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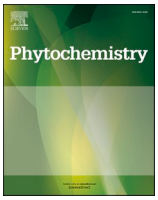

Genomic insights into the biosynthesis and physiology of the cyanobacterial neurotoxin 3-*N*-methyl-2,3-diaminopropanoic acid (BMAA)

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A B S T R A C T

Cyanobacteria are an ancient clade of photosynthetic prokaryotes, present in many habitats throughout the world, including water resources. They can present health hazards to humans and animals due to the production of a wide range of toxins (cyanotoxins), including the diaminoacid neurotoxin, 3-*N*-methyl-2,3-diaminopropanoic acid (β-*N*-methylaminoalanine, BMAA). Knowledge of the biosynthetic pathway for BMAA, and its role in cyanobacteria, is lacking. Present evidence suggests that BMAA is derived by 3-*N* methylation of 2,3-diaminopropanoic acid (2,3-DAP) and, although the latter has never been reported in cyanobacteria, there are multiple pathways to its biosynthesis known in other bacteria and in plants. Here, we used bioinformatics analyses to investigate hypotheses concerning 2,3-DAP and BMAA biosynthesis in cyanobacteria. We assessed the potential presence or absence of each enzyme in candidate biosynthetic routes known in *Albizia julibrissin*, *Lathyrus sativus* seedlings, *Streptomyces*, *Clostridium*, *Staphylococcus aureus, Pantoea agglomerans*, and *Paenibacillus larvae*, in 130 cyanobacterial genomes using sequence alignment, profile hidden Markov models, substrate specificity/active site identification and the reconstruction of gene phylogenies. Most enzymes involved in pathways leading to 2,3-DAP in other species were not found in the cyanobacteria analysed. Nevertheless, two species appear to have the genes *sbnA* and *sbnB*, responsible for forming the 2,3-DAP constituent in staphyloferrin B, a siderophore from *Staphylococcus aureus*. It is currently undetermined whether these species are also capable of biosynthesising BMAA. It is possible that, in some cyanobacteria, the formation of 2,3-DAP and/or BMAA is associated with environmental iron-scavenging. The *pam* gene cluster, responsible for the biosynthesis of the BMAA-containing peptide, paenilamicin, so far appears to be restricted to *Paenibacillus larvae*. It was not detected in any of the cyanobacterial genomes analysed, nor was it found in 93 other *Paenibacillus* genomes or in the genomes of two BMAA-producing diatom species. We hypothesise that the presence, in some cyanobacterial species, of the enzymes 2,3-diaminopropionate ammonia-lyase (DAPAL) and reactive intermediate deaminase A (RidA) may explain the failure to detect 2,3-DAP in analytical studies. Overall, the taxonomic distribution of 2,3-DAP and BMAA in cyanobacteria is unclear; there may be multiple and additional routes, and roles, for the biosynthesis of 2,3-DAP and BMAA in these organisms.

1. Introduction

3-*N*-methyl-2,3-diaminopropanoic acid (syn: α-amino-β-methyl-aminopropionic acid, MeDAP; β-*N*-methylaminoalanine, BMAA) is a neurotoxin that was first isolated from seed of *Cycas micronesica* K.D.Hill (Cycadaceae) (Vega and Bell, 1967; Nunn, 2017). BMAA is a non-encoded amino acid (i.e. not coded for by a codon in the genetic code). It has since been detected in a range of organisms, including some species of cyanobacteria (Cox et al., 2003, 2005; Banack et al., 2007; Downing et al., 2011; Downing and Downing, 2016). Interest in BMAA was generated by the possibility that it may be a contributory causative agent of the chronic neurological complex, amyotrophic lateral sclerosis/parkinsonism/dementia (ALS-PDC) of Guam (Nunn, 2017), but this remains a controversial matter (Chernoff et al., 2017; Dunlop et al.,

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2021). Ecological and physiological functions for BMAA have not been determined and metabolic pathways for the biosynthesis of BMAA in cyanobacteria are currently unknown. Among other benefits, knowledge of such pathways would allow targeting of specific biosynthesis genes by polymerase chain reaction (PCR)-based methods, which could be used as an early-warning system for the potential presence of cyanotoxins in water resources and other environments (Humbert, 2017; Kurmayer et al., 2017).

