© 2021. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/
The definitive publisher version is available online at https://doi.org/10.1016/j.resmic.2021.103852

1

2

3 # Cysteine biosynthesis contributes to β-methylamino-

4 # L-alanine tolerance in *Escherichia coli*

5

6 Carly J. Italiano[a], Lisa Pu[a], Jake P. Violi[a], Iain G. Duggin[b], Kenneth J. Rodgers [a]*

7

8 [a]School of Life Sciences, University of Technology Sydney, Ultimo, NSW 2007,

9 Australia

10 [b]The iThree institute, University of Technology Sydney, Ultimo, NSW 2007, Australia

11

12 Kenneth.Rodgers@uts.edu.au *Correspondence and reprints

13 Carly.J.Italiano@student.uts.edu.au

14 Lisa.Pu@student.uts.edu.au

15 Jake.Violi@uts.edu.au

16 Iain.Duggin@uts.edu.au

17

18 ## Abstract

19 In contrast to mammalian cells, bacteria such as *E. coli* have been shown to display
20 tolerance towards the neurotoxin β-methylamino-L-alanine (BMAA) suggesting that
21 these prokaryotes possess a way to metabolise BMAA or its products, resulting in their
22 export, degradation, or detoxification. Single gene deletion mutants of *E. coli* K-12 with
23 inactivated amino acid biosynthesis pathways were treated with 500 μg/ml BMAA
24 and the resulting growth was monitored. Wild type *E. coli* and most of the gene
25 deletion mutants displayed unaltered growth in the presence of BMAA over 12 hours.
26 Conversely, deletion of genes in the cysteine biosynthesis pathway, *cysE*, *cysK* or *cysM*
27 resulted in a BMAA dose-dependent growth delay in minimal medium. Through
28 further studies of the Δ*cysE* strain, we observed increased susceptibility to oxidative
29 stress from H_2O_2 in minimal medium, and disruptions in glutathione levels and
30 oxidation state. The cysteine biosynthesis pathway is therefore linked to the tolerance
31 of BMAA and oxidative stress in *E. coli*, which potentially represents a mechanism of
32 BMAA detoxification.

33

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

36

37 ## 1. Introduction

38 The non-protein amino acid (NPAA), β-methylamino-L-alanine (BMAA) has been
39 implicated as an environmental neurotoxin. Exposure has been associated with
40 development of neurodegenerative disease such as amyotrophic lateral sclerosis
41 (ALS) [1, 2]. Mechanisms behind the toxic effects of BMAA that could contribute to
42 neuronal damage include oxidative stress [3], excitotoxicity [4, 5], and proteotoxic
43 stress [6]. The toxicity of BMAA has been observed across various models including
44 mammalian cell lines [5, 7], rodents [8, 9], and primate models [1, 2].

45 BMAA is known to be produced by cyanobacteria; ubiquitous photosynthetic
46 microorganisms that inhabit a diverse range of aquatic and terrestrial environments
47 [10, 11]. This presents a growing public health concern, as the global problem of
48 increased cyanobacterial blooms brings with it the risk of increased levels of
49 cyanotoxin production, including BMAA [12]. So far, researchers have been unable to
50 identify a common taxonomic group or geographical characteristic shared by BMAA-
51 producing species of cyanobacteria [13]. The conditions which promote cyanobacterial
52 production of BMAA have seen more research in recent years, though the mechanism
53 of production is unidentified [14]. Other aquatic organisms such as diatoms also
54 appear to be capable of BMAA production [15, 16]. However, it is still unknown how
55 BMAA is produced and how it is metabolized by different species. Due to the many
56 potential routes of exposure and its putative links to fatal neurodegenerative disease,
57 it is imperative that the metabolism of BMAA be explored.

58 Despite being producers of BMAA, cyanobacterial responses to BMAA have
59 included growth defects and changes to nitrogen and amino acid metabolism [17-21].
60 To date, only two studies in bacteria other than cyanobacteria have been published,
61 both showing common gut microorganisms, including *Escherichia coli*, appear to
62 tolerate BMAA without obvious ill effects that are observed in cyanobacteria and
63 mammalian cells [22, 23]. With BMAA exerting toxic effects across many species
64 including those that produce it, the observation that *E. coli* is seemingly unaffected by
65 BMAA toxicity makes its response prudent to investigate. The mechanism of tolerance
66 or effects that BMAA may have on *E. coli* could reveal important information that can
67 be used to understand how BMAA is produced, processed, or detoxified in
68 prokaryotic species generally, including cyanobacteria.

69 As many NPAAs structurally resemble protein amino acids, this allows them to
70 act as antimetabolites and displace or mimic canonical amino acids in physiological
71 systems (reviewed in [24]). Various cellular pathways that involve amino acids may
72 be influenced by the presence of NPAAs, including protein synthesis, and amino acid
73 synthesis and metabolism [25]. This enables NPAAs to exert different kinds of toxic
74 effects on the organism exposed. However, due to its links to human disease BMAA
75 research has understandably focused on mammalian systems, with prokaryotic
76 systems receiving less study. Prokaryotic systems have previously been used to reveal
77 amino-acid mimicking ability and protein misincorporation as a mechanism of action
78 for other NPAAs including azetidine-2-carboxylic acid [26, 27], canavanine [27], and
79 *meta*-tyrosine [28]. BMAA influences amino acid metabolism in other cell models [29]

80 and might have additional effects on cells that are not typical of many standard-
81 amino-acid mimicking NPAAs.

