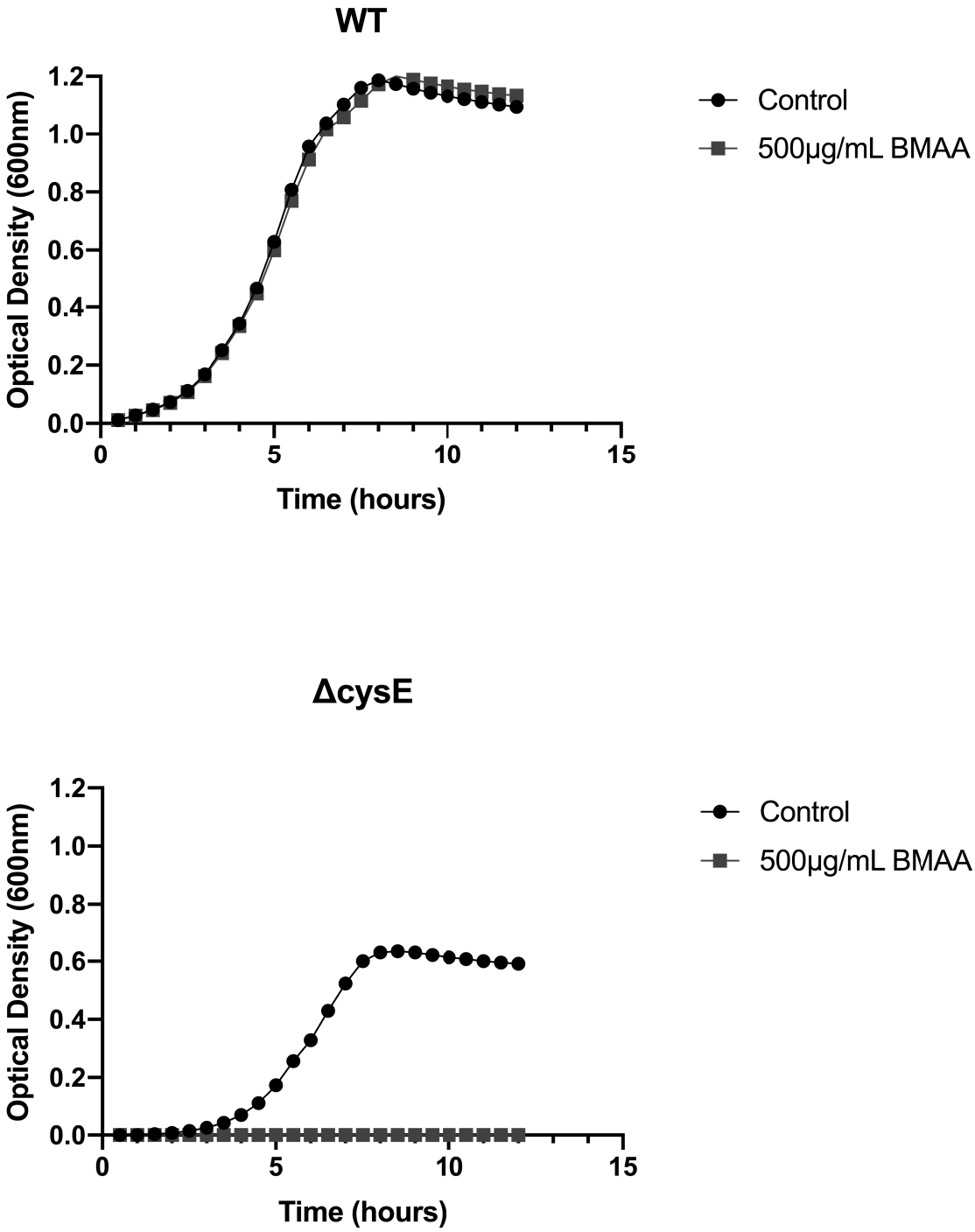


Figure 3. Effect of BMAA on the growth of wild type *E. coli* and cysteine deletion mutant.

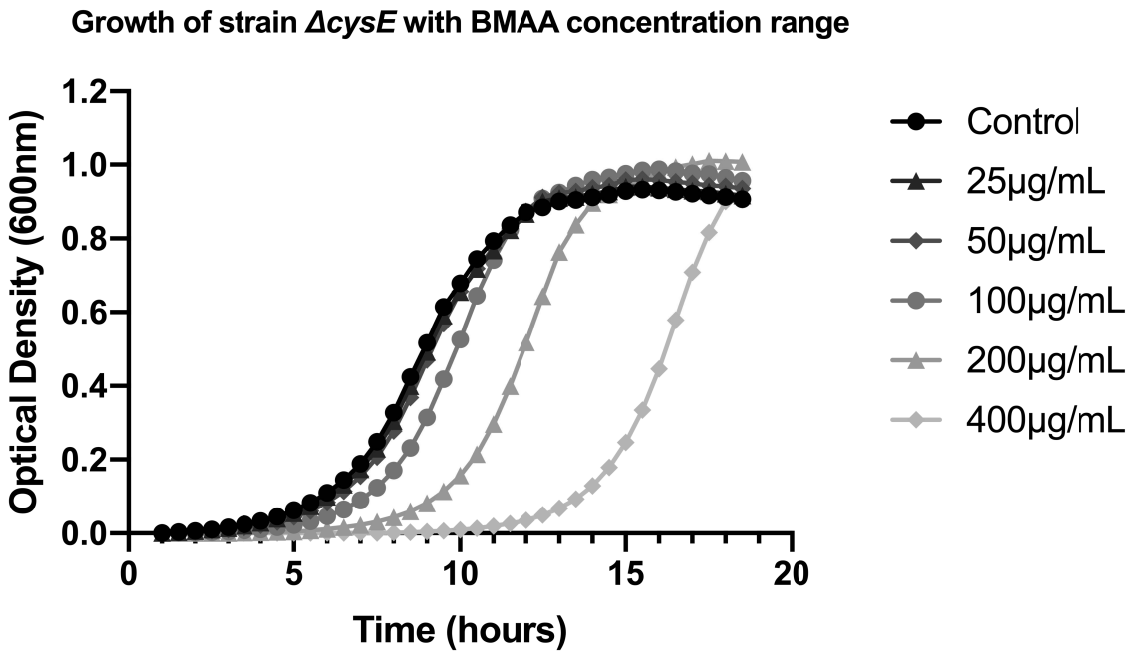


Figure 4. Growth time-course of *ΔcysE* mutant exposed to a range of BMAA concentrations.

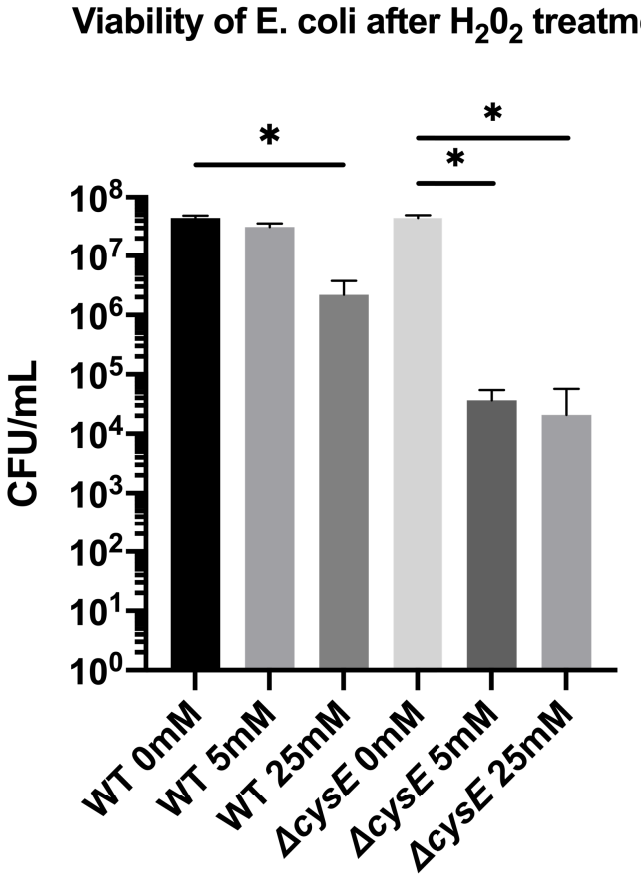
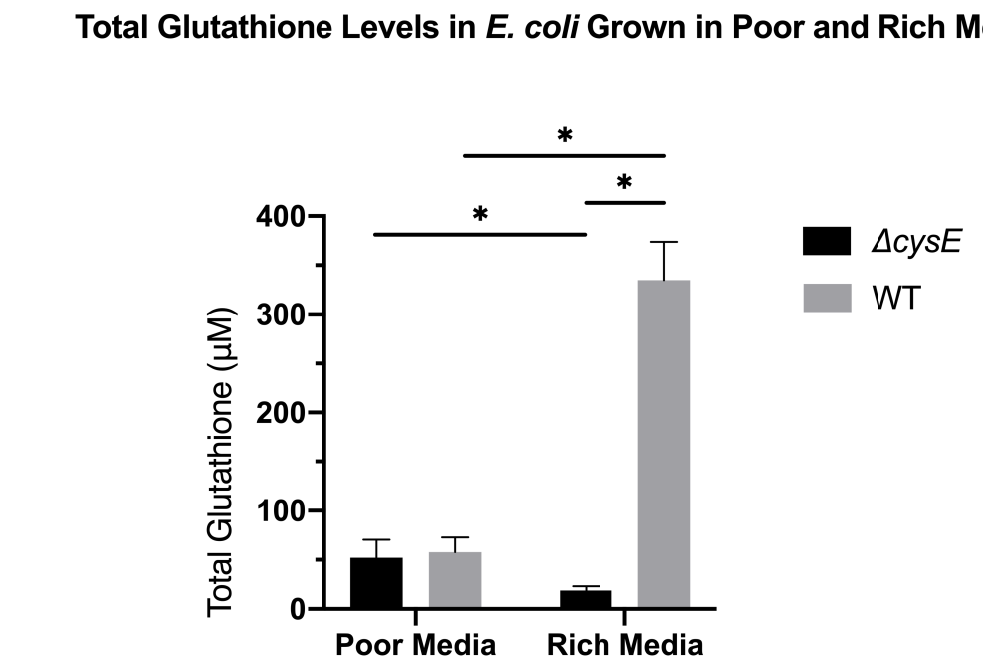


Figure 5. Viability of *E. coli* strains treated with H_2O_2.

a)



b)

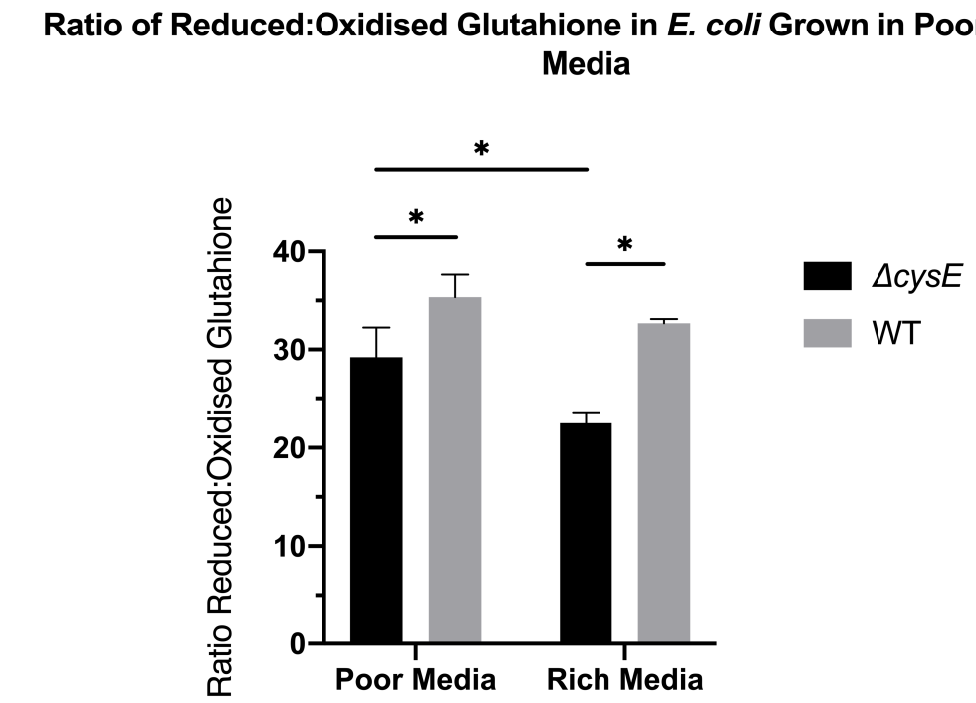


Figure 6. Measurement of glutathione in *E. coli* grown in nutrient poor and nutrient rich media.

Chapter Three – Investigation into the connection between cysteine biosynthesis genes of *E. coli* and BMAA-induced growth delays.

Chapter Three – Investigation into the connection between cysteine biosynthesis genes of *E. coli* and BMAA-induced growth delays.

3.1 Introduction

Due to the identification of the cysteine biosynthesis gene *cysE* being linked to BMAA tolerance in *E. coli* (Chapter 2), investigations into other genes within the cysteine biosynthesis pathway were necessary to begin to understand the significance of its involvement in BMAA tolerance. Cysteine biosynthesis comprises of a two-step pathway that is conserved across bacteria, plants, and cyanobacteria, but is absent in humans [116-119]. The enzymes serine-O-acetyltransferase (SAT) encoded by *cysE* and O-acetylserine sulfhydrylase (OASase) encoded by *cysK*, work sequentially to produce cysteine from serine via the intermediate O-acetylserine (OAS) (Figure 2).

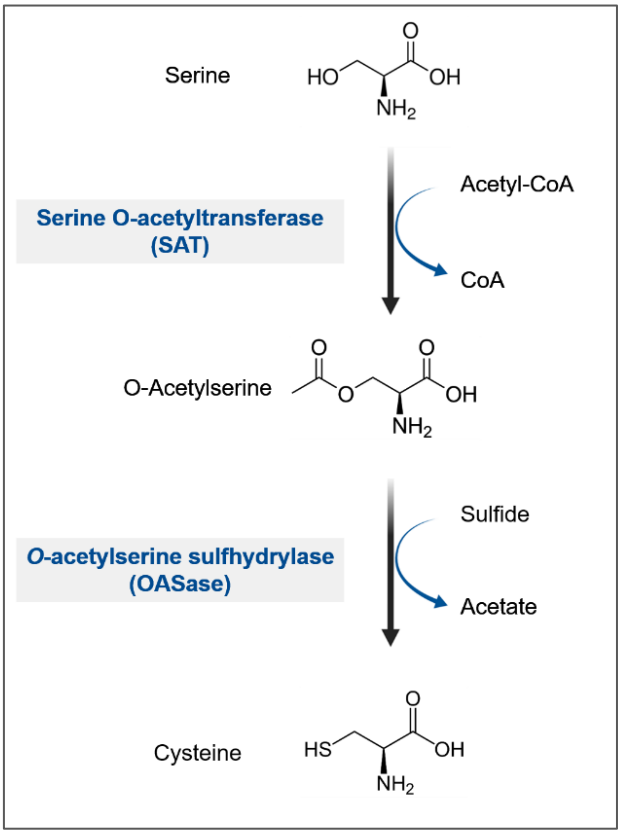


Figure 2. The cysteine biosynthesis pathway of plants and bacteria. In E. coli, the enzymes SAT and OASase work sequentially to produce the amino acid cysteine from serine, with an O-acetylserine intermediate. For cysteine production, sulfide acts as a nucleophilic donor during the second step with OASase.

An isoform of the OASase enzyme encoded by *cysM* is also present in *E. coli* and is termed OASase B, it is thought to be expressed under differing conditions to OASase A and to have less involvement in cysteine biosynthesis [120]. While OASase B shares 43% sequence similarity with OASase A, it contributes to less than 20% of cellular cysteine biosynthesis [121], suggesting an alternate role. Both OASase enzymes have moonlighting properties and variable substate specificity, signifying additional functions [122]. Some already described alternative processes involving OASase enzymes include involvement in contact-dependent growth inhibition [123], and regulating activity of transcription factor *CymR* [124]. Though *cysE* was identified as a gene of interest in our previous research (Chapter 2), the suggestion of alternative functions for *cysK* and *cysM* enzymes means they are also of interest to investigate

when considering BMAA tolerance. Additionally, the essential link between the first and second steps of cysteine biosynthesis means that deletion of *cysE*, impacts directly on the secondary enzymes both for cysteine generation and also on a transcriptional level [125]. Such a connection means that all components within the cysteine biosynthesis pathway should be investigated to further understand any impact they may have on BMAA tolerance in *E. coli*. As there is limited information of BMAA metabolism in *E. coli,* tolerance may be mediated by several factors including both direct and indirect involvement of the cysteine biosynthesis pathway. Characterisation of a tolerance mechanism originating from this pathway could provide new information to progress the field of BMAA research into the area of BMAA detoxification.