BMAA occurs free and as two bound forms (Vega and Bell, 1967; Polsky et al., 1972; Dossaji and Bell, 1973; Murch et al., 2004; Faassen et al., 2016) but the chemical complexes in which BMAA is incorporated (corresponding to the soluble bound form and TCA-precipitated bound form) have not been elucidated. All three fractions of BMAA may or may not be present in organisms shown to produce BMAA (Rosén et al., 2016) and it cannot be dismissed that free and bound BMAA moieties may be derived from different metabolic precursors (Nunn and Codd, 2017).

The extent of BMAA biosynthesis throughout the Cyanoprokaryota has not been systematically determined due, to some extent, to analytical limitations (Faassen, 2014; Mantas et al., 2021). It is widely accepted that analytical methods using tandem mass spectrometry (MS/MS) methods are the most suitable for the identification and quantification of BMAA (Cohen, 2012; Faassen et al., 2012; Faassen, 2014). However, variation still exists regarding sample processing and separation methods (Faassen, 2014) and inter-laboratory comparisons are scarce (Faassen et al., 2016). Despite such problems, the ability of axenic, single strain cultures of some cyanobacteria to biosynthesise BMAA has been unequivocally confirmed (Eriksson et al., 2009; Downing et al., 2011; Downing and Downing, 2016).

In this study, we used bioinformatics tools to investigate hypotheses concerning BMAA biosynthesis in cyanobacteria through an assessment of the presence or absence of enzymes in six known potential metabolic pathways, across 130 cyanobacterial genomes. We show that most enzymes involved in pathways leading to the putative precursor of BMAA (2,3-diaminopropanoic acid, 2,3-DAP) in other species, were not found in cyanobacteria. Genes coding for SbnA and SbnB, by whose concerted action the biosynthesis of 2,3-DAP is known to occur in *Staphylococcus aureus* Rosenbach 1884 (Staphylococcaceae), were found, though limited to a subset of cyanobacterial species. We highlight the potential physiological role of 2,3-DAP in siderophore formation in some cyanobacterial species and show that the *pam* gene cluster, responsible for directing the biosynthesis of peptide-bound BMAA in *Paenibacillus larvae* (White 1906) Ash et al. 1994 (Paenibacillaceae), was not detected in 130 cyanobacterial species, nor was it found in 93 genomes of *Paenibacillus* Ash et al. 1994 (other than *P. larvae*) or two diatom species. We also show that the presence, in some cyanobacterial species, of genes putatively encoding the enzymes 2,3-diaminopropionate ammonia-lyase (DAPAL, EC 4.3.1.15) and reactive intermediate deaminase A (RidA, EC 3.5.99.10) could explain the failure to detect 2,3-DAP in analytical studies. The biosynthesis of 2,3-DAP in cyanobacteria appears to be either restricted to a small subset of cyanobacterial species, or there may be multiple, additional, routes for the biosynthesis of this amino acid.

2. Routes to the biosynthesis of 2,3-DAP and BMAA in other taxa and potential relevance in cyanobacteria

2.1. The biosynthesis of BMAA from 2,3-diaminopropanoic acid (2,3-DAP)

The simplest explanation for the biosynthesis of BMAA is through the 3-*N* methylation of 2,3-DAP, found free and as simple derivative forms in plant species (Gmelin, 1959; Nunn and Codd, 2017) (Fig. 1).

However, mechanistic support for this pathway (Brenner et al., 2003) is lacking in cyanobacteria. First, although the possible genes encoding cysteine synthase-like and methyltransferase enzymes occur in cyanobacteria (Aráoz et al., 2010), the existence of such enzymes with specificity for the proposed substrates remains speculative. Second, since this pathway requires ammonium, it may be difficult to reconcile with the results of Downing et al. (2011), who showed that BMAA levels in the non-nitrogen-fixing cyanobacterium *Microcystis* PCC 7806 (Microcystaceae) increase under conditions of nitrogen (ammonium/nitrate) starvation and decrease when ammonium is added. Finally, this pathway assumes that BMAA is biosynthesised by direct methylation of free 2,3-DAP, which has not been found in any cyanobacterial species or in cycads, which can accommodate cyanobacteria, to date.