82 This study aimed to investigate the effects of BMAA on *E. coli*, to begin to
83 understand the mechanism of tolerance. Auxotrophic strains that each have one
84 amino acid biosynthetic pathway inactivated were used to identify any essential
85 interactions between BMAA and the biosynthesis of specific protein amino acids. As
86 toxic NPAAs tend to mimic specific canonical amino acids, identification of
87 interactions between BMAA and certain pathways may give insight into the complex
88 effects and metabolism of BMAA in prokaryotic cells. Additionally, as BMAA can
89 cause toxicity via oxidative stress mechanisms [3], investigations continued into the
90 oxidative defenses of *E. coli* and susceptibility of strains to oxidative stress.
91

92 ## 2. Materials and methods

93 *2.1 Materials*

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

97 *2.2 Bacterial strains*

98 *E. coli* strains from the Keio collection were obtained from the Coli Genetic Stock
99 Centre (CGSC) and derived from parent strain BW25113 [30]. Strains and their
100 relevant phenotypes are listed in Table 1. Strains were stored at -80 °C in 16% glycerol
101 and samples were revived on LB agar medium as needed. An auxotrophic strain for
102 alanine was not available as this deletion is non-viable. In addition, deletions to create
103 auxotrophy for glycine, asparagine, aspartic acid, and glutamic acid could not be
104 grown sufficiently in minimal medium, despite amino acid supplementation, and
105 these strains were excluded from the screening study.

106 *2.3 Growth screening of amino acid auxotrophic strains with BMAA*

107 Strains were streaked onto LB agar and incubated for 18 hours at 37°C, single
108 colonies were then inoculated into 3 ml of M9 minimal medium with glucose as the
109 carbon source [31]. 10 μg/ml uracil and a trace-elements solution were included in
110 minimal medium [32]. Auxotrophic strains were supplemented with 100 μg/ml of
111 their corresponding required amino acid. *E. coli* were initially cultured for 16-18 hours
112 at 37°C with shaking at 150 rpm. These starter cultures were then used to inoculate 10

113 ml of fresh M9 medium of the same composition to an OD_{600} of 0.05 and growth was
114 continued until exponential phase (OD_{600} 0.5-0.6) before cells were washed in M9
115 medium lacking amino acids. Cells were then re-suspended to OD_{600} 0.1 in M9
116 medium with 100 μg/ml of required amino acid for each auxotrophic strain or no
117 amino acids for the wild type. These cultures were transferred to 96-well plates with
118 BMAA (500 μg/ml final concentration), whereas controls received the same volume of
119 10 mM HCl. Washed cultures not supplemented with amino acids served as negative
120 controls and confirmed the auxotrophy of the strains. *E. coli* were grown at 37 °C with
121 constant shaking in a microplate reader (PowerWave HT Microplate
122 Spectrophotometer, BioTek), with absorbance measurements taken at 30-minute
123 intervals (at 600 nm) for 12 hours. Final absorbance measurements were appropriately
124 blanked to correct for background absorbance and the data were used to generate
125 growth curves for all cultures. For growth of the cysteine deletion strain JW3582-2
126 with varied BMAA concentrations, the same procedure was followed but for 18 hours
127 total growth time and with concentrations of BMAA at 25, 50, 100, 200, and 400 μg/ml.

128 *2.4 Growth of the wild-type and cysteine biosynthesis gene deletion strains with BMAA*

129 For experiments comparing the response of wild type *E. coli* and cysteine deletion
130 strain JW3582-2 to 500 μg/ml of BMAA in rich and poor medium, M9 minimal and LB
131 media were employed. Strains were grown and treated with BMAA as described
132 above, with either M9 or LB used throughout growth and treatment. For experiments
133 comparing susceptibility of various cysteine biosynthesis gene deletions, strains
134 JW3582-2, JW2407-1, and JW2414-3, with deletions in *cysE*, *cysK* and *cysM*,

135 respectively, were prepared as described in section 2.3 and grown for 24 hours with
136 500 μg/ml BMAA.

137 *2.5 Hydrogen peroxide (H_2O_2) treatment of wild type and cysteine auxotrophic E. coli*

138 Wild-type *E. coli* and the cysteine auxotrophic strain JW3582-2 were grown to
139 exponential phase as described in section 2.3. Strain JW3582-2 was supplied with 100
140 μg/ml cysteine throughout. Exponentially growing cultures were diluted to OD_{600} of
141 0.1 in additional medium pre-warmed to 37 °C. H_2O_2 was added at a final
142 concentration of either 5 mM or 25 mM, and then incubated at 37°C for 15 minutes. 1
143 ml of cells were washed in 30 ml of PBS and pelleted at 4000 x g for 15 minutes. Pellets
144 were re-suspended in 1 ml PBS and serially diluted before spotting onto LB agar
145 plates. Agar plates were then incubated at 37°C for 18 hours and subsequent colonies
146 counted and used to calculate CFU/ml of original culture.

147 *2.6 Glutathione analysis of wild type and cysteine auxotrophic E. coli*

148 Wild-type *E. coli* and the cysteine auxotrophic strain JW3582-2 were grown to
149 exponential phase as described previously in 30 ml of nutrient poor M9 minimal or
150 nutrient rich LB medium. Cultures were centrifuged at 4000 x g for 15 minutes and
151 washed in 30 ml of PBS before further centrifugation. Pellets were stored at -80°C prior
152 to glutathione analysis. During sample preparation, pellets were reconstituted in 3 ml
153 of 80% ice cold methanol and probe sonicated twice on ice. Samples were then
154 centrifuged at 3900 x g for 10 minutes and the supernatant transferred into new tubes
155 for glutathione analysis while the pellet was kept for protein quantification. 2 ml of

156 0.1% Triton X-100 was added to the pellet and vortexed before performing the
157 bicinchoninic acid assay (BCA) for protein quantification [33].