3.2 Aims

Experiments within this Chapter aimed to examine the connection between deletion of various genes within the cysteine biosynthetic pathway of *E. coli* and sensitivity towards BMAA. In addition, overexpression of genes in deletion backgrounds was undertaken to explore potential protective effects of gene restoration on BMAA-induced growth inhibition.

3.3 Methods

3.3.1 Plasmids used for Genetic Manipulation and Overexpression

Plasmids used in this project are listed in Table 2 and include vectors for construction of gene deletion mutants and vectors for gene overexpression in deletion backgrounds (complementation).

Table 2. List of plasmids used in this project

Plasmid	Purpose	Source
pTAB1	Vector backbone for complementation. CmR.	Tamika A. Blair
pTAB1-*cysE*	pTAB1 vector carrying *cysE* from MG1655 *E. coli*. IPTG inducible. CmR.	This project
pTAB1-*cysK*	pTAB1 vector carrying *cysK* from MG1655 *E. coli*. IPTG inducible. CmR.	This project
pTAB1-*cysM*	pTAB1 vector carrying *cysM* from MG1655 *E. coli*. IPTG inducible. CmR.	This Project
pFLP2	Flippase (*Flp*) recombinase-expressing plasmid for excision of genomic DNA flanked by recombinase target (FRT) sequences. AmpR.	Tayla A. Corocher
pKD46	Lambda Red recombinase expression plasmid. AmpR.	Duggin Lab Group

Cysteine synthesis genes *cysE*, *cysK* and *cysM* were amplified from genomic DNA of *E. coli* strain MG1655 via polymerase chain reaction (PCR). For complementing deletion mutants with plasmid borne genes, the low copy number vector pTAB1 was used. The pTAB1 vector constructed by Tamika A. Blair was derived from a backbone vector of pACYC184, and contains the multi-cloning site, lac promoter, and *laqIq* repressor of pNDM220. PCR amplified fragments of *cysE*, *cysK* and *cysM* were inserted between BamHI and XhoI restriction sites of pTAB1 to create plasmids pTAB1-*cysE*, pTAB1-*cysK* and pTAB1-*cysM* respectively. All constructed plasmids were verified via Sanger Sequencing conducted at the Australian Genome Research Facility (Westmead, NSW).

3.3.2 Construction of Cysteine Deletion Mutants

E. coli mutants with gene deletions in the first and second step of cysteine synthesis included *ΔcysE* from the Keio collection and a double knockout *ΔcysKΔcysM* which was constructed using lambda red recombination. Briefly, Keio strain JW2414-3 (*ΔcysM*) which has a kanamycin resistance cassette (KmR) flanked by Flp recombinase target (FRT) sites in place of the *cysM* gene, was transformed with plasmid pFLP2 encoding Flp recombinase. Excision of the KmR cassette in this strain by Flp recombinase was followed by removal of the pFLP2 vector via sucrose selection, rendering the strain JW2414-3 susceptible to kanamycin. The newly kanamycin susceptible strain was then transformed with pKD46 carrying the lambda red recombinase system in preparation for the deletion of *cysK* from the genome. The KmR region, along with upstream and downstream FRT flanking sites from Keio strain JW2407-1 (*ΔcysK*) was amplified via PCR. The amplified fragment was transformed via electroporation into the previously prepared kanamycin sensitive JW2414-3 (*ΔcysM*) strain harbouring pKD46. Via the lambda red recombinase enzymes expressed by pKD46, homologous recombination of the PCR amplified KmR fragment from JW2407-1 (*ΔcysK*) into the genome of host strain proceeded, producing the double knockout strain *ΔcysKΔcysM* possessing kanamycin resistance. This new strain was given the designation CJI001 and was observed to display cysteine auxotrophy. The *ΔcysE*, *ΔcysK*, *ΔcysM* and *ΔcysKΔcysM* strains were used for further growth studies with BMAA.

3.3.3 Growth of Cysteine Deletion Mutants with BMAA

Scrapings from frozen glycerol stocks of *ΔcysE*, *ΔcysK*, and *ΔcysM* were streaked into LB agar plates and incubated for 18 hours at 37°C. Single colonies were inoculated into 3 mL of M9 minimal media supplemented with cysteine. A cysteine concentration of 35 µg/mL was selected for all further growth experiments with these strains. These cultures were grown at 37°C, 150 rpm for 16-18 hours and then diluted into 10 mL of fresh media to an OD_{600} of 0.05 before being incubated further until OD_{600} reached 0.5. Cultures were diluted in fresh media added to the wells of a 96-well plate containing either 500 µg/mL BMAA or 10 mM HCl control. The cultures were grown for 24 hours

at 37 °C with constant shaking in a microplate reader with OD_{600} measurements taken at 30-minute intervals. Absorbance measurements were appropriately blanked and used to construct growth curves for each condition.

3.3.4 Complementation of Cysteine Deletion Mutants with Cysteine Synthesis Enzymes

For complementation of deleted genes, strain *ΔcysE* was transformed with pTAB1-*cysE* and newly created strain *ΔcysKΔcysM* was transformed with pTAB1-*cysK* or pTAB1-*cysM*. The double knockout mutant was used in place of single mutants to minimise overlapping effects which may occur due to the similarity of OASase A and B (encoded by *cysK* and *cysM* respectively). The strains harbouring pTAB1 were included as empty vector controls. For testing of growth changes when exposed to BMAA, all strains were grown on LB agar plates for 18 hours at 37°C, plasmids were maintained with 35 µg/mL chloramphenicol (Cm). Single colonies were then inoculated into 3mL of M9 minimal media containing 35 µg/mL Cm and supplemented with 35 µg/mL cysteine. Cultures were grown for 16-18 hours at 37°C with shaking at 150 rpm. These starter cultures were diluted in fresh media and growth continued until an OD_{600} of 0.5 was reached. Cultures were then induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours at 37°C with shaking at 150 rpm to induce the expression of plasmid-encoded cysteine biosynthesis enzymes. Induced cultures were diluted to OD_{600} of 0.05 in fresh media of the same composition and transferred to a 96-well plate containing 500 µg/mL BMAA or 10mM HCl for controls. The cultures were grown at 37°C with constant shaking in a microplate reader with OD_{600} measurements taken at 30-minute intervals for 24 hours. Absorbance measurements were blanked and used to construct growth curves for each condition.

3.4 Results

3.4.1 Growth of Cysteine Deletion Mutants with BMAA

The finding of delayed growth in the *ΔcysE* mutant in the presence of BMAA suggests that some or all the elements of the cysteine biosynthesis pathway are required for tolerance towards BMAA. To explore how perturbations in the second step of the cysteine biosynthesis pathway affect growth during BMAA exposure, knockout strains *ΔcysK* and *ΔcysM* were grown with BMAA present for 24 hours. All single deletion mutants for each step in the cysteine biosynthesis pathway showed an extended lag phase when grown with BMAA present (Figure 3). Variability in the duration of lag phase was observed in strain *ΔcysM* which is unable to express OASase B, an enzyme sharing 43% sequence similarity with OASase A but is likely to have a range of roles in the cell unrelated to cysteine biosynthesis [126, 127]. Duration of lag was variable between strains, but most prominent for the strain *ΔcysK* which is missing the gene encoding OASase A, the predominate enzyme involved in conversion of OAS to cysteine (Figure 3b).

3.4.2 Complementation of Cysteine Deletion Mutants with Cysteine Synthesis Enzymes

To confirm the involvement of cysteine biosynthesis enzymes in BMAA tolerance, knockout strains *ΔcysE* and *ΔcysKΔcysM* were transformed with plasmids bearing their respective missing genes. Induction of plasmid borne genes and subsequent growth with 500 µg/mL BMAA for 24 hours shows that expression of *cysK* via a plasmid in strain *ΔcysKΔcysM* mostly alleviates the lag in growth otherwise caused by BMAA (Figure 4b). Therefore, OASase A expression in *E. coli* cells results in restoration in growth which is close to comparable to when *E. coli* are grown in the absence of BMAA. Consistent with previous results showing the variable effect of *cysM* deletion on BMAA tolerance, restoration of *cysM* via plasmid resulted in an inconsistent recovery of growth which does not display a reduction in lag as strong as what is observed for *cysK* (Figure 4c).

When strain *ΔcysE* is complemented with the plasmid pTAB1-*cysE* under the condition of BMAA exposure, a lag of 15 hours is seen for most cultures (Figure 5b). The lack of ability to reduce growth lag suggests *cysE* is not likely to be directly involved in BMAA tolerance and the lag produced in the absence of *cysE* may be related to other mechanisms. This strain also failed to produce a lag when grown with BMAA and harbouring empty vector pTAB1, which is inconsistent with previous results for this strain (Figure 5a).

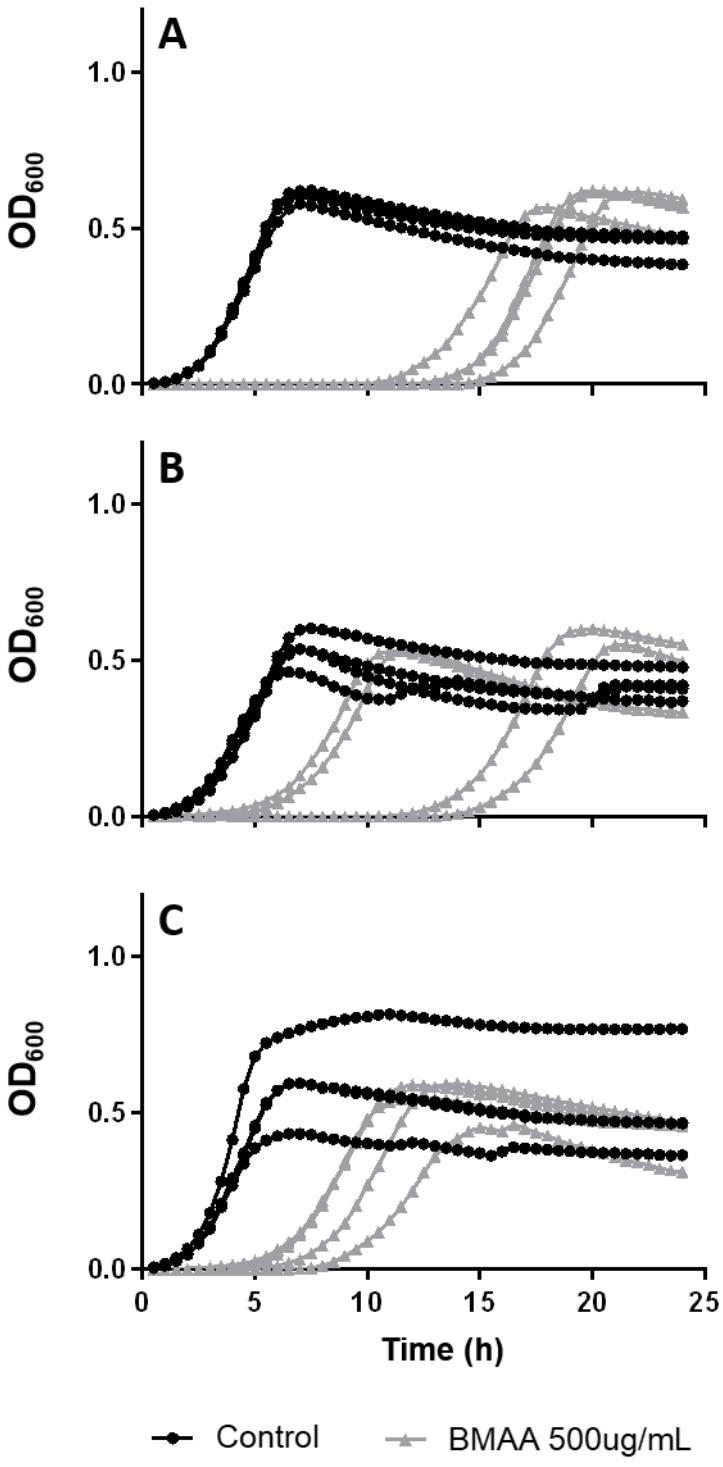


Figure 3. Effect of BMAA on growth of *E. coli* cysteine deletion mutants. Panel A shows the growth over 24 hours of strain *ΔcysE* treated with 500 µg/mL BMAA (grey) or untreated control (black) in M9-minimal medium supplemented with 35 µg/mL cysteine. Panel B and Panel C show strain *ΔcysK* and *ΔcysM* respectively under the same conditions described. Biological replicates for each strain and treatment are plotted individually (n=4).

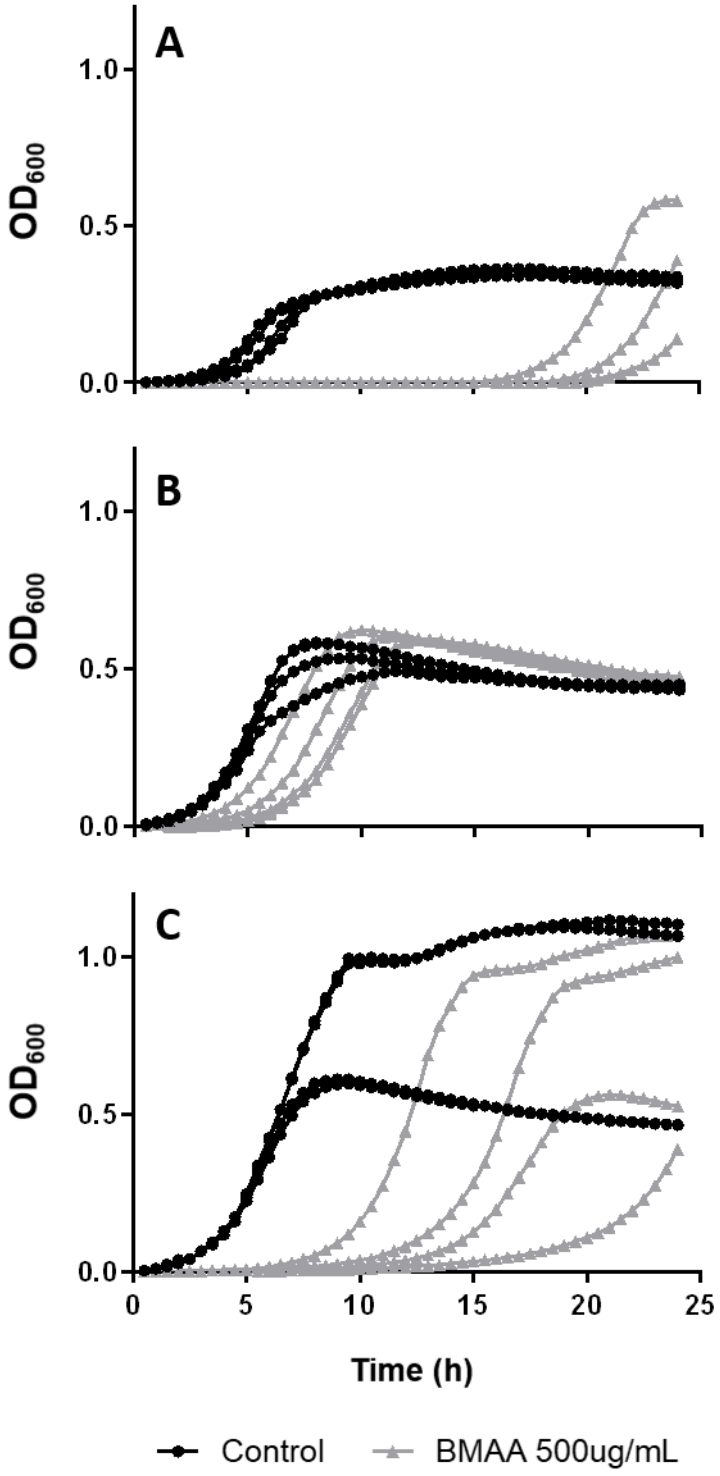


Figure 4. Growth of *ΔcysKΔcysM E. coli* in BMAA containing media with and without expression of *cysK* or *cysM*. Panel A shows strain *ΔcysKΔcysM* with empty vector pTAB1 grown in the presence (grey) and absence (black) of 500 µg/mL BMAA. Panel B and C show the same strain harboring pTAB1-*cysK* or pTAB1-*cysM* respectively. Cultures were induced with 0.5 mM IPTG for 3 hours at 37°C before treatment with BMAA. Growth was monitored by optical density readings at 600 nm, biological replicates are plotted individually (n=4).