There are at least four known biosynthetic routes to 2,3-DAP. Two are complex pathways in plants, and the others are simpler mechanisms found in *Streptomyces* Waksman and Henrici 1943 (Streptomycetaceae) and *Staphylococcus* Rosenbach 1884 (Staphylococcaceae).

2.2. Biosynthesis of 2,3-DAP from uracil in Albizia julibrissin

2,3-DAP biosynthesis from uracil occurs in plants such as *Albizia julibrissin* Durazz. (Fabaceae), which produce albizziine (3-*N*-ureido-2,3-diaminopropanoic acid) from uracil as part of the pyrimidine degradation pathway (Brown and Turan, 1995, 1996). This pathway encompasses five steps and the enzymes dihydrouracil dehydrogenase (syn: dihydropyrimidine dehydrogenase, DUD, EC 1.3.1.2), dihydropyrimidinase (DHP, EC 3.5.2.2), and β-ureidopropionase (syn: β-alanine synthase, *N*-carbamoyl-β-alanine amidohydrolase, βUP, EC 3.5.1.6) (Fig. 2). In theory, the methylation reaction resulting in the formation of BMAA could occur at any step in the metabolic pathway; 1-methyluracil, a potential methylated primary precursor, does not appear to be a natural product. Albizziine formation is almost entirely confined to the Mimosoideae (Seneviratne and Fowden, 1968), and there is no indication of its presence in cyanobacteria.

2.3. Biosynthesis of 2,3-DAP from β-(isoxazolin-5-on-2-yl) in Lathyrus sativus seedlings

Lathyrus sativus L. (Fabaceae) biosynthesises the amino acid β-*N*-oxalyl-2,3-diaminopropanoic acid (β-ODAP) (Rao et al., 1964). In the pathway to β-ODAP, β-(isoxazolin-5-on-2-yl)-S-alanine (BIA), identified

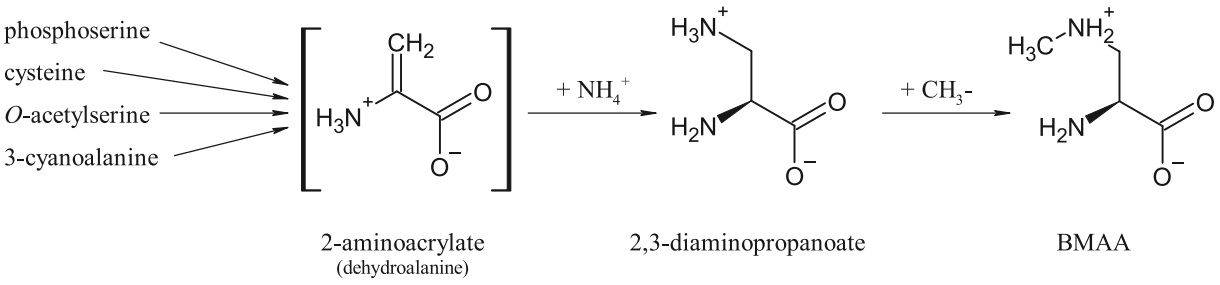

Fig. 1. Biochemical pathway to free 2,3-diaminopropanoate and BMAA as proposed by Brenner et al. (2003). A cysteine synthase-like enzyme catalyses the synthesis of 2,3-diaminopropanoate from S–*O*-acetylserine, phosphoserine, cysteine or 3-cyanoalanine, and ammonium. A likely intermediate in this reaction is 2-aminoacrylate (dehydroalanine). S-adenosyl-*S*-methionine (SAM) donates the 3-*N*-methyl group to 2,3-diaminopropanoate, yielding BMAA.