158 *2.7 Glutathione analysis by LC-MS/MS*

159 Glutathione reduced (GSH) and oxidised (GSSG) were analysed using the Agilent
160 Technologies 6490 triple quadrupole mass spectrometer (TQMS) coupled with an
161 Agilent 1290 Infinity UHPLC. Liquid chromatography was performed using a Waters
162 Cortecs® UPLC C18 column (150 x 2.1 mm, 1.6 μM particle size, 90 Å pore size).
163 Solvent A consisted of ultrapure water + 0.1% (v/v) formic acid (FA) with solvent B
164 consisting of LC-MS grade methanol + 0.1 % FA (v/v). GSH and GSSG were
165 chromatographically separated and eluted via gradient elution (Fig. 6c). Flow rate was
166 set to 0.25 ml/min with the column temperature being maintained at 20°C. Solvent B
167 conditions were as follows: 0.0 min 0%, 2.0 min 0%, 2.1 min 50%, 5.0 min 50%, 5.1 min
168 100%, 7.0 min 100%, 7.1 min 0%, 9.5 min 0%.

169 The TQMS was operated with an electrospray ionisation (ESI) source in positive
170 mode. The operating settings for the TQMS were as follows: 250°C drying gas
171 temperature at 14 l/min, 250°C sheath gas temperature at 11 l/min, 20 psi nebuliser
172 pressure, 3000 V capillary voltage and 1500 V nozzle voltage. Retention time and
173 multiple reactions monitoring (MRM) ion transitions were used to identify GSH and
174 GSSG (Table 2). Each MRM transiton had a dwell time of 200 ms. Quanitification of
175 GSH was conducted on the most abundant transition (308 m/z > 75.9 m/z). In-source
176 fragmentation was observed for GSSG, resulting in its most abundant ion transiton to
177 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.

3. Results

3.1 Analysis of the growth of E. coli amino acid auxotrophs in the presence of BMAA

To explore potential requirement for individual amino acid biosynthetic pathways for BMAA tolerance in *E. coli*, growth studies of single-gene knockout strains exposed to BMAA were undertaken. The final optical density (OD_{600}) of BMAA-exposed cultures after 12 hours growth was compared to that of untreated control cultures and a relative percentage of growth was calculated. BMAA was added to a final concentration of 500 μg/ml (4.23 mM), giving a ratio of NPAA to the auxotrophic protein amino acid of around 5:1. Wild type *E. coli* treated with BMAA attained the same density of growth as BMAA-free controls (Fig. 1). Much like the wild-type strain, most of the gene knockout mutants also showed no or minimal growth inhibition in media containing BMAA. The exception to this pattern was the cysteine auxotrophic strain, which carries a deletion in the gene *cysE*, encoding serine O-acetyltransferase for the first step in cysteine biosynthesis. This strain failed to grow during the 12-hour exposure to BMAA (Fig. 1).

Growth of the wild type and *cysE* mutant during BMAA exposure was then compared over time in both nutrient rich and nutrient poor media. Growth with BMAA over time was uninhibited and comparable to BMAA-free controls when strains were in nutrient rich conditions (Fig. 2cd). In nutrient poor conditions, represented by M9 minimal medium, wild type growth remained unaffected by the presence of BMAA (Fig 2a). In contrast, cultures of the Δ*cysE* mutant, which has a non-

218 functional cysteine biosynthesis pathway, failed to produce any noticeable change in
219 optical density during the 12-hour exposure to BMAA in nutrient poor conditions, yet
220 grew well in BMAA-free medium (Fig. 2b).

221 *3.2 Characteristics of growth inhibition by BMAA in cysteine biosynthesis mutants*

222 We next sought to determine a BMAA minimal inhibitory concentration by
223 monitoring Δ*cysE* cultures containing 25 µg/ml to 400 µg/ml BMAA. However, we
224 observed a dose-dependent lag in the growth of cultures exposed to BMAA (Fig. 3),
225 rather than complete inhibition of growth that was apparent at the 12-hour time-point
226 with 500 µM BMAA (Fig. 2b). At all concentrations of BMAA tested, the *E. coli* cultures
227 reached similar optical densities to control cultures by 18 hours (Fig. 3). Additional
228 cysteine (up to 1.6 mM) was unable to reverse the inhibitory effect of BMAA on Δ*cysE*
229 (data not shown). Therefore, instead of indefinite inhibition of growth, or death of the
230 whole culture, the pattern was one of a dose-dependent growth lag preceding a return
231 to normal growth rates and final yield.

232 The response of other cysteine biosynthesis mutants to BMAA was observed
233 alongside strain Δ*cysE,* to understand whether BMAA tolerance is linked to *cysE* alone
234 or required the entire cysteine biosynthesis pathway. Strains with deletions of *cysK* or
235 *cysM,* which encode O-acetylserine sulfhydrylase enzymes for the second and final
236 step of L-cysteine biosynthesis, were grown for 24 hours in minimal medium with 500
237 µg/ml BMAA. The Δ*cysK* and Δ*cysM* strains also displayed the pattern of growth lag
238 as seen for Δ*cysE* upon BMAA exposure (Fig. 4bc); cultures eventually recovered
239 normal growth rates and final yields. However, with these extended time courses, we

240 observed that the duration of the growth lag was variable in replicate cultures of all
241 three mutants, with lag durations of up to 15 hours (Fig. 4). Δ*cysE* strains collected
242 after growth in 500 μg/ml BMAA exhibited similar lag characteristics when subjected
243 to a second round of growth and BMAA treatment (data not shown), indicating that
244 the lag pattern was not caused by the appearance of genetically acquired BMAA
245 resistance during the initial exposure. Overall, the dose-dependent growth
246 characteristics of the cysteine biosynthesis mutants in minimal medium suggest that
247 these cells require time to metabolize BMAA or its products.