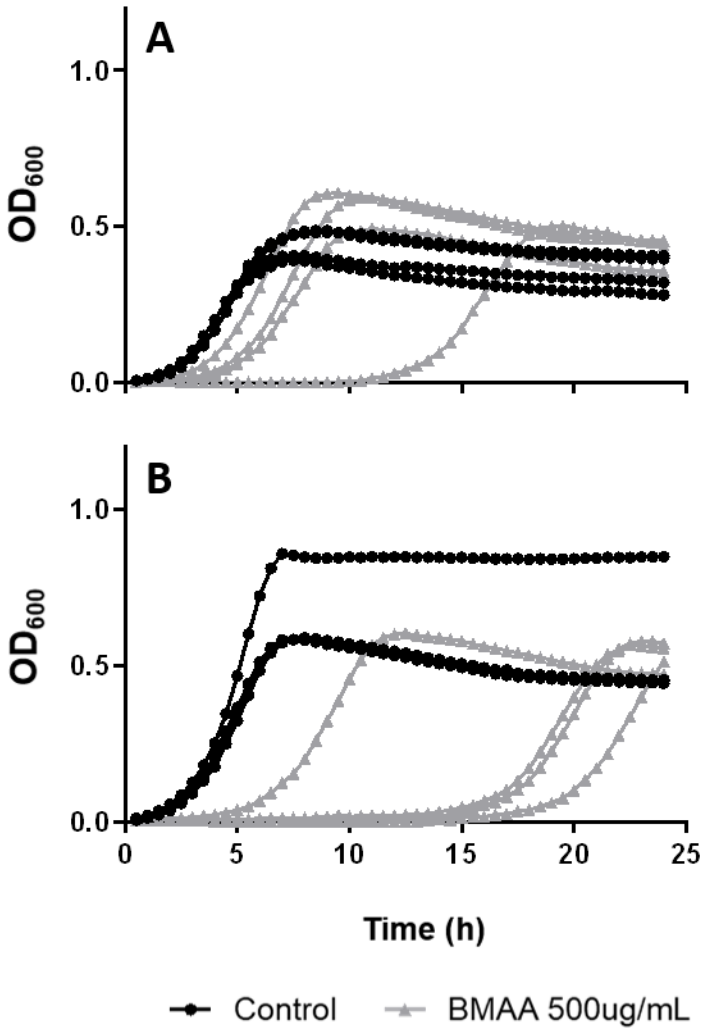


Figure 5. Growth of *ΔcysE E. coli* in BMAA containing media with and without expression of *cysE*. Panel A shows growth of strain *ΔcysE* harboring pTAB1 with 500 µg/mL BMAA present (grey) or unexposed controls (black). Panel B displays strain *ΔcysE* harboring complementation plasmid pTAB1-*cysE* with (grey) and without (black) 500 µg/mL BMAA in the growth media. Cultures were induced with 0.5 mM IPTG for 3 hours at 37 °C prior to growth with BMAA. Growth was monitored by optical density readings at 600 nm, biological replicates are plotted individually (n=4).

3.5 Discussion

This study has shown that the cysteine biosynthesis pathway of *E. coli* is involved in BMAA tolerance and deletion of any of the three key genes involved is detrimental to the cells ability to cope with BMAA, as displayed by delays in growth of gene deletion strains *ΔcysE*, *ΔcysK*, and *ΔcysM* (Figure 3). Delays in growth for all the gene deletion mutants implies that disruption of the pathway generally is detrimental to the cells ability to cope with BMAA and continue to grow. While there is an initial inhibition of growth with BMAA, this can be overcome when cells are given enough time. The time-based recovery in all three single gene deletion strains suggests that disruption to an essential process like cysteine biosynthesis disturbs homeostatic balance of the cell, that is required for BMAA tolerance. The eventual recovery of growth and the inconsistent duration of lag time before restoration of growth (Figure 3) supports the idea that *E. coli* require balance within cysteine homeostasis for BMAA tolerance and when this is disrupted, cells must circumnavigate the disruption and adapt to dealing with BMAA in an alternative way. Cysteine is an important precursor for many bio-molecules such as thiamine, biotin, and antioxidants such as glutathione and thioredoxin [106]. Cysteine biosynthesis also plays an important role in sulfur metabolism and generation of iron-sulfur clusters which are essential for the activity for many enzymes [128]. Cysteine levels in bacteria are often tightly regulated [129] and as an important amino acid, disruption of cysteine biosynthesis potentially has important carry-on effects in other aspects of cell metabolism, such as sulfur metabolism and oxidative stress defences [106, 130]. BMAA has been described previously as causing oxidative stress [22, 71, 115]. In wild type *E. coli,* there may be a scenario where growth is possible with BMAA due to adequate oxidative stress defences, as displayed by the lack of growth inhibition observed by our group and others [67, 68]. However, for cysteine deletion mutants oxidative defences may be impaired due to the importance of cysteine in oxidative stress defence. Chapter 2 shows that deletion of *cysE* did indeed cause perturbation in glutathione levels and sensitivity to oxidative stress, which supports this theory. In addition, Chapter 4 addresses this further by analysing the transcriptomic response of wild type *E. coli*

exposed to BMAA to see potential mechanisms being employed to generate a tolerance or metabolism mechanism.

During overexpression of cysteine biosynthesis genes in deletion background strains, there were signs of growth restoration during overexpression of *cysK* and *cysM* (Figure 4b and 4c). The enzyme OASase A encoded by *cysK* is interesting to investigate as its absence causes the most severe lag in growth, which is alleviated by overexpression (Figure 4b). Overexpression of *cysM* displayed a more varied change to duration of lag but did work to reduce lag (Figure 4c). This contrasts with overexpression of the first step of cysteine biosynthesis *cysE*, which did not alleviate BMAA-induced lag despite its apparent importance in BMAA tolerance (Figure 5b). Within the second step of cysteine biosynthesis, the OASase enzymes encoded by *cysK* and *cysM* may have a more direct effect on BMAA tolerance. An alternative function of OASase separate to its role in cysteine biosynthesis is its ability to degrade cysteine (Figure 6).

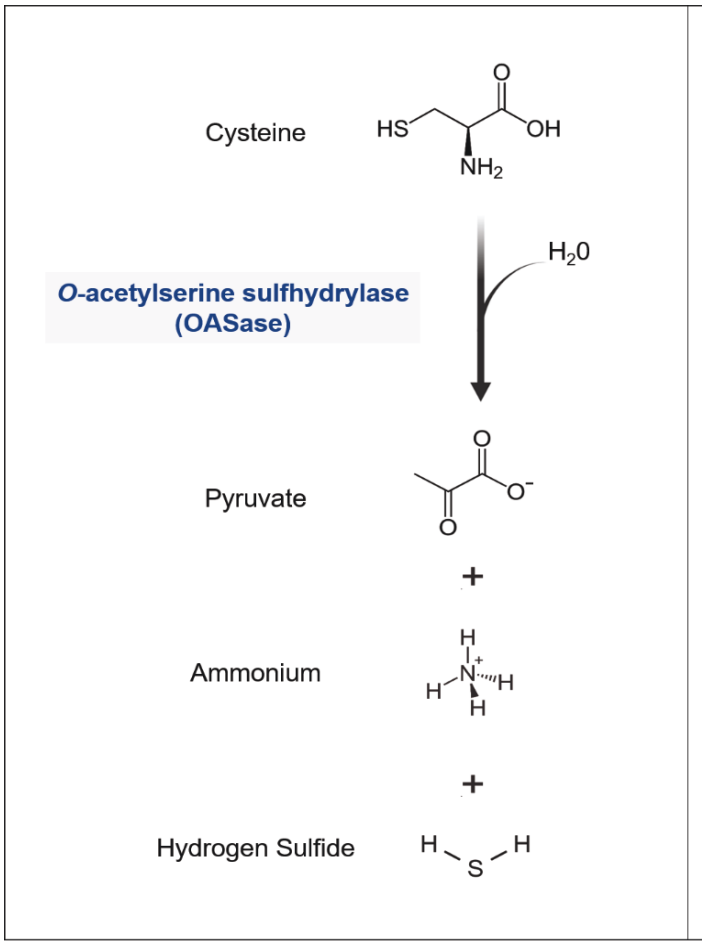


Figure 6. The cysteine degradation pathway in *E. coli*. OASase enzymes encoded by genes *cysK* and *cysM* possesses cysteine desulfhydrase activity allowing for degradation of cysteine into pyruvate, ammonium, and hydrogen sulfide

As OASase enzymes can accept alternative substrates, it is possible that BMAA may take the place of cysteine in this reaction leading to its degradation. Mimicry and competition with canonical amino acids is a well characterised mechanism of toxicity for non-protein amino acids [15], and as a toxin belonging to this group, BMAA may also be participating in this mechanism with OASase. The suspected products from OASase action on BMAA are methylamine and pyruvate. Interestingly, methylamine has been described as a by-product of BMAA degradation [131]. Detoxification of BMAA via OASase enzymes could explain why toxic effects of BMAA are often seen in eukaryotic systems and less so in prokaryotes [67, 68], as OASase enzymes are conserved across plants and prokaryotic species, but not present in mammals such as humans which obtain cysteine from dietary sources [132]. The possibility of OASase enzymes playing a role in BMAA detoxification is explored further in Chapter 5.

In addition to the connection between the presence of OASase and BMAA tolerance, deletions in *cysE* also produce a lag in growth when BMAA is present, though complementation with plasmid-expressed SAT cannot reverse this (Figure 5). SAT produces the intermediate OAS which is used by OASase in a forward reaction to produce cysteine, but this forward reaction is unlikely to be related to direct detoxification of BMAA. Detoxification of BMAA via OASase would theoretically proceed independently of SAT or OAS involvement, and so *cysE* deletion also producing lag in growth may be due to overall impact of the cysteine pathway on downstream processes in the cell required for BMAA tolerance. This supports the potential scenario described earlier whereby the cysteine biosynthesis pathway mediates BMAA tolerance in an indirect way due to its influence on downstream processes which may be important for BMAA tolerance. In addition, it is also possible that the lag observed in the *ΔcysE* mutant is a result of its influence on *cysK* and *cysM*. This can be explained via understanding the transcriptional regulation of the cysteine synthesis pathway. Removal of *cysE* means that no SAT is present and therefore no OAS is produced by the cell (Figure 2). OAS is not only an intermediate but also a signalling molecule which spontaneously isomerises into N-Acetylserine (NAS), which binds to transcriptional activator *cysB* resulting in transcription of *cysK*, *cysM*, and

other genes related to sulfur metabolism [133]. Due to this, it is possible that removal of *cysE* and therefore OAS would result in *cysK* and *cysM* not being expressed adequately by the cell via *cysB* transcriptional activation. NAS itself is also a known antioxidant and its absence in the *ΔcysE* mutant may therefore reduce defences against reactive oxygen species [134, 135]. Subsequent low levels of OASase could mean the cells are ill equipped to detoxify BMAA via this enzyme, resulting in a lag in growth. This would also explain why the lag produced upon *cysE* deletion is not as severe as when *cysK* and *cysM* are fully removed from the genome and zero levels of OASase are expressed (Figure 3a and Figure 4a). This principle applies to both *cysK* and *cysM* which are under control of transcription activator *cysB*. Although the two enzymes share 43% sequence similarity, the role of OASase B is not well understood [136]. For OASase B, several other roles in cellular metabolism which are unrelated to cysteine biosynthesis and could be having unknown effects on response to BMAA. As OASase B is less well characterised compared to OASase A, it would be interesting to investigate its ability to detoxify BMAA.

There is the possibility that the expression system used in complementation studies may be influencing the growth of the strains, as evidenced by the difference in growth seen when cells are harbouring these plasmids compared to when they are absent, particularly for *ΔcysE* (Figure 5). Despite this, the current results would suggest that OASase enzymes could be involved in detoxification or at least some sort of modulation of BMAA toxicity in cells. The gene *cysE* seems unlikely to be involved, as its behaviour so far can be explained by its influence on the cysteine operon or by the overall importance of cysteine biosynthesis to other metabolic functions of the cell. OASase B encoded by *cysM* shares some similarities with OASase A, and the variability in responses suggests it should also be investigated as a potential detoxification means of BMAA. In addition, as there are more unknowns associated with this enzyme it is hard to predict its influence completely. Isolation of OASase B and testing of detoxification of BMAA would reveal if it carries out a degradation reaction.

There are a limited number of studies investigating the effects of BMAA in prokaryotic systems such as *E. coli*. However, the results of this work are consistent with a previous report which showed that growth wild type *E. coli* is unaffected by concentrations of

100 µM of BMAA in the medium [67]. In addition, the authors of that study determined that cellular respiration was not decreased, nor was there an increase in reactive oxygen species generation upon BMAA exposure. Overall, the tolerance of *E. coli* towards BMAA is somewhat unique when compared to the multitude of toxicity studies which have repeatedly demonstrated the damaging effects of BMAA in mammalian systems [11, 24, 69, 70]. This suggests that wild type *E. coli* have the necessary metabolic requirements to counteract toxicity of BMAA. Due to the increased importance of understanding the chemistry and metabolism of BMAA in living systems, the discovery of a detoxification pathway in an organism which tolerates BMAA is critical information. Work from this Chapter and Chapter 2 have provided two main paths of future research into this tolerance phenomenon in wild type *E. coli*. Firstly, it has highlighted that there should be investigation into the overall defences of the cell that allow for BMAA tolerance as cysteine biosynthesis influences several processes such as antioxidant production and sulfur metabolism. Secondly, OASase enzymes involved in cysteine biosynthesis should be investigated for ability to detoxify BMAA. These two avenues of research are explored in the remaining Chapters of this thesis. Identification of a BMAA detoxifying system derived from the observed tolerance in *E. coli* has far reaching implications for BMAA research and points the way towards a future where bioremediation may be possible, potentially reducing the number of people exposed to this toxin.

Chapter Four - Analysis of Genomic and Transcriptomic changes in *E. coli* Exposed to BMAA.

Chapter Four - Analysis of Genomic and Transcriptomic changes in *E. coli* Exposed to BMAA.

4.1 Introduction

Since the discovery of the possible link between BMAA and neurodegenerative diseases, a range of studies have been conducted to reveal the mechanism by which this non-protein amino acid could lead to neurodegeneration. This includes numerous toxicity studies involving mammalian cell lines [24, 70], rodents [50, 51], and primate models [11, 12]. Despite extensive research into the mechanism by which BMAA causes detrimental effects on mammalian cells, there has been limited research on the effect BMAA has on prokaryotic species and bacteria [67, 81, 82]. This is particularly of interest because BMAA is known to be produced by prokaryotic cyanobacteria, though the production pathway is yet to be definitively shown [79, 80]. Bacteria such as *E. coli* do not appear to experience the toxic effects that BMAA is known to exert on mammalian cells. Notably, a study in 2015 showed lack of growth defects and the absence of protein incorporation in bacteria treated with BMAA [67]. This is in contrast to other toxic NPAAs such as canavanine and azetidine-2-carboxylic acid which exert similar toxics effects on bacteria as they do on mammalian cells, resulting in slow growth or death upon exposure (for reviews see [15] and [14]). As such, it is noteworthy that *E. coli* can tolerate BMAA with no observable effect on growth [67, 68]. However, results from our laboratory have demonstrated that strains with deletion in cysteine biosynthesis genes experience negative growth effects and susceptibility to BMAA (Chapter 2 and 3). The link between the cysteine biosynthesis pathway and BMAA tolerance is not clear and requires a more in-depth observation of the cells. The possibility of genetic mutations arising from BMAA exposure could explain the unpredictable growth responses that occur in cysteine auxotrophic strains when treated with BMAA (Chapter 3). To investigate this possibility, cysteine auxotrophic strains sensitive to BMAA were treated with BMAA for 20 hours and genome sequenced to determine if genetic mutations occurred. This was compared to

the same cells grown for an additional 20 hours in the absence of BMAA. This approach was used to determine if any mutations that occur to restore growth while BMAA is present are transient or passed onto daughter cells even in the absence of BMAA as a stimulus.