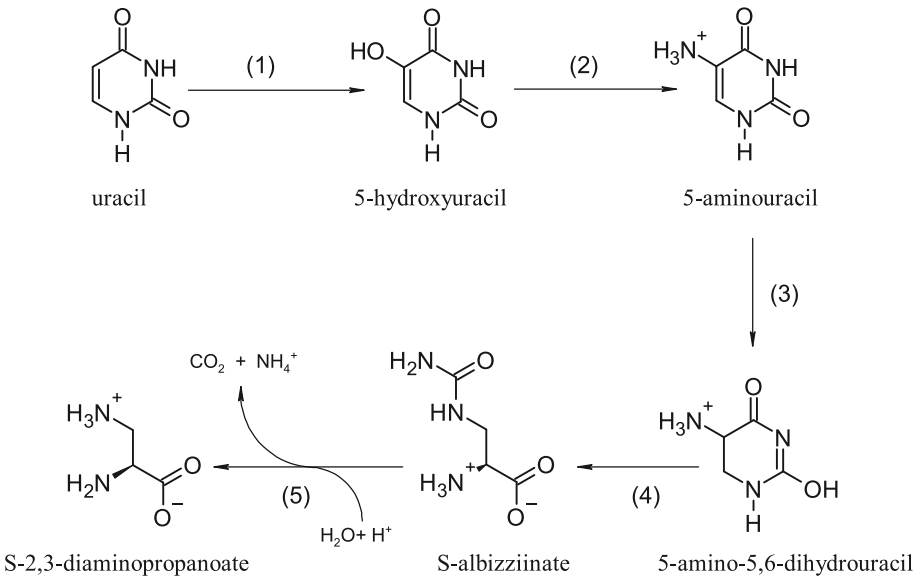

Fig. 2. Biochemical pathway to S-2,3-diaminopropanoate from uracil in *Albizia julibrissin*. Redrawn from Brown and Turan (1996). Uracil serves as a precursor for 5-hydroxyuracil formation through (1) unspecified hydroxylase activity and (2) an amination reaction, forming 5-aminouracil. (3) Dihydrouracil dehydrogenase (syn: dihydropyrimidine dehydrogenase, DUD, EC 1.3.1.2) reduces 5-aminouracil, in an NADPH-dependent reaction, to 5-amino-5, 6-dihydrouracil, which serves as substrate for (4) dihydropyrimidinase (DHP, EC 3.5.2.2). Following cleavage of the pyrimidine ring, catalysed by DHP, S-2,3-diaminopropanoate is formed from S-albizziinate by hydrolysis, catalysed by (5) β-ureidopropionase (syn: β-alanine synthase, *N*-carbamoyl-β-alanine amidohydrolase, βUP, EC 3.5.1.6). The nomenclature is from www.brenda-enzymes.org. The charged forms of the amino acids are those predominating at physiological pH values.

by Kuo et al. (1998) in germinating seedlings of this species, is synthesised from isoxazolin-5-one and S–*O*-acetylserine via cysteine synthase (EC 2.5.1.47). Although BIA, and not 2,3-DAP, was detected in *L. sativus* seedlings, convincing evidence exists supporting the hypothesis that 2,3-DAP is the immediate precursor of β-ODAP (Malathi et al., 1970; Ikegami et al., 1999). Hence, it is suggested that 2,3-DAP, derived from BIA, is used to synthesise β-ODAP via S-2,3-diaminopropionate *N*-oxalyltransferase (EC 2.3.1.58) (Nunn and Codd, 2017) (Fig. 3). This enzyme has not been purified or sequenced. Since, in this pathway, 2, 3-DAP does not appear free in plant seedlings, it is hypothesised that substrate channelling may occur, i.e. that the 2,3-DAP intermediate remains bound to the catalytic site of the enzyme, until the following reaction coverts it to the final product (Nunn and Codd, 2017). This process occurs frequently in a broad spectrum of metabolic reactions (Agius, 1997; Jørgensen et al., 2005). Although there are no reports of 2, 3-DAP released from a bound form after hydrolysis of cyanobacterial cells, the fact that a 3-*N*-derivative of 2,3-DAP could be formed whilst the amino acid remains bound to the catalytic site of an enzyme could explain the failure to detect free 2,3-DAP in BMAA-producing cyanobacterial species (Nunn and Codd, 2017). Nevertheless, although isoxazolinones are widespread in plants (Lambein et al., 1986) and bacteria (Becker et al., 2017), there are no reports of their presence in cyanobacteria.