248 *3.3 Sensitivity of E. coli ΔcysE to oxidative stress*

249 Due to the BMAA susceptibility characteristics of Δ*cysE*, coupled with the links
250 between BMAA and oxidative stress [3], it was of interest to compare the tolerance of
251 the wild-type and Δ*cysE* strains to oxidative stress. With cysteine synthesis being
252 important for cellular redox reactions and maintaining cellular antioxidants [34, 35],
253 disruption of cysteine biosynthesis in Δ*cysE* could result in a weakening of oxidative
254 tolerance. The wild-type and Δ*cysE* strains were treated with 5 mM and 25 mM H_2O_2.,
255 a well-known inducer of oxidative stress, and resulting viability (CFU/mL) was
256 determined (Fig. 5). Wild type *E. coli* showed somewhat decreased viability with 25
257 mM H_2O_2, however the Δ*cysE* strain displayed greater reductions in viability at both
258 25 mM and 5 mM H_2O_2 (Fig. 5), indicating that Δ*cysE* causes hypersensitivity to
259 oxidative stress.

260 As glutathione is one of the major cellular antioxidants influenced by intracellular
261 cysteine availability, the total glutathione contents, and ratio of reduced (GSH) and

262 oxidised (GSSG) forms were examined in the wild-type and Δ*cysE* strains. As seen in
263 Fig. 6a, wild-type cells had significantly more total glutathione than the Δ*cysE* in rich
264 medium, whereas in nutrient poor media there was a similar lower level of total
265 glutathione in both strains. The relative amounts of reduced (GSH) and oxidised
266 (GSSG) glutathione were then determined using HPLC separation and mass
267 spectrometry identification and levels quantified against commercial standards. The
268 ratio of reduced to oxidised glutathione was lower in Δ*cysE* compared to wild type,
269 and this was consistent in both rich and poor medium (Fig. 6b).

270 **4. Discussion**

271 We initially observed that high level BMAA exposure (4.24 mM) did not inhibit
272 growth of wild type *E. coli*, consistent with previous work showing exposure to BMAA
273 (100 μM) resulted in no inhibition in growth over an 8-hour time period [22]. The
274 general tolerance of *E. coli* to BMAA suggests that it does not share the amino acid
275 mimicry mechanism of toxicity observed with other NPAAs such as azetidine-2-
276 carboxyllic acid and canavanine. Typically, inhibition of growth upon exposure to a
277 protein amino acid mimicking NPAA is relieved by addition of the competing 'parent'
278 amino acid [27, 36]. With the Δ*cysE* strain, which is defective in cysteine biosynthesis
279 [30], we observed a substantial delay of growth with BMAA, despite the presence of
280 100 μg/mL cysteine in minimal media. BMAA toxicity is well documented in
281 mammalian cell models [1, 5, 7, 29, 37], where competition with L-serine [6] and
282 interference with amino acid metabolism [29] have been suggested. Even
283 cyanobacteria, which are thought to be the main producers of BMAA, can still
284 experience growth inhibition and perturbations in metabolism [19, 21]. The lack of
285 BMAA toxicity in *E. coli* is at odds with the toxicity seen in mammalian and
286 cyanobacterial systems. The tolerance of *E. coli* towards BMAA raises the possibility
287 of a defense or detoxification mechanism possessed by *E. coli* that could be absent in
288 BMAA-susceptible mammalian cells.

289 A potential means to understand BMAA tolerance in *E. coli* lies within the
290 response we observed in cysteine auxotrophic strains. The substantial delays in

291 growth we observed for the cysteine auxotrophs upon exposure to 500 μg/ml BMAA
292 in minimal medium was in contrast to the relatively uninhibited growth of the other
293 amino acid auxotrophs. This suggests that cysteine biosynthesis, or its downstream
294 effects on the cell, are important for tolerance towards BMAA in *E. coli*. As the main
295 route of sulfur assimilation in cells, cysteine biosynthesis is central to many
296 downstream activities essential for cell survival [38]. Free cysteine can directly
297 contribute to the protection of cells from oxidative damage via oxidation to cystine,
298 which can be reduced back to cysteine, providing a reservoir of antioxidant activity
299 [39-42]. In addition, it is a precursor for several other important antioxidants such as
300 glutathione. As such, a lack of cysteine can result in reduced levels of a range of
301 antioxidants and subsequently an increased susceptibility to oxidative damage across
302 many species [43-45],including the susceptibility of *E. coli* to external stressors,
303 antibiotics and toxins [34, 35, 46].

304 Total glutathione levels for wild type and Δ*cysE* strains in minimal medium were
305 similar (Fig. 6a), likely due to the nutrient scarce nature of minimal medium.
306 Compared to poor medium, wild type *E. coli* generated higher levels of glutathione
307 when grown in rich medium. Yet, the cysteine disrupted mutant did not produce
308 comparable levels of glutathione (Fig. 6a), suggesting that the lack of intracellular
309 cysteine biosynthesis in Δ*cysE* limits the production of the higher levels of glutathione
310 produced in rich medium. Additionally, the ratio of reduced to oxidized glutathione
311 was lower for the *cysE* lacking strain (Fig. 6b), suggesting that the cysteine
312 biosynthesis mutant experiences more oxidative stress. Oxidative stress has

313 previously been shown to be a component of BMAA toxicity, with mechanisms
314 identified such as inhibition of cystine uptake in neuronal cells resulting in reduced
315 glutathione [3, 47]. Oxidative stress and influence on cysteine metabolism perhaps
316 explains why a lack of cysteine biosynthesis could render the strain more susceptible
317 to BMAA. This was supported by results showing that the Δ*cysE* strain is more
318 susceptible to oxidative stress (H_2O_2) than wild type *E. coli* (Fig. 5). This could indicate
319 that the normal defenses against oxidation are sufficient to deal with BMAA in wild
320 type *E. coli,* but impairment by disruption of cysteine metabolism weakens these
321 defenses. This is supported by a previous study showing cellular respiration was not
322 decreased, nor was there an increase in reactive oxygen species generation upon 100
323 μM BMAA exposure in wild type *E. coli* [22].