Investigations continued into the gene expression level, to look for changes in transcription in wild type *E. coli* upon exposure to exogenous BMAA that may account for the observed tolerance. The impacts of certain gene deletions on ability of *E. coli* to tolerate BMAA remain unexplained, and the mechanism behind tolerance in wild type strain is unknown. Analysis of gene expression changes aimed to provide more detailed information about the events occurring within *E. coli* when exposed to BMAA, that can be used to explain the phenotypic responses observed. To date, no study has been conducted into the mechanism by which *E. coli* are able to tolerate BMAA. In this study we aim to elucidate the changes that occur within *E. coli* when exposed to levels of BMAA that allow them to survive. We have used a combination of genomic and transcriptomic techniques to study the effects of BMAA on a common laboratory strain of *E. coli* BW25113 and BMAA sensitive strain *ΔcysE*.

4.2 Methods

Genomics

4.2.1 Growth Conditions, DNA Isolation and Sequencing

For the investigation into growth characteristics displayed by strain *ΔcysE* when grown with BMAA, cells were grown as described in Chapter 2 and 3. Briefly, cultures were grown in 96-well plate format in M9 medium supplemented with 35 µg/mL cysteine and 500 µg/mL BMAA. After a growth period of 20 hours, cells were transferred to 8 mL of fresh M9 media for a period of recovery growth. Recovery growth continued for 20 hours at 37°C with shaking. To investigate if genomic changes were occurring over the duration of growth with BMAA, a Bioline ISOLATE II Genomic DNA Kit was used to isolate genomic DNA from samples of the cultures at different time points. Genomic DNA was extracted from 200 µL of cell culture after 20 hours of exposure to BMAA, as well as non-exposed controls. Genomic DNA was also extracted from the same cultures following the 20-hour recovery growth period in the absence of BMAA. DNA was eluted in 90 µL of MilliQ H_2O heated to 70°C, quality and concentration was determined via agarose gel electrophoresis, NanoDrop 100 spectrophotometer and Qubit 2.0 fluorometer. Samples were processed by the UTS Core Sequencing Facility (ithree Institute, University of Technology Sydney). Library preparation was done via Nextera DNA Flex Library Preparation Kit and samples were sequenced on Illumina MiSeq sequencer. Sequence reads underwent quality control using FastQC software tool available on Galaxy V3.2 web platform [137]. Sequence mapping of *E. coli* genomes was conducted via Bowtie2 software tool (V3.2) and visualised using Integrative Genomics Viewer (IGV).

Transcriptomics

4.2.2 Growth Conditions

E. coli BW25113 was streaked from frozen glycerol stocks onto LB agar and incubated at 37°C for 16-18 hours. A single colony was issued to inoculate 3 mL of M9 minimal medium and grown for 16 hours at 37°C, 150 rpm. The starter culture was used to inoculate 50 mL of fresh media to OD_{600} 0.05. Growth was continued under the same conditions as described, until mid-exponential growth was reached (OD_{600} 0.4). 20 mL of culture was transferred to two 150 mL flasks, and either BMAA or 10 mM HCl blank were added to the culture. Cultures were allowed to continue growing at 37°C, 150 rpm for 1 hour, after which 10 mL of culture was pelleted at 4500g for 10 minutes. Supernatant was discarded and 100 µL of RNAlater was added to the pellet. Samples were stored at 4°C overnight before RNA extraction.

4.2.3 RNA Isolation and Sequencing

Total RNA was isolated using hot-phenol chloroform extraction followed by ethanol precipitation. RNAlater was removed and pellets were resuspended in 150 µL of ice-cold Solution 1 (10 mM Sodium Citrate; 10 mM Sodium Acetate pH 4.5; 2 mM EDTA). Suspended cells were then transferred to tubes with 400 µL phenol and 150 µL Solution 2 (10 mM Sodium Acetate pH 4.5; 2% SDS) pre-heated to 65°C.

The suspension was immediately vortexed then incubated at 65°C for 3-4 minutes with 1300 rpm orbital shaking. Samples were then frozen in liquid nitrogen (30-45 seconds) before being centrifuged for 5 minutes at 22,000g. The resulting aqueous phase was transferred to a fresh tube with 400 µL phenol and the process of incubating at 65°C was repeated. The final aqueous phase was transferred to a new tube with equal volumes of phenol and chloroform. Contents were vortexed then centrifuged at 22,000g for 5 minutes at 4°C. Further chloroform extractions were carried out as described before moving aqueous phase to a new tube. 2.5x volume of 100% ethanol, 1/10 sample volume of 3 M sodium acetate and 1 µg/µL of RNA-grade glycogen were added to sample and incubated overnight at -20°C.

Samples were centrifuged for 1 hour at 22,000g at 4°C and supernatant discarded. The RNA pellet was washed with 70% ethanol and centrifuged for 5 minutes, twice. The resulting RNA pellet was lightly air dried before being resuspended in 50 µL of RNAse-free water. Quality and concentration of RNA was determined via bleach agarose gel electrophoresis [138], NanoDrop 100 spectrophotometer, and Qubit 2.0 fluorimeter. RNA was aliquoted and stored at -80°C.

Samples were processed by the UTS Core Sequencing Facility (ithree Institute, University of Technology Sydney) and treated with DNAse. Library preparation was done via Tecan Universal Prokaryotic RNA-Seq Library Preparation Kit and samples were single end 1x150 bp sequenced on Illumina MiSeq V3 sequencer.

4.2.4 Analysis

RNA sequencing results were analysed using the Galaxy web platform [137]. Quality control and trimming of reads was done via the Cutadapt (Ver 1.16) and FastQC (Ver 0.11.8) tools and visualised using Integrative Genomics Viewer (IGV) [139]. Bowtie2 (Ver 2.3.4.3) was used to align reads to the BW25113 reference genome from NCBI and visualised in IGV. The software tools htseq-count (Ver 0.9.1) and DEseq2 (1.22.1) were then applied to determine differentially expressed transcripts in the samples. Results were uploaded to Uniprot [140] and resulting gene names and protein features assigned to each transcript. Microsoft Office Excel 2016 was used to sort and filter entries with log2 fold change of >1 or <-1 that were statistically significant ($p<0.05$). Panther [141] and STRING [142] platforms were used to map gene associations across the differentially expressed genes.

4.3 Results and Discussion

4.3.1 Genomics

Results from genomic sequencing of wild type BW25113 and mutant *ΔcysE* after 20 hours exposure to BMAA showed no changes compared to unexposed controls. This was also the case for samples taken after 20-hour recovery growth post-BMAA exposure. Across all samples tested including wild type and strain *ΔcysE* for BMAA exposed and control cultures, genetic changes were identified in *proA*, which encodes glutamate-5-semialdehyde dehydrogenase (Table 3). Mutation to *proA* via deletion or single nucleotide polymorphism (snp) was consistent across all samples irrespective of BMAA treatment yet differs from the reference genome for *E. coli* BW25113. This mutation results in the replacement of alanine at position 383 in the resulting enzyme with glutamic acid. This mutation has been previously identified to occur in *E. coli* when under nutrient stress, such as during growth in minimal medium and allows for glutamate-5-semialdehyde dehydrogenase to synthesise additional amino acids rather than solely proline for which it is usually responsible [143, 144]. As all strains tested were grown in minimal media this common mutation across all conditions is not specific to BMAA treatment. It is also expected due to the growth of these strains in minimal media conditions, which is the condition described to promote this mutation [143]. Lack of BMAA-induced changes in the genome of *E. coli* suggest that the irregular growth patterns of cysteine auxotrophic mutants in response to BMAA is not due to mutations at the genomic level and is likely that changes on the gene expression or protein level are responsible. This also supports the idea that *E. coli* are metabolising BMAA to some extent and this is what allows for tolerance to occur, as gene presence is not directly responsible for tolerance but rather the cellular effects of those genes which would be altered on a transcriptional or protein level. Knowledge of this information provides new directions for BMAA research which may have otherwise not have been investigated. This includes the exploration of BMAA metabolism within bacteria generally, which could lead to exciting information regarding detoxification or bioremediation of BMAA from the environment. Previous research has indicated that BMAA is protein associated initially in wild type *E. coli*, before being found in non-protein associated from during later growth [68]. The

transient protein association was found to not occur in protein extract, only in protein isolated from living cells, furthermore this association contrasts with the gradual increase in protein association by BMAA observed in eukaryotic proteins [68]. With this information is it likely that *E. coli* funnel BMAA into a metabolic process and that this may be connected to the tolerance they possess. Whether this is purposeful, or a side effect of other cellular processes is still unknown. The difference between BMAA association in prokaryotic cells and eukaryotic also supports the pattern of susceptibility in eukaryotic cells which is not apparent in bacteria. If a mechanism for detoxification or metabolic processing of BMAA is present in bacteria and lacking in mammalian cells, its identification could lead to protective mechanisms being developed to combat BMAA toxicity.

Table 3. Genomic changes observed in *E. coli* irrespective of BMAA treatment

Mutation Type	**Change**	**Strand**	**Gene**	**Effect**	**Product**
snp	C → A	+	*proA*	Missense variant Ala383Glu	Glutamate-5-semialdehyde dehydrogenase E383A
deletion	AGCGGC CGCA → AA	+	*proA*	Intragenic variant delGCGGCCGC	Glutamate-5-semialdehyde dehydrogenase E383A

4.3.2 Transcriptomics

RNA sequencing indicated that levels of transcripts from two genes were significantly upregulated in BMAA treated BW25113 *E. coli (exbB, feoC)* and four genes were significantly downregulated (*thrL, dkgA, yqhD, malM*). In addition, there were a number of genes which were changed yet did not meet the cut off P-value of <0.05 (Table 4).

Table 4. Differentially expressed genes in *E. coli* treated with BMAA

"*" significantly different P-value < 0.05

Genes included up to P-value < 0.15

Gene Symbol	Description	log2 (FC)	P-value
***thrL** **	thr operon leader peptide (thr operon attenuator)	-1.73	1.72E-05
***exbB** **	Biopolymer transport protein ExbB	1.27	4.08E-03
***dkgA** **	2,5-diketo-D-gluconic acid reductase A	-1.15	0.01
***yqhD** **	Alcohol dehydrogenase YqhD	-1.11	0.01
***malM** **	Maltose operon periplasmic protein	-0.91	0.02
***feoC** **	Probable [Fe-S]-dependent transcriptional repressor FeoC (Fe(2+) iron transport protein C)	0.92	0.03
ymdB	O-acetyl-ADP-ribose deacetylase (Regulator of RNase III activity)	0.65	0.06
preA	NAD-dependent dihydropyrimidine dehydrogenase subunit PreA	-0.80	0.07
hemX	Protein HemX	0.78	0.07
purE	N5-carboxyaminoimidazole ribonucleotide mutase	-0.76	0.08

xdhB	Putative xanthine dehydrogenase FAD-binding subunit XdhB	-0.74	0.08
mazF	Endoribonuclease toxin MazF (mRNA interferase MazF)	-0.69	0.09
mukF	Chromosome partition protein MukF (Protein KicB)	0.75	0.09
cysN	Sulfate adenylyltransferase subunit 1	-0.75	0.09
trmH	tRNA (guanosine(18)-2'-O)-methyltransferase	-0.68	0.10
entH	Proofreading thioesterase EntH (Enterobactin synthase component H)	0.58	0.10
adeP	Adenine permease AdeP	0.71	0.11
sufS	Cysteine desulfurase (Selenocysteine beta-lyase) (Selenocysteine reductase)	0.64	0.12
yjjY	Uncharacterized protein YjjY	-0.68	0.12
wbbI	Beta-1,6-galactofuranosyltransferase	-0.69	0.12
hicA	Probable mRNA interferase toxin HicA (Endoribonuclease)	0.50	0.12
ydhV	Uncharacterized oxidoreductase	-0.63	0.13
nrdA	Ribonucleoside-diphosphate reductase 1 subunit alpha (Protein B1)	-0.59	0.14
lamB	Maltoporin (Lambda receptor protein) (Maltose-inducible porin)	-0.56	0.14
csgF	Curli production assembly/transport component	0.64	0.14

ftnA	Bacterial non-heme ferritin (Ferritin-1)	-0.61	0.14
rimM	Ribosome maturation factor RimM	0.57	0.14
cspI	Cold shock-like protein CspI	-0.47	0.15
radD (yejH)	Putative DNA repair helicase RadD	-0.64	0.15
trmD	tRNA (guanine-N(1)-)-methyltransferase	0.64	0.15

4.3.2.1 Iron Depletion

In BMAA treated *E. coli*, several genes involved in iron homeostasis were altered. Significantly upregulated was the gene *ExbB,* a component of the *TonB*-dependent energy-dependent transport of various receptor-bound substrates [145]. This system allows for essential and scarce nutrients to traverse the bacterial outer membrane via active transport [146]. Important nutrients transported this way include vitamin B-12 and iron-siderophore complexes. Other things that are taken up by this system include colicins [147], carbohydrates, and nickel [148]. Transcription of *ExbB* is regulated by the availability of iron and is upregulated in response to metal ion depletion [149, 150]. As such, its upregulation suggests that iron limitations may be present for cells when BMAA is present in the growth medium. Also upregulated is the gene *feoC*, representing a small protein that is a part of the 3 genes of the *feo* uptake system responsible for transporting ferrous iron (Fe 2^{+}) into the cell, *feoABC* [151]. *feoC* represents a ferrous (Fe 2^{+}) iron uptake pathway which exists in addition to the system that acquires ferric (Fe 3^{+}) iron and uses *ExbB*. Like *ExbB*, the *feo* system is also upregulated when iron levels are low [152].

The significant upregulation of both *ExbB* and *feoC* in BMAA treated *E. coli* points towards a disturbance in iron metabolism. These genes represent two independent avenues of iron uptake for *E. coli,* and both are known to be upregulated when iron levels are low, suggesting iron availability is scarce under BMAA conditions. In addition, upregulation of *feo* genes and *ExbB* have been observed in *E. coli* treated with volatile

organic compounds [153]. This suggests that upregulation of iron acquisition genes is part of a more general stress response, with iron uptake also reported to help *E. coli* deal with stressful environments [153]. In addition to the significant upregulation of iron acquisition genes *ExbB* and *feoC*, there were changes in other iron related genes which did not meet the cut-off for significance, but still point toward a lack of iron under BMAA conditions.

Other genes involved in iron homeostasis were changed but below the cut-off significance level including *HemX, entH, ftnA,* and *nrdA*. In *E. coli HemX* has yet to be assigned detailed function, but its homologue in *Staphylococcus aureus* is involved in the biosynthesis of the essential iron containing molecule heme [154]. *HemX* regulates the levels of *HemA* which is the first step of heme biosynthesis [155]. Its upregulation may indicate an attempt to compensate for lowered iron levels by increasing acquisition options such as heme. Additionally, there was modest upregulation of *EntH* which encodes a proofreading enzyme involved in synthesis of the iron acquisition siderophore enterobactin [156, 157]. Previous research has shown that when iron is scarce, *entH* levels increase so that the cell may acquire more iron from the environment [156]. Downregulation of *ftnA* which encodes the iron storage protein ferritin A also occurred with BMAA treatment [158]. As the major iron storage molecule for *E. coli*, expression of ferritin A is induced by iron so that *E. coli* may sequester any free iron and store it, thereby protecting the cell from damage caused by free iron [159]. As iron triggers transcription of *ftnA*, the downregulation of *ftnA* in BMAA treated *E. coli* suggests that iron levels are low or not sufficient to induce expression of *ftnA* compared to untreated cells. Finally, the gene *nrdA* is involved in DNA biosynthesis via production of deoxyribonucleotide precursors, it is an iron-dependent enzyme which functions under conditions of sufficient iron and has a magnesium reliant counterpart which takes over under iron starvation conditions [160, 161]. As it is downregulated this also supports to the possibility of iron deprivation, as it is most functional when iron is plentiful.