2.4. Biosynthesis of 2,3-DAP from serine in Streptomyces, Clostridium and plants

2,3-DAP biosynthesis from serine has been unequivocally shown to occur during the production of the antibiotics tuberactinomycin (TUB) and viomycin, in *Streptomyces* (Carter et al., 1974), and also in the biosynthesis of the similar peptide antibiotic, capreomycin, in *Streptomyces capreolus* A250 (Streptomycetaceae/Pseudonocardiaceae) (Wang

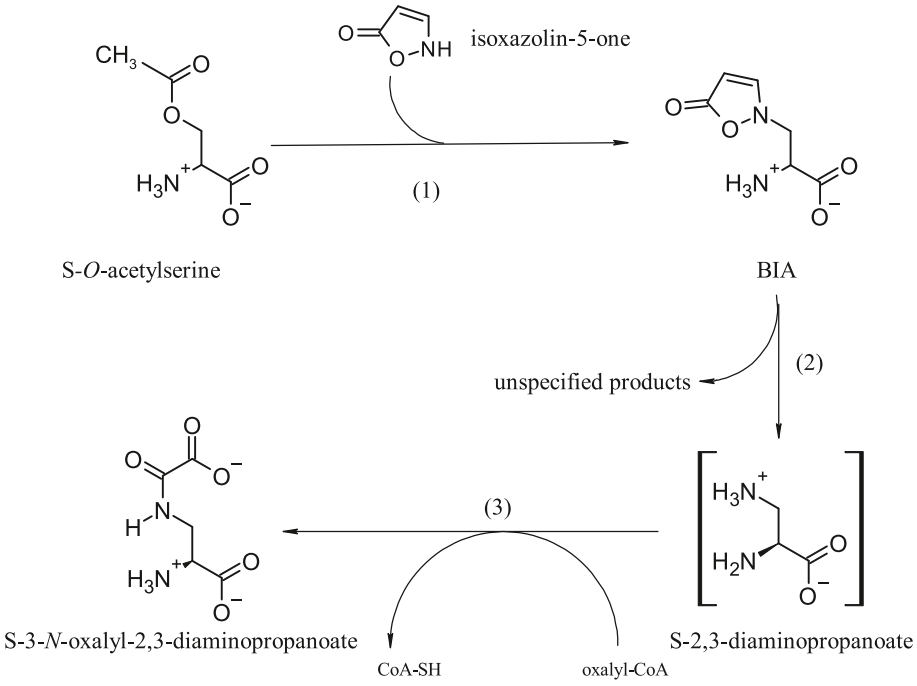

Fig. 3. Biochemical pathway to S-2,3-diaminopropanoate from β-(isoxazolin-5-on-2-yl) in *Lathyrus sativus* seedlings. Redrawn from Ikegami et al. (1999). The nomenclature is from www.brenda-enzymes.org; Enzyme Commission numbers are: (1) cysteine synthase: EC 2.5.1.47; (2) S-2,3-diaminopropionate *N*-oxalyltransferase: EC 2.3.1.58. The charged forms of the amino acids are those predominating at physiological pH values. S-2,3-diaminopropanoic acid is shown in square brackets as it is not released free from the enzyme during the reaction