324 In apparent contrast to the data presented here, previous work has indicated that
325 bacterial cysteine mutants were more successful at surviving brief exposure to H_2O_2
326 than wild type [48, 49]. However, the differing results may be explained by the
327 increased exposure time used here (15 minutes) or our use of minimal medium as
328 opposed to the rich medium used previously. Our BMAA results indicate that *E. coli*
329 is only sensitive to BMAA in minimal medium and when impaired for cysteine
330 biosynthesis (with knockouts of either *cysE, cysK* or *cysM*). Thus, the correspondence
331 between sensitivity to H_2O_2 and BMAA suggests that *E. coli* is more sensitive in
332 minimal medium to the oxidative stress expected to be present in these conditions.

333 It is also clear from our data that the inhibition of growth BMAA caused for the
334 cysteine disrupted strains is not permanent. There was a BMAA concentration

335 dependence in growth lag time, but maximum growth rate or final growth yield were
336 unaffected (Fig. 3, Fig 4). A possibility to explain this growth pattern is that BMAA is
337 metabolised using a process that may take some time for the *E. coli* to establish and
338 complete; for example, via neutralization of oxidative damage and the export and/or
339 prevention of further uptake of BMAA or its products.

340 The observed link between BMAA and the enzymes in the cysteine biosynthesis
341 pathway of *E. coli* also presents alternative possibilities for BMAA detoxification. In *E.*
342 *coli*, cysteine is synthesized by two sequential enzymes: serine O-acetyltransferase
343 (SAT; EC 2.3.1.30) encoded by *cysE*, and O-acetylserine sulfhydrylase (OASase; EC
344 4.2.99.8), encoded by the partially redundant *cysK* or *cysM* genes. The pyridoxal 5'-
345 phosphate-dependent OASase enzymes encoded by *cysK* and *cysM* have previously
346 been linked to NPAA metabolism, including enzymatic production of the toxic
347 NPAAs mimosine and β-Pyrazol-1-yl-L-alanine [50-52]. It is possible that the cysteine
348 biosynthesis enzymes help overcome BMAA by directly metabolizing it or its possible
349 products, rather than support the downstream antioxidant effects of cysteine
350 described above.

351 This is the first study to examine the effects of BMAA on a range of auxotrophic
352 *E. coli* strains and it presents new evidence from which to design future investigations.
353 Investigations into cyanobacteria have revealed that they are the likely source of
354 BMAA production, yet how they tolerate its toxic effects is not known. It has been
355 shown that BMAA can inhibit growth of certain cyanobacterial strains [19, 20]. It is
356 therefore interesting that *E. coli* is so tolerant of BMAA. As cyanobacteria share many

357 similarities to other prokaryotic species such as *E. coli*, further exploration of BMAA
358 metabolism in a range of bacteria may provide insights into mechanisms of toxicity
359 and detoxification. For example, it has already been documented that cyanobacteria
360 carry additional copies of cysteine biosynthesis enzymes homologous to the *cys* genes
361 of *E. coli,* in excess of what is required for cysteine production [53]. Exploration in this
362 area could pave the way for the discovery of metabolic processes in cyanobacteria that
363 involve BMAA, including mechanisms of production and protection. Such knowledge
364 would present the opportunity to develop methods for combating BMAA production
365 in these species and prevent BMAA release into the environment.
366

367 **Funding:** This research was funded by MND Australia grant number PRO-15-
368 0352.
369 C.J.I, L.P, and J.P.V are recipients of the Australian Government Research Training
370 Program Stipend, and I.G.D. was supported by an Australian Research Council Future
371 Fellowship (FT160100010).
372 **Conflicts of Interest:** The authors declare no conflict of interest.
373

374 ## 5. References

375 1. Spencer PS, Nunn PB, Hugon J, Ludolph AC, Ross SM, Roy DN, and Robertson
376 RC, *Guam amyotrophic lateral sclerosis-parkinsonism-dementia linked to a plant*
377 *excitant neurotoxin.* Science 1987;**237**(4814):517-22.
378 2. Cox PA, Davis DA, Mash DC, Metcalf JS, and Banack SA, *Dietary exposure to an*
379 *environmental toxin triggers neurofibrillary tangles and amyloid deposits in the brain.*
380 Proc Biol Sci 2016;**283**(1823).
381 3. Liu X, Rush T, Zapata J, and Lobner D, *beta-N-methylamino-l-alanine induces*
382 *oxidative stress and glutamate release through action on system Xc(-).* Exp Neurol
383 2009;**217**(2):429-33.
384 4. Rao SD, Banack SA, Cox PA, and Weiss JH, *BMAA selectively injures motor*
385 *neurons via AMPA/kainate receptor activation.* Exp Neurol 2006;**201**(1):244-52.
386 5. Chiu AS, Gehringer MM, Braidy N, Guillemin GJ, Welch JH, and Neilan BA,
387 *Excitotoxic potential of the cyanotoxin beta-methyl-amino-L-alanine (BMAA) in*
388 *primary human neurons.* Toxicon 2012;**60**(6):1159-65.
389 6. Dunlop RA, Cox PA, Banack SA, and Rodgers KJ, *The non-protein amino acid*
390 *BMAA is misincorporated into human proteins in place of L-serine causing protein*
391 *misfolding and aggregation.* Plos One 2013;**8**(9):e75376.
392 7. Liu XQ, Rush T, Ciske J, and Lobner D, *Selective death of cholinergic neurons*
393 *induced by beta-methylamino-L-alanine.* Neuroreport 2010;**21**(1):55-8.
394 8. Seawright AA, Brown AW, Nolan CC, and Cavanagh JB, *Selective degeneration*
395 *of cerebellar cortical neurons caused by cycad neurotoxin, L-beta-methylaminoalanine*
396 *(L-BMAA), in rats.* Neuropathol Appl Neurobiol 1990;**16**(2):153-69.
397 9. Wilson JM, Khabazian I, Wong MC, Seyedalikhani A, Bains JS, Pasqualotto BA,
398 Williams DE, Andersen RJ, Simpson RJ, Smith R, Craig UK, Kurland LT, and
399 Shaw CA, *Behavioral and neurological correlates of ALS-parkinsonism dementia*
400 *complex in adult mice fed washed cycad flour.* Neuromolecular Med 2002;**1**(3):207-
401 21.