Taken collectively, these various changes in expression of iron related genes point towards a condition of iron depletion in BMAA treated *E. coli.* In order to scavenge

more iron for the cell there are transcriptional changes in genes involved in various iron acquisition pathways (*ExbB, feoC, EntH*), iron storage proteins (*hemX, ftnA*) and iron dependent enzymes (*nrdA*). Interestingly, similar gene expression changes have been observed in *E. coli* undergoing iron starvation induced by a metal chelator [162]. Upregulation of *ExbB, entH* and *feo* genes mirrors the results of BMAA treatment, as does *ftnA* downregulation. This study also detected changes in proteins that are iron dependent, suggesting that production of iron requiring enzymes is adjusted to cope with the lower iron availability [162]. This is consistent with the changes observed in *nrdA* for BMAA treated *E. coli* that as discussed earlier, encodes an iron dependent enzyme. Furthermore, other similarities can also be drawn between this iron starvation study and the BMAA results, including downregulation of cold shock protein *cspL* and transcriptional changes in sulfur and purine metabolism, which are discussed in later sections. In addition, genes involved in iron uptake have been shown to increase the tolerance of *E. coli* towards environmental stresses [163]. *E. coli* exposed to volatile organic compounds have also been shown to have upregulated genes involved in uptake of iron and siderophores (*feo* genes, *exbBD, yncD* and *fhuF*) [153]. And bacterial communities exposed to pollution have shown increased expression of genes such as *ExbB* [164].

One possible explanation for a scenario of iron depletion is that BMAA, may be chelating iron from the media leading to low levels in the cell. BMAA has previously been associated with metal ion chelation, and the metal chelation properties of BMAA have been suggested to contribute to disease development for BMAA associated neurodegenerative disease [165]. Iron and other trace metals have always been an important aspect when considering neurodegenerative disease, and on Guam the levels of iron and other metals were investigated alongside BMAA for involvement in ALS-PDC development [166]. BMAA is known to be a chelator of metals and to complex with zinc and copper in particular [165, 167]. It has been suggested that BMAA binds to iron and this complex is what allows it to cross the blood brain barrier - specifically, carbamate adducts of BMAA rather than free BMAA [168].

The transcriptional profile of cells treated with iron chelator to induce iron starvation parallels what is seen with BMAA exposed *E. coli* here, particularly the iron and sulfur

related genes, adding support to this possibility [162]. BMAA-induced iron depletion could have wider implications for cell metabolism, as many important enzymes require iron to function, including catalase and superoxide dismutase [169][170]. Low iron levels can also cause growth delays and interfere with general cellular processes [171]. In addition, iron metabolism is closely tied to sulfur metabolism, which also experienced changes in gene expression in response to BMAA (section 4.3.2.2).

The changes to iron homeostasis in BMAA-treated wild type *E. coli* could be linked to the tolerance observed. From the transcriptomic changes, there is an indication that iron depletion is occurring, yet wild type *E. coli* showed no obvious growth defects upon BMAA exposure. This could indicate that the changes that occur to the iron systems within *E. coli* are sufficient to handle the stress being caused by BMAA. In relation to cysteine biosynthesis gene disruptions leading to BMAA sensitivity, this may be linked to iron metabolism. Cysteine gene deletions may result in changes to iron homeostasis, which in turn makes it harder to deal with BMAA and leads to delays in growth that take time to overcome (Chapter 2 and 3).

As iron and trace metals are associated with neurodegenerative diseases generally, this link between iron and BMAA may be worth exploring further. The metal chelation effects of BMAA have been documented but the implications have not been exhaustively followed up. The changes that occur in bacteria may be a starting point for unravelling the pathways of BMAA metabolism on different systems.

4.3.2.2 Sulfur metabolism

Genes linked to sulfur metabolism were also altered in BMAA treated *E. coli* including *cysN, ydhV* and *sufS.* As sulfur and iron metabolism are closely linked [172], this is unsurprising considering the number of iron related genes being differentially expressed. Sulfur assimilation genes including *cysCDKN* are among those regulated by the transcription factor *cysB* which activates their expression when sulfide levels are low, to acquire more sulfide for processes such as cysteine biosynthesis [133, 173]. Sulfate comes from the environment and into cell, and *cysN* is part of the pathway to

turn sulfate into sulfite then sulfide which *cysK* uses to synthesise cysteine [174]. The last step in sulfide synthesis requires substantial amounts of NADPH, which may be an issue for the BMAA treated cells as discussed later. As typically genes like *cysN* are upregulated when the cell is low in sulfide, downregulation in BMAA treatment may indicate levels of sulfide are higher. However, the many other sulfur assimilation genes under the control of *cysB* do not appear changed. This may indicate that any underlying changes are not profound enough to appear in the transcriptomic analysis at the time point chosen or culture conditions used. The changes in cysteine biosynthesis associated genes here provides a link to the BMAA sensitive *E. coli* strains with deletions in *cysEKM* (described in Chapter 3). The regulation of these genes all fall under *cysB* regulation and this may provide an explanation as to why cysteine synthesis disruption also leads to BMAA sensitivity, but the exact mechanism still remains unclear. Despite this, the connection between cysteine biosynthesis and sulfur metabolism is clear and the influence on iron metabolism and BMAA tolerance could be significant.

Another gene related to sulfur metabolism and downregulated is *ydhV*. As an oxidoreductase YdhV contains a unique redox-active molybdenum cofactor involving four sulfur moieties in addition to cysteine and iron ions [175]. Its downregulation may be a way of sparing iron from other processes within the cell by reducing the amount of iron requiring enzymes. *SufS* however is upregulated, it is a PLP cofactor enzyme involved in iron sulfur cluster generation. Iron sulfur cluster proteins are important for a variety of metabolic functions within the cell including gene regulation and DNA repair. *sufSE* generates iron sulfur clusters, and is induced by perturbations in sulfur metabolism and iron depletion via the fur system, and oxidative stress under the *oxyR* system [176]. Fur uses iron as a corepressor and represses iron uptake gene expression such as siderophores, stopping additional iron uptake when iron is plentiful [177]. Iron storage gene *ftnA* mentioned in the previous section is also regulated by *Fur*, causing its activation when iron is high [159]. As discussed earlier, *ftnA* was downregulated under BMAA treatment conditions, suggesting iron depletion, this would be consistent with *Fur* failing to activate *ftnA* transcription due to low iron. Such conditions would also mean *Fur* is not repressing genes such as *sufS*, hence the upregulation observed

here. This is consistent with changes in sulfur metabolism that occurred in a study with *E. coli* treated with a metal chelator to induce iron starvation [176]. In this study, *sufS* was also upregulated as it is for the BMAA treated cells [176]. In a similar study of *E. coli* treated with iron chelator to induce iron starvation, *sufS* was also upregulated [162]. It is possible that the changes in sulfur related genes are merely an after effect of upstream changes to iron homeostasis induced by iron starvation. But there remains the possibility of a further connection with cysteine biosynthesis, the disruption of which leads to BMAA susceptibility in *E. coli*.

4.3.2.3 Methylglyoxal metabolism, NADPH, and amino acid biosynthesis

The downregulation of *YqhD* and *dkgA* reported here mirrors what happens when cells are stressed by the toxic aldehyde furfural. *YqhD* and *dkgA* are important for dealing with oxidative damage/stress in cells affected by reactive oxygen species (ROS). Such stress generates toxic aldehydes from lipid and carbohydrate oxidation, which can go on to cause protein and DNA damage [178]. Both *yqhD* and *dkgA* convert toxic aldehyde species into less toxic compounds, consuming NADPH in the process [179]. The process of detoxifying aldehydes via these enzymes leads to depletion of NADPH in the cell, causing disruption of amino acid biosynthesis [180]. When NADPH is funnelled into the pathways to remove aldehydes there is insufficient NADPH for essential processes such as amino acid biosynthesis, causing depletions and build-up of intermediates [180]. *cysN* is downregulated, and as mentioned in previous sections, it contributes to cysteine biosynthesis by participating in sulfur acquisition and transformation for use in cysteine biosynthesis. Cysteine is one of the more metabolically expensive amino acids due to the high requirements of NADPH for sulfur assimilation [181]. NADPH limitations therefore would lead to disruptions in amino acid biosynthesis but also sulfur metabolism. Threonine biosynthesis also relies heavily on NADPH availability, possibly explaining *thrL* downregulation due to scarce threonine levels. *thrL* is a leader peptide that attenuates expression of threonine biosynthesis genes [182, 183]. Speed of translation depends on threonine and isoleucine availability; low levels induce expression of the *thrABC* genes [184]. Threonine interconnected with many other amino acids and is degraded into methylglyoxal which

as previously mentioned is detoxified by *YqhD* using NADPH [185]. Downregulation suggests a disruption amino acid metabolism, possibly due to low threonine and/or isoleucine levels or a lack of NADPH.

The changes to amino acid metabolism as indicated by *thrL* and *cysN* transcriptional changes may indicate a depletion of NADPH, and thus downregulation of NADPH expensive processes such as those employed by *yqhD* and *dkgA*. BMAA may be causing oxidative stress to the cells, leading to generation of damaging electrophilic aldehydes which these enzymes then deal with. But the cell cannot upregulate these genes because it would cause further NADPH depletion and therefore perturbations in amino acid biosynthesis with particular impact on cysteine and threonine biosynthesis, hence a downregulation.

4.3.2.4 Slowing of cellular metabolism

lamB and *malM* are involved in carbohydrate transport and belong to the maltose regulon [186, 187]. Both *lamB* and *malM* are downregulated in *E. coli* exposed to BMAA. Typically the transcriptomic profile of *E. coli* under general stress conditions has a pattern of downregulation of central carbon utilization [188]. This is presumably to conserve energy in cells under stressful conditions so growth is slowed, and resources can be funnelled into survival. In a similar pattern, DNA biosynthesis, protein synthesis can also be slowed by cells under stressful conditions. Changes to genes involved in purine and pyrimidine metabolism (*purE, nrdA, xdhB, preA*) reflect this general stress response in *E. coli*, as do changes in ribosomal assembly genes (*rim, trmD, trmH*). This may indicate a general stress response is being stimulated by BMAA or that the previously discussed iron depletion is also causing a generalised stress response. Iron starvation has been shown to cause inhibition of cell division and growth [189]. So, it is unsurprising to see a similar pattern in BMAA treated *E. coli* when suspecting iron chelation effects. Downregulation of *mazF* which is a toxin-antitoxin system component also reflects a general stress response [190]. Toxin antitoxin systems work to degrade RNA and stimulate growth delays and cell death when the toxin component is not balanced by its antitoxin counterpart [191]. Under conditions of stress, toxin

component *mazF* becomes downregulated, presumably to prevent additional cell death under stressful conditions [192]. The downregulation of carbohydrate metabolic genes *lamB* and *malM* may also be connected to the upregulation of *mazF*, which has previously been identified to selectively modulate cellular processes to slow metabolic activity [190]. Studies of iron starvation on other prokaryotes such as *Bacillus subtillis* have also shown similar transcriptional changes in amino acid biosynthesis, purine biosynthesis, and pyrimidine metabolic pathways [193]. Overall, this would suggest that BMAA is causing stress to the cells but is being somewhat managed to the point that large growth defects are not apparent.

4.4 Summary and Conclusions

Tolerance by *E. coli* when challenged by exogenous BMAA is an unusual phenomenon which has yet to be fully understood. Cysteine biosynthesis has been identified as an important process required for this tolerance (Chapters 2 and 3), but the exact connection has been difficult to uncover. Results here have shown that on a genomic level, BMAA is not inducing changes or mutations that render *E. coli* able to grow when BMAA is present. A longer period of exposure to BMAA over multiple generations may have the potential to induce genetic adaptation in *E. coli* and may be a possibility to explore in future studies. However, the tolerance displayed here by wild type *E. coli* and the eventual recovery in growth by cysteine disrupted *E. coli* is unrelated to genetic adaptations in either of these strains. This presents the possibility that *E. coli* are processing BMAA using their current genomic makeup and that while much slower, the cysteine disrupted mutants are still able to utilise the tolerance process and eventually return to normal growth (Chapter 3). The connection between cysteine metabolism in this yet unidentified process, could reveal important aspects about BMAA metabolism in prokaryotic species generally.

On a gene expression level, much more information has been revealed about this process of BMAA tolerance in wild type *E. coli*. It appears that there are other systems being affected by BMAA which have connections to cysteine metabolism including sulfur metabolism and shared energy resources like NADPH. It also provides insight

into the effects BMAA is having on bacterial cells, which until now have remained unexplored. It has been previously reported that BMAA has no ill effects on *E. coli* that are observable via growth monitoring or other methods such as respiration measurements or ROS measurements [67, 68]. The transcriptomic analysis presented here suggests that there are changes occurring in response to BMAA that are indicative of stress response and iron depletion but are evidently being managed well enough by the cells that growth appears unaffected. The possibility of iron chelation by BMAA is interesting as it provides a connection to the neurodegenerative diseases that BMAA has been implicated in the development of. This may provide further evidence to help explain the mechanism behind BMAA related disease and metabolism of BMAA in other species.

Chapter 5 – Investigation into the ability of *E. coli* enzyme O-acetylserine sulfhydrylase to degrade BMAA in vitro

Chapter 5 – Investigation into the ability of *E. coli* enzyme O-acetylserine sulfhydrylase to degrade BMAA *in vitro*

5.1 Introduction

Cyanobacteria are distributed globally and often occur in high numbers in freshwater and marine environments [21]. Increases in frequency of cyanobacterial blooms due to elevated temperature and nutrient loads is contributing to increased levels of cyanotoxins in these environments [92, 194]. The neurotoxic non-protein amino acid BMAA is produced by cyanobacteria, enabling more opportunity for BMAA to be present in our environment when cyanobacterial loads are high [90, 91]. This therefore poses the risk of increased BMAA exposure and potential adverse effects, since exposure to BMAA has been associated with development of neurodegenerative disease such as amyotrophic lateral sclerosis [11, 12]. Past and present research has focused on the mechanism linking BMAA and disease development. Excitotoxicity [23, 24], oxidative stress [195], metal chelation [167, 168], and proteinopathy [25] have been described in mammalian systems treated with BMAA. Unfortunately, how these various mechanisms enable BMAA to contribute to neurodegenerative disease development is still not fully understood.

The conditions which cause production of BMAA and its role in cyanobacterial metabolism have seen more research in recent years [196]. However, the mechanism of BMAA production in these organisms is currently unknown. In addition, other organisms such as diatoms may also contribute to BMAA production suggesting a shared metabolic mechanism for BMAA production between species [85, 93]. Some studies have investigated the response of cyanobacteria exposed to BMAA with the hope of better understanding its role within cells. From these studies it has been shown that BMAA has links to nitrogen metabolism and may inhibit photosynthesis [79, 80, 82]. In addition, BMAA was shown to inhibit growth in certain cyanobacterial strains [82]. There have been attempts to make connections between species and

conditions that allow for BMAA production, but no clear metabolic role for BMAA has been determined, nor specific taxonomic group responsible for production. As toxin-producing organisms often have a strategy for escaping the effects of their own toxin, it would not be surprising to find that BMAA-producing cyanobacteria employ a mechanism to protect themselves from the toxic effects of BMAA. Knowledge of BMAA to date suggests that it is a somewhat reactive molecule that can undergo many changes within physiological systems. Movement of BMAA throughout different compartments of cells and organisms suggest that it is not passive within cells and can participate in metabolic routes [68, 197, 198]. As an amino acid it can participate in cellular pathways which other amino acids are involved in [25]. Despite this knowledge, a mechanism for BMAA metabolism or detoxification particularly in bacteria and cyanobacteria has yet to be discovered.

Investigations have uncovered that certain bacteria such as the common gut microbiota *E. coli*, appear to tolerate BMAA without obvious ill effects that are observed when mammalian cells are treated with BMAA [67]. This phenomenon of BMAA tolerance in *E. coli* has been corroborated by our lab [68]. In addition, screening of BMAA tolerance across a number of gene deletion mutant *E. coli* strains indicated that tolerance is diminished in strains with disruptions to the cysteine biosynthetic pathway (Chapters 2 and 3). This could indicate a connection between tolerance towards BMAA and cysteine biosynthesis in *E. coli*. As mentioned briefly in Chapter 3, cysteine biosynthesis in *E. coli* is conserved across bacteria, plants, and cyanobacteria, while humans use alternative pathways [116-119]. The two-step pathway in *E. coli* involves the enzymes Serine-O-acetyltransferase (SAT) and O-Acetyl-L-serine

sulfhydrylase (OASase) working sequentially to produce cysteine from serine via the intermediate O-acetyl-L-serine (OAS) (Figure 7a).

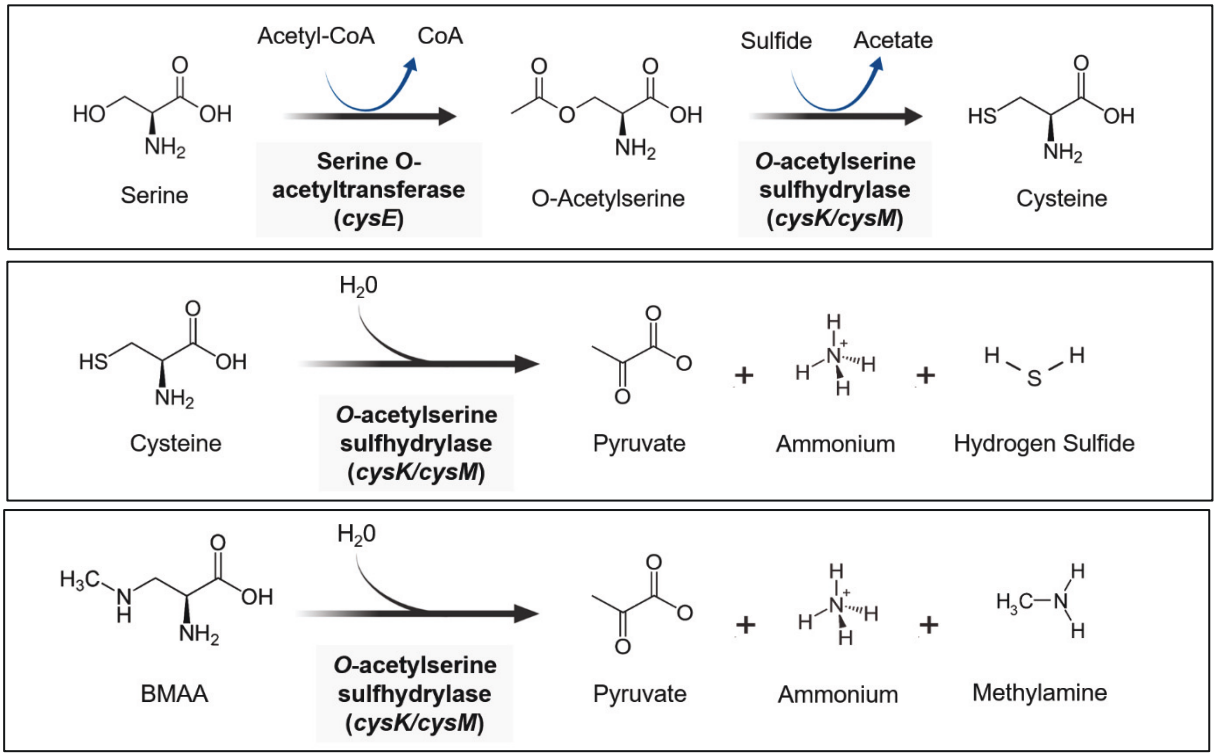


Figure 7. Cysteine biosynthesis and degradation in bacteria and theoretical BMAA degradation A) In *E. coli*, the enzyme SAT (encoded by the *cysE* gene) and the enzymes OASase A (*cysK*) or OASase B (*cysM*) produce the amino acid L-cysteine from precursor amino acid L-serine, with O-acetyl-L-serine being produced as an intermediate. For L-cysteine production, Sulfide acts as a nucleophilic donor during the second step with OASase. B) OASase also participates in cysteine degradation, producing pyruvate, ammonium, and hydrogen sulfide. C) As an amino acid, BMAA may be able to be catalytically converted by OASase into pyruvate, ammonium, and methylamine.

OASase enzymes from the second step of this pathway (OASase A encoded by *cysK* or OASase B encoded by *cysM*) also possesses cysteine desulfhydrase activity allowing for degradation of cysteine into sulphide and 2-aminoprop-2-enoate which spontaneously reacts to form pyruvate [199] (Figure 7b). Due to this degradation ability of OASase it is possible that as an amino acid, BMAA might take the place of cysteine in this reaction and be enzymatically degraded. Such degradation of BMAA would yield methylamine (Figure 7c). Methylamine has been described previously as a degradation product of BMAA in a non-enzymatic reaction with pyridodxal-5'-phosphate (PLP) [131]. However, the conversion of BMAA to methylamine was slow and not complete after 24 hours [131]. In addition, incubation of BMAA with rat liver and kidney preparations yielded methylamine, yet this did not occur for brain homogenates [131]. This presents the possibility of an enzyme present in these tissues which uses PLP to degrade BMAA, resulting in methylamine production. Degradation ability may be more concentrated in the liver and less so in the kidneys but lacking in brain tissue based on the results of this research [131]. It is of interest that the OASase enzyme of *E. coli* uses PLP as an essential cofactor. Coupled with the sensitivity of *E. coli* towards BMAA when genes for OASase enzymes are removed, this is a good indication that OASase should be investigated as a potential detoxifying mechanism for BMAA. Results from Chapter 3 show that deletion of genes from any part of the *E. coli* cysteine biosynthesis pathway is detrimental to growth with BMAA. This would indicate that cysteine biosynthesis overall has an impact on the cell which mediates BMAA tolerance, and this was supported by gene expression analysis showing that cysteine reliant processes in *E. coli* are impacted by BMAA (Chapter 4). However, there was also indication that BMAA-induced growth inhibition could be alleviated by overexpression of OASase enzymes in *E. coli* biosynthesis (Chapter 3). The direct influence of these enzymes on BMAA susceptibility led to the hypothesis that they may be directly involved in BMAA modification or degradation. OASase is conserved across cyanobacteria and bacteria yet absent in mammalian cells [200], and this may explain why mammalian cells appear to suffer the most toxic effects from BMAA. In addition, it has been documented that some cyanobacterial species possess plasmids that carry additional OASase genes, in excess of what would be needed for standard cysteine biosynthesis [201]. Understanding of the interactions BMAA has with prokaryotic enzymes and the

changes it undergoes can lead to information about how to reduce BMAA production in the environment or even lead to bioremediation of BMAA in existing locations. In this study, we aim to isolate OASase from *E. coli* and incubate it with BMAA to determine if it can degrade BMAA into methylamine.

5.2 Methods

5.2.1 Materials

All reagents were analytical grade or cell culture grade and sourced commercially. Amino acids including BMAA were sourced from Sigma-Aldrich (St. Louis, MO, USA). Restriction enzymes used for DNA manipulations were purchased from New England Biolabs. HisTrap HP and HiLoad® 26/600 Superdex® 200 pg were products of GE Healthcare Life Sciences.

5.2.2 Overexpression and purification of OASase

Expression strain BL21 (DE3) was transformed with expression vector plasmid pET28a carrying *cysM* from *E. coli* MG1655. Transformed colonies were inoculated into 40 mL of 2TY media (16 g/L Tryptone, 10 g/L yeast extract, 5 g/L NaCl) with 30µg/mL kanamycin in a 250mL flask. To assist growth and supress expression prior to induction, glucose was included in the culture medium at 1%. Cultures were grown at 37°C with shaking at 150 rpm for 1 hour, these starter cultures were then used to inoculate 1.6 L of additional media in a 5 L flask. Growth continued until an OD_{600} of 0.5 was reached, after which the cultures were cooled for 15 minutes at 18°C. Induction was initiated by the addition of 1 mM IPTG, cultures were induced for 18 hours at 18°C with shaking at 150 rpm. Cells were pelleted at 9300g for 30 minutes at 4°C and stored at -80°C prior to protein purification. All steps involved in the enzyme purification were performed on ice or at 4°C, including chromatography procedures.

Cells were resuspended in 40 mL of lysis buffer (50 mM sodium phosphate buffer; pH 8.0; 300 mM NaCl; 1 mM DTT; 10% glycerol) and disrupted by sonication on ice at 50% power with 30 second bursts followed by 1 minute rest. Lysate was centrifuged at 18,800g for 30 minutes at 4°C. Supernatant was collected and 0.05 mg/mL Pyridoxal-5' phosphate was added to the solution and incubated for 1 hour at 4°C with stirring. The crude protein solution was applied to a nickel affinity chromatography column (HisTrap HP, 5 mL, GE Healthcare Life Sciences) equilibrated with running buffer (50 mM sodium phosphate buffer; pH 8.0; 300 mM NaCl) using an ÄKTA Pure chromatography system. Bound protein was eluted off the column using elution buffer (50 mM sodium

phosphate buffer; pH 8.0; 300 mM NaCl; 1 mM DTT) with an imidazole gradient ranging from 100 mM to 250 mM. Elution was collected in 2 mL fractions and those showing high *cysM* concentration on SDS-PAGE were pooled. Pooled OASase fractions were concentrated with Vivaspin® 20, 10,000 molecular weight cut-off ultrafilters and applied to a HiLoad® 26/600 Superdex® 200 pg size exclusion chromatography column (GE Healthcare Life Sciences) equilibrated with storage buffer (25 mM Tris-HCl; pH 8.0, 300 mM NaCl, 1 mM DTT, 1 mM EDTA). Elutions were collected in 2 mL fractions and those containing OASase were pooled and concentrated to 12 mg/mL via ultrafiltration. Protein concentration was quantified via BCA assay. Concentrated protein was dispensed in aliquots and stored at -80°C for later use in enzyme assays.

5.2.3 Cysteine production assays with OASase

Assays were carried out in 50 mM Sodium Phosphate Buffer pH 7.0; detection of cysteine was carried out via acid ninhydrin method based on the work of Gaitonde [202]. All reagents were prepared fresh on the day of use. O-acetyl-L-serine substrate and sodium sulphide nucleophilic donor were added to the reaction at a final concentration of 2 mM each. The reaction was initiated with the addition of various enzyme concentrations (1 µg/mL, 5 µg/mL, 0.5 µg/mL) and the reaction monitored over 10 minutes at room temperature. The reaction was terminated by the addition of an equal volume of acetic acid to a 50 µL aliquot of reaction. 50 µL of acid ninhydrin solution (125 mg ninhydrin in 3 mL acetic acid and 2 mL HCl) was added to the tube followed by an incubation at 95°C for 10 minutes. Samples were cooled on ice for 2 minutes before being diluted with 95% ethanol. Diluted samples were transferred to a 96-well plate and absorbance at 560 nm was measured within 10 minutes. Samples were blanked against no-enzyme controls and the resulting absorbance was plotted against time for each enzyme concentration. Initial velocity (Vo) was calculated based on absorbance values from a concentration curve of cysteine detected via the same acid ninhydrin method.

5.2.4 BMAA Degradation assays with OASase

Reactions were carried out in 50 mM Sodium Phosphate Buffer pH 7.0 at room temperature. PLP solution was prepared fresh before use. Three reactions were prepared: buffer only control, OASase only, and PLP only, each containing BMAA at a final concentration of 95 µg/mL. OASase and PLP were at a final concentration of 5 µg/mL and 0.1 mM respectively. Samples from the buffer-only control reaction were taken at time zero, 2 hours, and 24 hours. Samples from OASase and PLP reactions were taken at 2 hours and 24 hours. Briefly, samples were diluted in H_2O and filtered with 0.2 µm filters before being derivatised with AccQ Tag Ultra Derivatisation Kit (Waters Corporation). Derivatised samples were kept at room temperature to avoid tag precipitation and analysed by LC-TQMS.

5.2.5 Liquid chromatography triple quadrupole mass spectrometry (LC-TQMS)

Liquid chromatography triple quadrupole mass spectrometry (LC-TQMS) was performed on a Shimadzu Nexra X2 UHPLC coupled to a Shimadzu 8060 triple quadrupole mass spectrometer. Reverse-phase liquid chromatography was employed on a Waters Cortecs C18+ column (2.1×100 mm, 1.6 µm particle size). Flow rate was set to 0.35 mL/min with a column oven temperature of 30°C. Solvent A consisted of ultrapure water + 0.1% Formic acid (FA), solvent B consisted of LC-MS grade methanol (Burdick & Jackson brand LC-MS grade, Chemsupply, Port Adelaide, South Australia, Australia) + 0.1% FA. AQC derivatised BMAA and methylamine were eluted via gradient elution (separation shown in Figure 7), Solvent B conditions were as follows: 0.00 min 11%, 3.00 min 16%, 11.00 min 16%, 11.10 min 30%, 13.00 min 11%, 13.10 min 11%, 15.50 min 11%. The Shimadzu 8060 TQMS with an electrospray ionisation source was run in positive mode with the following source parameters: 2 kV interface voltage, 300°C interface temperature, 250°C desolation line temperature and 400°C heat block, 1.5 L/min nebulising gas flow, 15 L/min heating gas flow, and 5 L/min drying gas flow. Multiple reaction monitoring (MRM) ion transitions were established prior to analysis

for both AQC derivatised BMAA and methylamine (Table 5), each with a dwell time of 30 msec. An 11-point calibration curve was run alongside samples to allow for quantification (0.5 µg/L, 1 µg/L, 5 µg/L, 10 µg/L, 25 µg/L, 50 µg/L, 100 µg/L, 250 µg/L, 500 µg/L, 1000 µg/L, 2000 µg/L). Injection volume was set to 5 uL with no carryover being observed following the highest concentration of standard used (2000 µg/L). Repeatability was determined via a calculation of %RSD of 5 repeat injections from one point in the standard curve with calculated values for both analytes being < 10%. AQC-BMAA was found to have a linear range from 0.5 µg/L to 2000 µg/L, while AQC-methylamine was found to be linear from 0.5 µg/L to 250 µg/L. Limit of detection (LOD) and Limit of quantification (LOQ) was calculated with a signal-to-noise ratio of 3.3 and 10 respectively, with AQC-BMAA having an LOD of 0.036 µg/L and LOQ of 0.110 µg/L, and AQC-methylamine having an LOD of 0.015 µg/L and LOQ of 0.046 µg/L. All data analysis was conducted on Shimadzu LabSolutions.

Table 5. MRM ion transitions for all targeted compounds. *denotes transition used for quantification.

Analyte	Collision energy	MRM transition (m/z)
AQC-BMAA	-26 -17	459.00 → 171.05* → 289.20
AQC-Methylamine	-39	202.00 → 171.05*

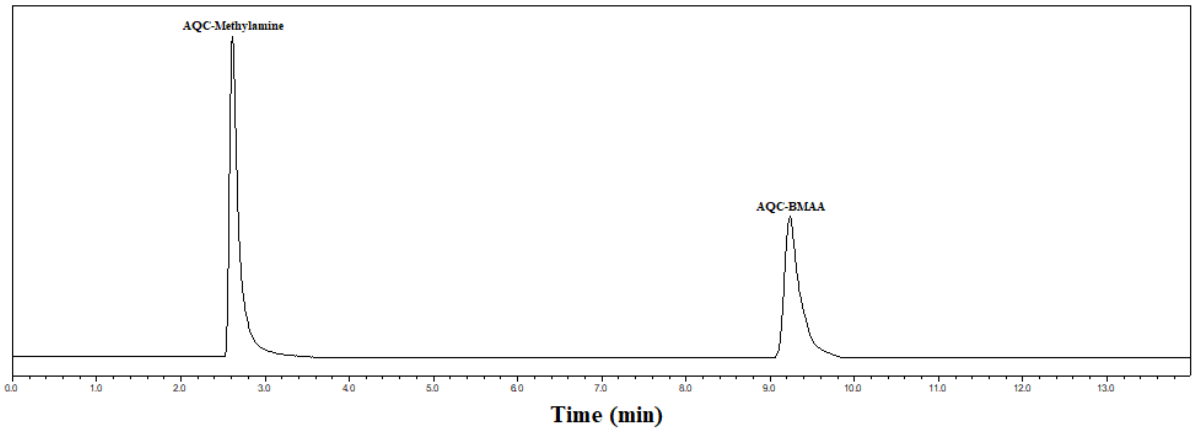


Figure 8. Example total ion chromatogram (TIC) containing both AQC-Methylamine and AQC-BMAA.

5.3 Results

5.3.1 Purification of OASase B

OASase B was purified to homogeneity on SDS-polyacrylamide gel (Figure 9) following nickel affinity and size exclusion chromatography. 1.6L of bacterial culture yielded a total of 132mg of purified protein, approximately half of the maximum yield reported in other studies [117]. A small amount of purified enzyme is present as the dimeric form with a molecular weight of 64KDa, the remaining enzyme is in monomeric form with a corresponding molecular weight of 32KDa (Figure 9). Purification produced a clear yellow solution which it typical of enzymes which use PLP as a cofactor.