402 10. Hitzfeld BC, Lampert CS, Spaeth N, Mountfort D, Kaspar H, and Dietrich DR,
403 *Toxin production in cyanobacterial mats from ponds on the McMurdo ice shelf,*
404 *Antarctica.* Toxicon 2000;**38**(12):1731-48.
405 11. Craighead D, Metcalf JS, Banack SA, Amgalan L, Reynolds HV, and Batmunkh
406 M, *Presence of the neurotoxic amino acids beta-N-methylamino-L-alanine (BMAA)*
407 *and 2,4-diamino-butyric acid (DAB) in shallow springs from the Gobi Desert.*
408 Amyotroph Lateral Scler 2009;**10 Suppl 2**:96-100.
409 12. Paerl HW and Huisman J, *Climate change: a catalyst for global expansion of harmful*
410 *cyanobacterial blooms.* Environ Microbiol Rep 2009;**1**(1):27-37.
411 13. Cox PA, Banack SA, Murch SJ, Rasmussen U, Tien G, Bidigare RR, Metcalf JS,
412 Morrison LF, Codd GA, and Bergman B, *Diverse taxa of cyanobacteria produce*
413 *beta-N-methylamino-L-alanine, a neurotoxic amino acid.* Proc Natl Acad Sci U S A
414 2005;**102**(14):5074-8.
415 14. Downing S and Downing TG, *The metabolism of the non-proteinogenic amino acid*
416 *beta-N-methylamino-L-alanine (BMAA) in the cyanobacterium Synechocystis*
417 *PCC6803.* Toxicon 2016;**115**:41-8.
418 15. Violi J, Mitrovic S, Colville A, Main B, and J Rodgers K, *Prevalence of β-*
419 *methylamino-L-alanine (BMAA) and its isomers in freshwater cyanobacteria isolated*
420 *from eastern Australia*. Vol. 172. 2019. 72-81.
421 16. Jiang L, Eriksson J, Lage S, Jonasson S, Shams S, Mehine M, Ilag LL, and
422 Rasmussen U, *Diatoms: a novel source for the neurotoxin BMAA in aquatic*
423 *environments.* Plos One 2014;**9**(1):e84578.
424 17. Downing TG, Phelan RR, and Downing S, *A potential physiological role for*
425 *cyanotoxins in cyanobacteria of arid environments.* Journal of Arid Environments
426 2015;**112**:147-51.
427 18. Scott LL, Downing S, Phelan RR, and Downing TG, *Environmental modulation of*
428 *microcystin and beta-N-methylamino-L-alanine as a function of nitrogen availability.*
429 Toxicon 2014;**87**:1-5.

430 19. Downing S, van de Venter M, and Downing TG, *The effect of exogenous beta-N-*
431 *methylamino-L: -alanine on the growth of Synechocystis PCC6803.* Microb Ecol
432 2012;**63**(1):149-56.
433 20. Berntzon L, Erasmie S, Celepli N, Eriksson J, Rasmussen U, and Bergman B,
434 *BMAA inhibits nitrogen fixation in the cyanobacterium Nostoc sp. PCC 7120.* Mar
435 Drugs 2013;**11**(8):3091-108.
436 21. Koksharova OA, Butenko IO, Pobeguts OV, Safronova NA, and Govorun VM,
437 *The First Proteomics Study of Nostoc sp. PCC 7120 Exposed to Cyanotoxin BMAA*
438 *under Nitrogen Starvation.* Toxins (Basel) 2020;**12**(5):310.
439 22. van Onselen R, Cook NA, Phelan RR, and Downing TG, *Bacteria do not*
440 *incorporate beta-N-methylamino-L-alanine into their proteins.* Toxicon 2015;**102**:55-
441 61.
442 23. Main BJ, Italiano CJ, and Rodgers KJ, *Investigation of the interaction of beta-*
443 *methylamino-L-alanine with eukaryotic and prokaryotic proteins.* Amino Acids
444 2018;**50**(3-4):397-407.
445 24. Rodgers KJ and Shiozawa N, *Misincorporation of amino acid analogues into*
446 *proteins by biosynthesis.* Int J Biochem Cell Biol 2008;**40**(8):1452-66.
447 25. Fowden L, Lewis D, and Tristram H, *Toxic Amino Acids: Their Action as*
448 *Antimetabolites*, in *Advances in Enzymology and Related Areas of Molecular Biology*.
449 1967, John Wiley & Sons, Inc. p. 89-163.
450 26. Fowden L and Richmond MH, *Replacement of proline by azetidine-2-carboxylic acid*
451 *during biosynthesis of protein.* Biochim Biophys Acta 1963;**71**:459-61.
452 27. Schachtele CF and Rogers P, *Canavanine death in Escherichia coli.* J Mol Biol
453 1965;**14**(2):474-89.
454 28. Rodgers KJ, Wang H, Fu S, and Dean RT, *Biosynthetic incorporation of oxidized*
455 *amino acids into proteins and their cellular proteolysis.* Free Radical Biology and
456 Medicine 2002;**32**(8):766-75.
457 29. Engskog MK, Ersson L, Haglof J, Arvidsson T, Pettersson C, and Brittebo E,
458 *beta-N-Methylamino-L-alanine (BMAA) perturbs alanine, aspartate and glutamate*

metabolism pathways in human neuroblastoma cells as determined by metabolic profiling. Amino Acids 2017;**49**(5):905-19.

30. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, and Mori H, *Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection.* Mol Syst Biol 2006;**2**:2006 0008.
31. Sambrook J, Fritsch EF, and Maniatis T, *Molecular cloning: A Laboratory Manual*. 2nd ed. Vol. 3. 1989, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
32. Duggin IG, Aylett CH, Walsh JC, Michie KA, Wang Q, Turnbull L, Dawson EM, Harry EJ, Whitchurch CB, Amos LA, and Lowe J, *CetZ tubulin-like proteins control archaeal cell shape.* Nature 2015;**519**(7543):362-5.
33. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, and Klenk DC, *Measurement of protein using bicinchoninic acid.* Analytical Biochemistry 1985;**150**(1):76-85.
34. Mironov A, Seregina T, Nagornykh M, Luhachack LG, Korolkova N, Lopes LE, Kotova V, Zavilgelsky G, Shakulov R, Shatalin K, and Nudler E, *Mechanism of H₂S-mediated protection against oxidative stress in Escherichia coli.* Proceedings of the National Academy of Sciences 2017;**114**(23):6022-27.
35. Shatalin K, Shatalina E, Mironov A, and Nudler E, *H₂S: A Universal Defense Against Antibiotics in Bacteria.* Science 2011;**334**(6058):986-90.
36. Grant MM, Brown AS, Corwin LM, Troxler RF, and Franzblau C, *Effect of L-azetidine 2-carboxylic acid on growth and proline metabolism in Escherichia coli.* Biochim Biophys Acta 1975;**404**(2):180-7.
37. D'Mello F, Braidy N, Marcal H, Guillemin G, Rossi F, Chinian M, Laurent D, Teo C, and Neilan BA, *Cytotoxic Effects of Environmental Toxins on Human Glial Cells.* Neurotox Res 2017;**31**(2):245-58.
38. Guédon E and Martin-Verstraete I, *Cysteine Metabolism and Its Regulation in Bacteria*, in *Amino Acid Biosynthesis ~ Pathways, Regulation and Metabolic*

488 *Engineering*, V.F. Wendisch, Editor. 2007, Springer Berlin Heidelberg: Berlin,
489 Heidelberg. p. 195-218.
490 39. Agarwal SM, Jain R, Bhattacharya A, and Azam A, *Inhibitors of Escherichia coli*
491 *serine acetyltransferase block proliferation of Entamoeba histolytica trophozoites.* Int J
492 Parasitol 2008;**38**(2):137-41.
493 40. Kumar S, Mazumder M, Dharavath S, and Gourinath S, *Single residue mutation*
494 *in active site of serine acetyltransferase isoform 3 from Entamoeba histolytica assists in*
495 *partial regaining of feedback inhibition by cysteine.* Plos One 2013;**8**(2):e55932.
496 41. Ohtsu I, Wiriyathanawudhiwong N, Morigasaki S, Nakatani T, Kadokura H,
497 and Takagi H, *The L-cysteine/L-cystine shuttle system provides reducing equivalents*
498 *to the periplasm in Escherichia coli.* J Biol Chem 2010;**285**(23):17479-87.
499 42. Ohtsu I, Kawano Y, Suzuki M, Morigasaki S, Saiki K, Yamazaki S, Nonaka G,
500 and Takagi H, *Uptake of L-cystine via an ABC transporter contributes defense of*
501 *oxidative stress in the L-cystine export-dependent manner in Escherichia coli.* Plos
502 One 2015;**10**(3):e0120619.
503 43. Vergauwen B, Pauwels F, and Van Beeumen JJ, *Glutathione and catalase provide*
504 *overlapping defenses for protection against respiration-generated hydrogen peroxide in*
505 *Haemophilus influenzae.* J Bacteriol 2003;**185**(18):5555-62.
506 44. Arkblad EL, Tuck S, Pestov NB, Dmitriev RI, Kostina MB, Stenvall J, Tranberg
507 M, and Rydstrom J, *A Caenorhabditis elegans mutant lacking functional*
508 *nicotinamide nucleotide transhydrogenase displays increased sensitivity to oxidative*
509 *stress.* Free Radic Biol Med 2005;**38**(11):1518-25.
510 45. Blaszczyk A, Brodzik R, and Sirko A, *Increased resistance to oxidative stress in*
511 *transgenic tobacco plants overexpressing bacterial serine acetyltransferase.* Plant J
512 1999;**20**(2):237-43.
513 46. Apontoweil P and Berends W, *Isolation and initial characterization of glutathione-*
514 *deficient mutants of Escherichia coli K 12.* Biochim Biophys Acta 1975;**399**(1):10-22.

515 47. Esterhuizen-Londt M, Wiegand C, and Downing TG, *beta-N-methylamino-L-alanine (BMAA) uptake by the animal model, Daphnia magna and subsequent oxidative stress.* Toxicon 2015;**100**:20-6.

518 48. Turnbull AL and Surette MG, *L-Cysteine is required for induced antibiotic resistance in actively swarming Salmonella enterica serovar Typhimurium.* Microbiology-Sgm 2008;**154**:3410-19.