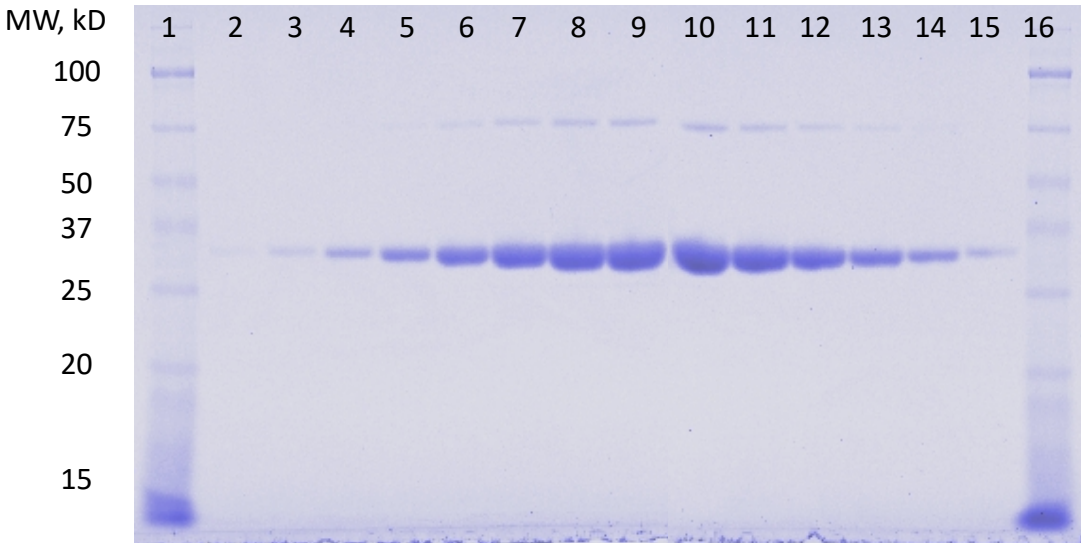


Figure 9. Coomassie stained 4-12% polyacrylamide gel of eluted fractions following size exclusion chromatography of *cysM*. Lanes 1 and 16 have molecular weight ladder, lanes 2 to 15 contain eluted fractions.

5.3.2 Cysteine production assays with *cysM* (OASase B)

Incubation of purified OASase B with precursor reaction components OAS and sulfide produced detectable levels of cysteine via acid ninhydrin (Figure 10). To promote the reaction OAS and sulfide were both provided at saturating concentrations of 2 mM each. Cysteine concentration was measured between 2 and 10 minutes and was found to increase over time, with maximum cysteine production of 5.5 mM. The enzyme concentration displaying linear kinetics over this period was 0.5 µg/mL, which corresponds to a Vo of 102.7 µM cysteine per minute at 25 degrees. Levels of cysteine for higher concentrations of enzyme tested plateaued within the 10-minute time period but were approximately three times higher with 2 µg/mL OASase.

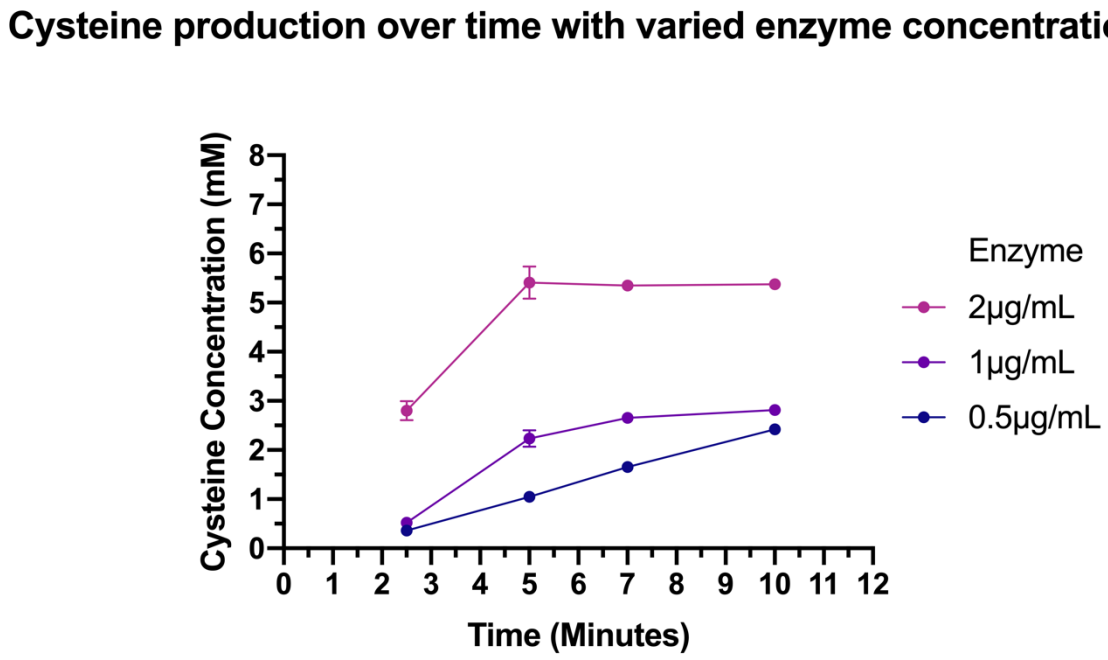


Figure 10. Cysteine production over time with varied enzyme concentration.

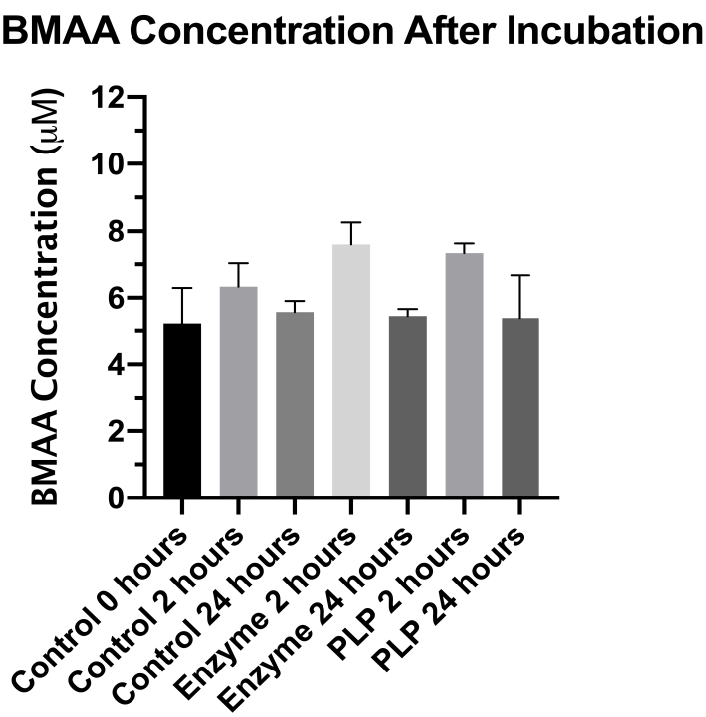
Purified OASase enzyme was incubated with OAS and sulfide and samples were taken over a 10-minute time period. Concentration of cysteine at each time point was determined via acid ninhydrin detection and a cysteine concentration curve. N=3

5.3.3 BMAA degradation assays with *cysM* (OASase B)

As the suspected breakdown product of BMAA, methylamine levels were examined across several time points for samples containing BMAA. Levels of methylamine appeared stable at low background levels across all samples as expected, the exception to this is in PLP samples (Figure 11b) At both 2 hours incubation and 24 hours, the levels of methylamine in PLP containing samples spiked above the baseline levels, with the highest amount detected after 24 hours (Figure 11b). This is in direct contrast to samples without additional PLP, including OASase incubations, as OASase does not appear to have produced any noticeable increases in methylamine levels after 2- or 24-hours incubation.

Levels of BMAA in the same samples indicate that across the time course the amount of BMAA within no-enzyme and no-PLP controls was maintained at a similar amount (Figure 11a). Samples incubated with PLP for 24 hours appear to have less BMAA than at time zero, reflecting the large increase in methylamine seen from those same samples. However, the amount of BMAA in all these samples is large and any conversion of BMAA into methylamine may not be efficient or significant enough to make a noticeable change in the levels of BMAA enough to be detected accurately, resulting in the inconsistent of BMAA levels detected via the method used. A future approach to address this could include repetition of the experiment with $^{15}N_2{}^{13}C_3$-BMAA where the radiolabels may be detected and tracked.

a)



b)

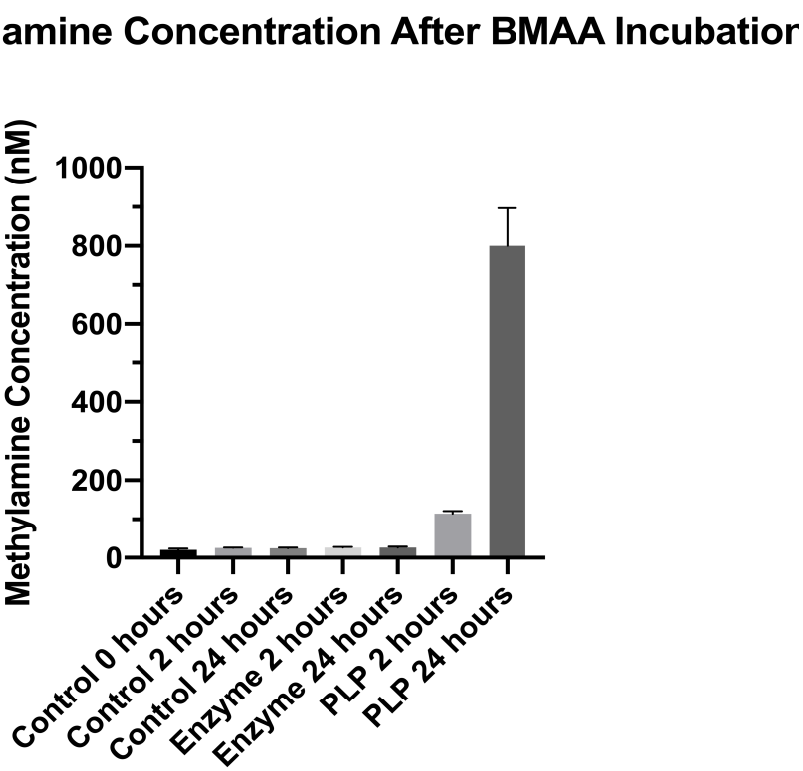


Figure 11. Concentrations of BMAA and Methylamine before and after incubation with OASase enzyme. BMAA was incubated with OASase for 24 hours at room temperature. (a) Levels of BMAA at 0, 2, and 24 hours of enzyme incubation. (b) levels of methylamine in the same samples. (n=4)

5.4 Discussion

As the growing problem of increased cyanobacterial blooms leads to greater contamination of the environment with cyanotoxins, it is prudent to investigate phenomena which may aid in the reduction and/or removal of these toxins. Previous research indicated the PLP-dependent enzyme OASase of *E. coli* may have potential as a detoxifying tool for BMAA (Chapter 3). The isolation procedure of bacterial OASase proved successful with a correct molecular weight characteristic of this enzyme [117, 203] (Figure 9). Additionally, the isolated OASase enzyme proved functional as it produced cysteine when given the precursor components OAS and sulfide (Figure 10) that was consistent with previous reports [117, 118, 203, 204]. However, when considering degradation ability of BMAA by OASase, incubation under the same conditions did not appear to catalyse the degradation reaction of BMAA into methylamine. OASase failed to produce any noticeable change in levels of the proposed substrate BMAA, nor did it produce detectable methylamine by-product (Figure 11). This suggests that despite the evidence for the importance of this enzyme in BMAA tolerance in *E. coli*, it does not appear to act directly on BMAA to degrade it into methylamine, at least not at a level that would be physiologically relevant or could explain the requirement of this gene for growth in BMAA exposed cells (Chapters 2 and 3). There is a possibility that the enzyme does indeed participate in BMAA degradation using the cofactor, but the reaction is not favourable, and we are unable to see conversion of small amounts using the current detection methods. The more likely scenario arising from these results, is that the importance of this enzyme lies with its downstream effects on cell metabolism, which is negatively impacted when it is removed from the genome and subsequently impairs the ability of *E. coli* to tolerate BMAA, as described in Chapter 4.

However, the PLP cofactor of this enzyme did produce an increase in methylamine by-product when incubated with BMAA under the same conditions and in the absence of enzyme (Figure 11b). This supports previous work of Nunn who described the same phenomenon when BMAA is incubated with PLP [131]. As PLP is a cofactor for many enzymes aside from OASase, it may be possible that an enzyme does exist that degrades BMAA but has not yet been identified as participating in this reaction. There

are also many homologues of OASase that differ slightly between bacterial species [205, 206], archaea [207], plants [208], and cyanobacteria [209]. The redundancy of cysteine synthesis enzymes and the presence of multiple isoforms means some may have a greater propensity for BMAA degradation. In addition, isoforms such as *cysM* found in *Salmonella typhimurium* and *E. coli* are suspected to be involved in moonlighting activities [122]. OASase enzymes have been implicated in resistance to oxidative stress inducting toxins such as potassium tellurite [210] and chromate [211]. While research into the alternative roles of OASase enzymes is continuing, it is likely there are many yet unidentified functions these enzymes participate in. However, the connections already made to their requirement in toxin tolerance and resistance to oxidative stress as described in the previous studies makes them interesting targets of BMAA detoxification research. *cysM* is of particular interest as unlike OASase encoded by *cysK*, *cysM* OAsase does not form a complex with SAT to produce cysteine and only contributes 2-20% of cysteine producing ability within the cell [121]. Additionally, *cysM* has a larger active site and accepts bigger substrates than *cysK* including a variety of nucleophilic donors [212, 213]. Curiously, the wider range of nucleophilic donors accepted by *cysM* has made it the subject of several studies which employ *cysM* to produce non-protein amino acids including mimosine and β-(Pyrazol-1-yl)-L-alanine [116-118]. As such, if degradation of BMAA was to take place via one of these enzymes, OASase B would be the more likely candidate due to both is wider substrate specificity and suspected moonlighting activities. OASase is not present in mammals and thus has been the subject of research for the development of new antibiotics [214, 215]. This makes it a unique target in bacterial species as its absence in humans means its inhibition will not be detrimental in mammalian systems. Due to the relevance of this enzyme in bacteria, it may be of interest to investigate homologous enzymes in other species for ability to degrade BMAA and compare it to the results seen here with OASase B. This includes other OASase enzymes from species such as cyanobacteria, which may be more likely to be tailored to BMAA acceptance due to the presence of BMAA in cyanobacterial metabolism.

Degradation of BMAA via PLP in could prove to be an important aspect of protection against BMAA. As the active form of vitamin B6, PLP is a widespread cofactor participating in many essential cellular reactions, particularly amino acid metabolism [216-218]. PLP forms a Schiff base linkage to a specific lysine residue within the active site of PLP-dependent enzymes forming an internal aldimine. Upon the entrance of substrate (usually an amino acid), PLP forms a new Schiff base linkage to the incoming substrate to form an external aldimine linkage. The reaction then diverges based on the type of enzyme involved and may continue into a diverse range of reactions including transamination, racemization, decarboxylation, or elimination/substitution reactions [219, 220]. The type of reaction is influenced by the enzyme in question, however there is often opportunity for alternate reactions to occur with PLP and these side reactions may be prevalent enough to be physiologically relevant [220]. As PLP is a very reactive molecule it is unsurprising that it is known to catalyse reactions alone, albeit at a slower rate than enzyme catalysed reactions [221]. Reactivity of PLP with amino acids in the absence of an enzyme counterpart is markedly slow in comparison to enzyme catalyzed reactions [221]. PLP-dependent enzymes account for 1.5% of prokaryotic genes [222] and 4% of all enzyme activities [223] making PLP one of the most diverse cofactors used across all forms of life. PLP-dependent enzymes are grouped into five categories determined by their fold type with OASase belonging to fold type II [221]. With the large diversity of PLP-dependent enzymes across multiple fold change groups, species, and domains of life, there is the possibility that one or more of these enzymes is suitable for degradation of BMAA. Variations in sequences and active sties of enzymes dictates their propensity for side reactions which may favour BMAA degradation. As an amino acid, BMAA is suitable substrate for PLP mediated reactions. Likely candidates for BMAA receptive enzymes would be found in cyanobacteria, the producers of BMAA and plants due to their high number of PLP-dependent enzymes relative to bacteria and mammals [223].