521 49. Turnbull AL and Surette MG, *Cysteine biosynthesis, oxidative stress and antibiotic resistance in Salmonella typhimurium.* Research in Microbiology 2010;**161**(8):643-50.

524 50. Mino K, Yamanoue T, Ohno K, Sakiyama T, Eisaki N, Matsuyama A, and Nakanishi K, *Production of beta-(pyrazol-1-yl)-L-alanine from L-serine and pyrazol using recombinant Escherichia coli cells expressing serine acetyltransferase and O-acetylserine sulfhydrylase-A.* Biotechnology Letters 2001;**23**(24):2051-55.

528 51. Zhao C, Kumada Y, Imanaka H, Imamura K, and Nakanishi K, *Cloning, overexpression, purification, and characterization of O-acetylserine sulfhydrylase-B from Escherichia coli.* Protein Expr Purif 2006;**47**(2):607-13.

531 52. Zhao C, Ohno K, Sogoh K, Imamura K, Sakiyama T, and Nakanishi K, *Production of nonproteinaceous amino acids using recombinant Escherichia coli cells expressing cysteine synthase and related enzymes with or without the secretion of O-Acetyl-L-Serine.* Journal of Bioscience and Bioengineering 2004;**97**(5):322-28.

535 53. Nicholson ML, Gaasenbeek M, and Laudenbach DE, *Two enzymes together capable of cysteine biosynthesis are encoded on a cyanobacterial plasmid.* Mol Gen Genet 1995;**247**(5):623-32.

538 ## 6. Legends to figures

539 **Figure 1. Knockout the *E. coli cysE* gene inhibits tolerance to BMAA.** Cultures
540 of wild-type *E. coli* BW25113 and amino-acid biosynthetic gene knockout strains were
541 incubated for 12 hours at 37°C with shaking in M9-minimal medium with or without

542 500 μg/ml BMAA. Auxotrophy of the knockout strains was alleviated by
543 supplementation with 100 μg/ml of the required amino acid. Maximum growth yield
544 (OD_{600}) is plotted as a percentage of growth relative to the untreated control. Error
545 bars represent standard deviation from triplicate cultures.

546 **Figure 2. Effect of BMAA on the growth of wild type *E. coli* and cysteine**
547 **deletion mutant Δ*cysE* in nutrient rich and nutrient poor media**. *E. coli* were treated
548 with 500 μg/mL BMAA (grey) or untreated control (black) for 12 hours in nutrient rich
549 LB medium or nutrient poor M9 minimal medium. (a) Wild type *E. coli* treated with
550 BMAA in M9 medium. (b) *cysE* deletion strain treated with BMAA M9 medium
551 supplemented with 100 μg/ml L-cysteine. (c) Wild type *E. coli* treated with BMAA in
552 LB medium. (d) *cysE* deletion strain treated with BMAA in LB medium. Figures are
553 representative of curves generated from triplicate cultures.

554 **Figure 3. *E. coli* Δ*cysE* exhibits a BMAA dose-dependent growth lag in minimal**
555 **medium.** Mean optical density measured at 600 nm of *E. coli* cultures grown in 96-
556 well plates with shaking at 37°C. Δ*cysE* mutant *E. coli* growth over 18 hours in M9
557 minimal media is shown with indicated BMAA concentrations. Cysteine auxotrophy
558 in this strain was alleviated by supplementation with 100 μg/ml cysteine. Figures are
559 representative of curves generated from triplicate cultures.

560 **Figure 4. Effect of 500 μg/ml BMAA on growth of *E. coli* mutants of the cysteine**
561 **biosynthesis pathway**. (A) The growth over 24 hours of strain *ΔcysE* treated with 500
562 μg/mL BMAA (grey) or untreated control (black) in M9-minimal medium
563 supplemented with 100 μg/mL L-cysteine. Panels (B) and (C) show strain *ΔcysK* and

564 *ΔcysM*, respectively, under the same conditions. Replicate cultures for each strain and
565 treatment are plotted individually (n=4).

566 **Figure 5. Viability of *E. coli* Δ*cysE* and wild-type strains treated with H_2O_2.** Wild
567 Type and *cysE* deletion mutant *E. coli* were treated with 5 mM and 25 mM H_2O_2 for 15
568 minutes at 37°C in M9 minimal medium. Serially diluted cultures were plated on LB
569 agar and resulting colonies were used to calculate CFU/ml. Cysteine auxotrophic
570 strain Δ*cysE* was supplemented with 100 μg/ml L-cysteine. Error bars represent
571 standard deviation from the mean (n=3). * - Significantly different ($p < 0.05$) using
572 Student's t-test.

573 **Figure 6. Measurement of glutathione in *E. coli* grown in nutrient poor and**
574 **nutrient rich media.** (a) Quantification of total glutathione levels in *E. coli* grown in
575 nutrient poor (M9 minimal) and nutrient rich (LB) media during exponential growth.
576 Concentration is normalized to protein as detected by BCA assay. (b) Ratio of reduced
577 to oxidised glutathione in *E. coli* strains grown in nutrient poor and nutrient rich
578 medium. Glutathione content was determined using triple quadrupole mass
579 spectrometer (TQMS) coupled with UHPLC, n=4. * Significantly different ($p < 0.05$)
580 using T-test. (c) Representative chromatogram of GSH and GSSG from one of the
581 standards used for the calibration curve.

582

583

584

585

586

587

7. Tables

588

Table 1. List of *E. coli* strains used in this study.

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
JW2407-1	***ΔcysK743::kan***	Cysteine Synthesis Mutant
JW2414-3	***ΔcysM750::kan***	Cysteine Synthesis Mutant

589

590

591

592

593

594 **Table 2.** Retention times and MRM ion transitions for all targeted compounds.

595 *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

596

597

598

8. Figures