While degradation via the cysteine biosynthesis enzymes of *E. coli* may not necessarily be the reason why *E. coli* devoid of this enzyme are susceptible to BMAA, its ties to sulfur metabolism, oxidative stress management, protein production and amino acid metabolism may be the link that is important to further research (Chapter 4). So, while

this enzyme may not be physically and directly responsible for BMAA tolerance in *E. coli*, it may affect some downstream tolerance mechanism which is yet to be explored. Further research may yet uncover this mechanism which would be useful in understanding the metabolism of BMAA and how to combat its toxic effects. In addition, the multitude of similar enzymes which rely on PLP as their cofactor means there is a possibility of an enzyme which does participate in BMAA degradation, possibly as a side reaction divergent from the main reaction the enzyme is used for. The conversion of BMAA into methylamine via PLP could represent an important aspect of BMAA metabolism within cells and should be explored further. Analysis of genes encoding PLP-dependent enzymes should be explored in BMAA producing cyanobacteria, as there may be potential candidate for BMAA degradation in this family of enzymes.

Chapter 6 – General Discussion and Future Directions

Chapter 6 – General Discussion and Future Directions

6.1 General Discussion

Since its discovery as the suspected causative agent of ALS-PDC on Guam, BMAA has received increasing amounts of interest relating to its connection with development of neurodegenerative disease. Neurodegenerative diseases have profound impacts on individuals and society and research into the factors that contribute to their development becomes increasingly important as our population ages [224]. As neurodegenerative disease is terminal and without a cure, prevention is the most ideal goal to strive for. A number of different environmental risk factors have been examined over time, and interest in BMAA as an environmental factor came about due to its links to ALS-PDC on Guam [40]. While on Guam researchers were able to track the content of BMAA throughout the food web of the island [47], BMAA exposure in other parts of the world seemed unlikely outside of these specific circumstances. When researchers discovered that BMAA was produced by cyanobacteria [45], this exposed a previously unidentified route of exposure for populations globally. Cyanobacteria inhabit extremely diverse ecosystems ranging from aquatic to terrestrial environments [225]. And overpopulation of waterways with cyanobacterial growth can occur from increased nutrient loads due to wastewater runoff and increased temperatures [226]. The characteristic 'blooms' of cyanobacteria that occur under these conditions are not limited to small water bodies and can cover large areas of oceans and lakes which are visible via satellite [227]. Increased cyanobacterial blooms are already a concern and with global temperatures expected to keep increasing, exposure to cyanobacteria and their toxins will occur more frequently [92, 194].

Investigations into BMAA have focused largely on its effects in mammalian cells, particularly neuronal-like cells to gauge the potential mechanisms behind neurodegeneration. As such there is a wealth of studies focusing on this topic that has revealed mechanisms such as excitotoxicity [228, 229], oxidative stress [22], mitochondrial disfunction [230], and effects on protein/amino acid metabolism [25,

26]. A number of animal studies have also been used with varying success (com comprehensively reviewed by Karamyan and Speth [231]). Less information is available on the effects BMAA has on prokaryotes, such as bacteria and cyanobacteria. A number of studies have shown that BMAA interferes with growth, nitrogen metabolism, and amino acid metabolism on cyanobacterial species [78, 79, 81-83]. However, it is yet to be discovered the purpose of BMAA and its role in these microorganisms. Other NPAAs such as *meta*-tyrosine and canavanine are used by the organisms that produce them for defence purposes and to compete for survival [17, 232]. While other NPAAs such as L-DOPA are simply by-products of common metabolic pathways [233, 234]. The purpose of BMAA remains unclear, but its ability to interact with and influence cellular processes, particularly in prokaryotes is an interesting avenue of exploration. The production pathway for BMAA has yet to be confirmed and more data is suggesting that cyanobacteria are not the only ones capable of BMAA production [20]. There has been some interest into the environmental conditions which promote cyanobacterial BMAA production [235], but to date it is still not possible to predict which species of cyanobacteria will produce BMAA and under what conditions. In other bacterial species, BMAA appears to have no obvious effects on growth or respiration [67]. Considering that producers of BMAA appear to suffer growth inhibitory effects and metabolic disruptions due to the presence of BMAA, the lack of susceptibility of these bacterial species is unusual. As cyanobacteria were one of the earliest groups to branch out in early life on earth, they share common pathways with both bacteria and plants, with much overlap occurring [236]. Bacterial mechanisms and metabolic pathways are often homologues on cyanobacteria. As such, it is possible that understanding of how BMAA effects bacteria on a basic level may begin the groundwork for future understanding of cyanobacterial metabolism of BMAA. As it is still uncertain what conditions are required for BMAA production, it is also possible that some bacterial species may also be capable of BMAA synthesis but have not been identified. As the systems and techniques to grow and genetically manipulate a common lab bacterium such as *E. coli* are much faster and simpler to carry out than in cyanobacteria, using *E. coli* as a model for BMAA exposure experiments has potential to gather much needed knowledge of bacterial responses to BMAA which may be translatable. However, while *E. coli* provide an excellent model

for investigation, they lack the photosynthetic ability that cyanobacteria possess which may also be important for BMAA metabolism.

The experiments in this thesis aimed to understand some basic responses that are occurring in *E. coli* exposed to BMAA and identify potential tolerance mechanisms. From previous research of *E. coli* treated with BMAA two unique responses were observed. Firstly, the lack of growth inhibition upon BMAA exposure [67], and secondly, the association of BMAA with protein of live *E. coli* which spiked at one hour of treatment then declined over 8 hours [68]. The lack of growth inhibition or death suggests a tolerance or defence mechanism against BMAA, and protein association that changes over time suggests that *E. coli* are undertaking some active process to rid themselves of BMAA. These interesting possibilities led to the experiments covered in Chapter 2, where a range of *E. coli* mutants were exposed to BMAA to observe growth patterns. The identification of the cysteine biosynthesis pathway in *E. coli* for BMAA tolerance built on the previous knowledge of bacterial responses to BMAA and gave rise to a new opening for further research. Chapter 3 expanded upon this information about cysteine biosynthesis in *E. coli*. Through deletion and complementation studies with plasmids, it was shown that all three genes in the cysteine biosynthesis pathway impacted in BMAA tolerance. However, the mechanism of how tolerance was occurring from the presence of these genes was unknown. Simply replacing the genes via plasmid induction of the deleted gene was not effective in restoring growth to full tolerance levels, meaning there may have been a more complex mechanisms at work. Two main possibilities arose from this information, giving rise to the experiments of Chapter 4 and Chapter 5 respectively.

Due to the inability of plasmid borne cysteine biosynthesis genes to fully restore BMAA tolerance in *E. coli*, and the variable time taken by strains to recover normal growth following BMAA induced “lag”. The possibility of suppressor mutations rising from BMAA exposure was considered. In Chapter 4 genome sequencing of *E. coli* exposed to BMAA for variable durations of time, including a recovery period without BMAA showed that no mutations arose due to the presence of BMAA, nor the cysteine biosynthesis gene deletion. This meant that like the results of our lab previously which

showed *E. coli* seeming to remove BMAA from protein association [68], a metabolic effect was likely taking place which could explain the variability in lag time upon BMAA exposure. If *E. coli* required time to mobilise different resources and cellular machinery to combat BMAA this could lead to a lag duration of minimal growth while cellular metabolism was adjusted to deal with BMAA, this could even include the use of BMAA as a substrate in other cellular activities. Such as response would likely result in changes on a gene expression level, and as such the remainder of Chapter 4 details the transcriptomic investigation onto the changes that occur in *E. coli* exposed to BMAA. Many interesting findings came from this data including the indication that iron depletion was occurring in the BMAA treated *E. coli*, a finding which has not previously been discovered. BMAA has been reported to chelate metals, most notably copper and zinc ions [165, 167]. Iron could be of great importance especially considering the significance of iron in prokaryotic species survival [172]. Metals generally are considered to have important links concerning development of neurogenerative disease [237] and this discovery could be an important aspect to consider in further research of BMAA. In addition, general cellular stress responses were observed which is in contrast with the lack of growth inhibition seen when *E. coli* are exposed to BMAA. These changes on a transcriptional level indicate that the cells were experiencing some stress due to the presence of BMAA, yet growth remained unaffected. This may indicate that the mechanism by which *E. coli* tolerate or process BMAA is efficient enough to not impede growth, but still causes some stress to the cell. Amino acid metabolism and sulfur metabolism were also altered during BMAA exposure, this is consistent with reports in cyanobacteria where proteomic investigations took place with similar perturbations in amino acid metabolism [83]. BMAA has also been shown to interfere with amino acid metabolism in mammalian cells [76], meaning this may be a shared mechanism across all species and not limited to prokaryotes or eukaryotes exclusively. Chapter 4 represents the first data on genomic and transcriptomic changes to *E. coli* in the presence of BMAA and offers interesting insights into the internal events occurring in these BMAA-tolerant organisms.

The alternative hypothesis which arose from the results of Chapter 2 and 3 was that a specific enzyme involved in the cysteine biosynthesis was directly involved in BMAA tolerance. Chapter 5 covers the investigation into OASase, a PLP-dependent enzyme and the second step of cysteine biosynthesis, encoded by *cysK* and *cysM* in *E. coli* and conserved across plant, bacterial, and cyanobacterial species. Of the three enzymes involved in cysteine biosynthesis in *E. coli* serine acetyltransferase (SAT) is the first step of biosynthesis and is encoded by the gene *cysE*. SAT catalyses the reaction of serine with acetyl-CoA to form the intermediate o-acetyl-serine. The SAT enzyme was first looked at as a candidate for BMAA transformation as BMAA has been shown to mimic serine during eukaryotic protein synthesis and therefore it was possible that BMAA would be a substrate for this enzyme [25]. A noteworthy study from an earlier period of BMAA research found that injection of BMAA into rats resulted in excretion of acetylated BMAA [238]. The enzyme responsible for acetylation of BMAA in rats was not identified, but it is perhaps possible that within *E. coli* SAT may be involved in BMAA acetylation. However, the structural observation of this potential reaction meant that it was unlikely to occur and instead focus turned to the enzymes of the second step in cysteine biosynthesis, OASase. OASase is a PLP-dependent enzyme and has been used previously in other areas of NPAA research to produce a variety of synthetic and naturally occurring NPAAs [116-120, 239]. Its cysteine desulfurase activity allows it to degrade cysteine yielding pyruvate, ammonium, and sulphide. Due to the importance of cysteine biosynthesis pathway in BMAA tolerance, it was hypothesised that OASase from *E. coli* either encoded by *cysM* or *cysK* could potentially degrade BMAA yielding methylamine. As OASase enzymes contain a PLP cofactor, this boosted support for the hypothesis that PLP acts as means of degrading BMAA into methylamine [131]. Furthermore, as an NPAA, BMAA has the potential to mimic other amino acids, so the possibility of it misplacing cysteine in this OASase catalysed reaction was investigated. OASase B (encoded by *cysM*) is known to have a larger and more accepting active site [212, 213]. It also has a less defined role in the cell, often participating in moonlighting functions apart from cysteine biosynthesis [122]. Due to these factors, it was chosen as the ideal candidate to test for BMAA degradation. Isolation of OASase yielded pure and functional enzyme, able to produce cysteine. However, incubation of the enzyme with BMAA failed to produce a

methylamine by-product, suggesting that this enzyme does not participate in BMAA degradation. However, the cofactor PLP did produce methylamine when incubated with BMAA in enzyme-free controls. This agrees with previous findings of BMAA degradation via PLP and suggests that there is indeed an enzyme or enzymes either in bacteria or other species which are PLP-dependent and capable for catalysing this reaction. Identification of such an enzyme is an interesting aspect of BMAA research going forward.

With the increasing need for mechanistic information about BMAA, further studies in bacteria and cyanobacteria are warranted. Understanding of how BMAA interacts with these organisms has the potential to explain overlapping mechanisms that also apply to mammalian systems, but also can reveal important aspects unique to bacterial species. As cyanobacteria are the main BMAA producers identified to date, a further understanding of bacterial interaction with BMAA could yield essential information such as tolerance and detoxification mechanisms, or even production methods. Knowledge of these aspects of BMAA chemistry could lead to better environmental control of BMAA as a preventive measure for BMAA exposure. Overall, the components of this thesis have investigated the responses of *E. coli* to BMAA and investigated multiple possibilities to explain these responses. This work has explored different options for *E. coli* metabolism in BMAA, and in doing so has opened future opportunities for further research.

6.2 Future directions

While this project covered many different areas of BMAA research in *E. coli* there were limitations to the depth of studies that could be done and the follow up investigations that could be conducted. Future research could build in this current data and probe further into different mechanisms. For example, it is likely there are other PLP-dependent enzymes that would work to degrade BMAA. However, screening and identifying candidates is a potentially large project, which should cover not only bacterial and cyanobacterial enzymes, but also those of plants which have a high abundance of PLP-dependent enzymes [223]. As OASase enzymes have been found in

cyanobacterial plasmids beyond what is required for cysteine biosynthesis [201], there may be a purpose for these enzymes related to BMAA, especially as these enzymes are known for having alternative roles [122]. An approach which analyses the OASase homologues in various cyanobacterial strains may be able to identify candidates which have a greater likelihood of interacting with BMAA. Screening could also consider whether particular strains are known producers of BMAA. This could lead to the isolation of select enzymes to be tested for BMAA interaction and perhaps identification of PLP-dependent enzymes in cyanobacteria which modify BMAA. As research into cyanobacterial metabolism of BMAA is increasingly important, insights such as these could open new routes of cyanobacterial research.

It is particularly interesting that PLP-dependent OASase enzymes are used to synthesise NPAAs in higher plants which naturally produce NPAAs [239]. In fact, by supplying alternative nucleophilic donors to corresponding bacterial OASase enzymes, researchers have synthetically produced many NPAAs successfully [117, 118]. While BMAA production has not been attempted in these studies, the use of methylamine as a nucleophilic donor for BMAA production with OASase is an interesting possibility to explore. While it is beyond the scope of this particular project, investigation into the NPAA production capabilities of OASase enzymes in regard to BMAA specifically may be worthwhile. Additionally, the production of plant derived NPAAs by equivalent bacterial enzymes highlights the conserved nature of these enzymes across plants and bacteria. The implication being that if production occurs via OASase enzymes in one species, it may represent a universal pathway of production across other species, as OASase enzymes have homologs across many forms of life. This may also explain why other organisms are increasingly being identified as BMAA producers, including diatoms, dinoflagellates, and plants, as they all share related OASase enzymes [20, 84, 85, 240].

Additionally, the metabolism of BMAA in bacteria is complex and metabolically driven. An important follow up study for the transcriptomic changes would be deeper level analysis, such as metabolomics and proteomics. In addition, analysis of multiple strains and BMAA exposure conditions would reveal more information about the bacterial response to BMAA, which was unable to be conducted in this project due to time and

resource constraints. There are already similarities that can be drawn between cyanobacterial response to BMAA and the results of the transcriptomic investigation from Chapter 4. This includes changes to carbon and amino acid metabolism but most notably, changes to cysteine metabolism in cyanobacteria treated with BMAA [83]. In addition, proteomic and metabolomic investigations could serve as a comparison to similar studies conducted in mammalian cells (see [76, 241]), possibly revealing important overlapping aspects of BMAA metabolism across differing forms of life.

Lastly, transcriptomic investigations conducted as part of this project have revealed an important aspect of BMAA chemistry which deserves further attention. Iron chelation abilities of BMAA are suggested to explain the iron starvation response displayed by *E. coli* treated with BMAA. Prior to this investigation it was unlikely that research would focus on iron starvation in BMAA exposed bacteria, but now there is potential for future investigation. Due to the essential nature of iron in bacterial metabolism and the importance of trace metals in the aetiology of neurodegenerative disease, this is an aspect of BMAA chemistry that should be the focus of continued research. For example, future projects may choose to focus on the trace metal content within cells exposed to BMAA and to investigate how disruptions in iron homeostasis systems may affect susceptibility of cells to BMAA.

In summary, the work presented here has shed light on previously unexplored aspects of BMAA metabolism in bacteria and represents an alternate focus of BMAA research which has received less attention. Many new investigative opportunities have arisen from the data presented here, with the potential to instigate new research into the complex toxin that is BMAA.

Chapter 7 - References

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