and Gould, 1993; Felnagle et al., 2007), although this was recently contested by Hsu et al. (2020). TUBs contain several unusual amino acids including β-ureidodehydroalanine, β-lysine, S-capreomycidine and 2,3-DAP, and are believed to be biosynthesised by a non-ribosomal peptide synthesis (NRPS) mechanism (Thomas et al., 2003). Following the identification of the viomycin biosynthetic gene cluster from *Streptomyces* sp. ATCC 11861, it became apparent that the biosynthesis of 2,3-DAP relied on the concerted action of two enzymes: VioK and VioB (Thomas et al., 2003). VioK is an ornithine cyclodeaminase (OCD) (EC 4.3.1.12) and VioB (syn: 2,3-diaminopropanoic acid synthetase) is a putative *O*-acetyl-S-serine sulfhydrylase (OASS), homologous to serine dehydratases and cysteine synthases. However, while cysteine synthases use sulfur from sulfide in the addition reaction, VioB is thought to use the nitrogen from ammonium, released from S-ornithine by VioK (Thomas et al., 2003). The combined action of VioK and VioB enables the direct transfer of ammonium needed to form the 3-*N* amino group of 2,3-DAP, by-passing the generally low intracellular concentration of ammonium and driving the reaction towards 2,3-DAP formation (Fig. 4). Similar mechanisms operate in *Bacillus cereus* Frankland and Frankland 1887 (Bacillaceae) and *Bacillus thuringiensis* subsp. *kurstaki* YTP-1520 for the biosynthesis of the 2,3-DAP-containing peptide antibiotic, zwittermicin A (Emmert et al., 2004; Zhao et al., 2008). However, although multiple enzymes can catalyse β-replacement reactions involving ammonium (Parmeggiani et al., 2018), the rate of diffusion of ammonia inside a cell can be a limiting factor (Beasley et al., 2011).

2.5. Biosynthesis of 2,3-DAP in Staphylococcus aureus

Under iron-limiting conditions, *Staphylococcus aureus* synthesises two carboxylate-type siderophores, staphyloferrin A (SA) (Konetschny-Rapp et al., 1990) and staphyloferrin B (SB) (Beasley et al., 2011). To date, SB is the only iron-chelator known to contain 2,3-DAP (Cheung et al., 2009). *S. aureus* contains a nine-gene *sbn* siderophore biosynthesis operon (*sbnA-I*) in which SbnA and SbnB are responsible for 2,3-DAP biosynthesis (Thomas et al., 2003; Beasley et al., 2011; Kobylarz et al., 2014) (Fig. 5). SbnA (*N*-(2-amino-2-carboxyethyl)-S-glutamate synthase, EC 2.5.1.140) is a pyridoxal 5′-phosphate (PLP)-dependent enzyme similar to VioB (Heine et al., 2004) and commonly annotated as cysteine synthase (Kobylarz et al., 2014). However, unlike all previously known sulfhydrylases, SbnA uses S–*O*-phosphoserine (OPS) instead of OAS as substrate, with S-glutamate as nitrogen donor, instead of sulfur (Kobylarz et al., 2016). SbnB (*N*-[(2S)-2-amino-2-carboxyethyl]-S-glutamate dehydrogenase, EC 1.5.1.51) is homologous to the OCD protein family (including VioK) and amino acid dehydrogenases (Beasley et al., 2011). Nevertheless, Kobylarz et al. (2014) showed that SbnB uses NAD^+ as substrate, rendering it a closer relative of NAD^+-dependent amino acid dehydrogenases than to ornithine cyclodeaminase (OCD).

2.6. Biosynthesis of peptide-bound BMAA

Although free forms of BMAA in cyanobacteria could be biosynthesised directly by a pathway analogous to that of Brenner et al. (2003), another possibility is that free BMAA is released from a polymeric structure by metabolic turnover of small molecules or macromolecule assemblies (Tripathi and Gottesman, 2016). Galantins and paenilamicins are examples of peptide-bound BMAA that might act as reservoirs for the neurotoxin and release it upon metabolic turnover (Nunn and Codd, 2017). These peptides are either hypothesised (galantins) or known (paenilamicins) to be synthesised via a NRPS mechanism (Müller et al., 2014). Several classes of other cyanotoxins are produced via the same mechanisms (Moffitt and Neilan, 2004; Mbedi et al., 2005; Kellmann et al., 2008; Mihali et al., 2008) and it is possible that an as yet unidentified specialised metabolite cluster exists for

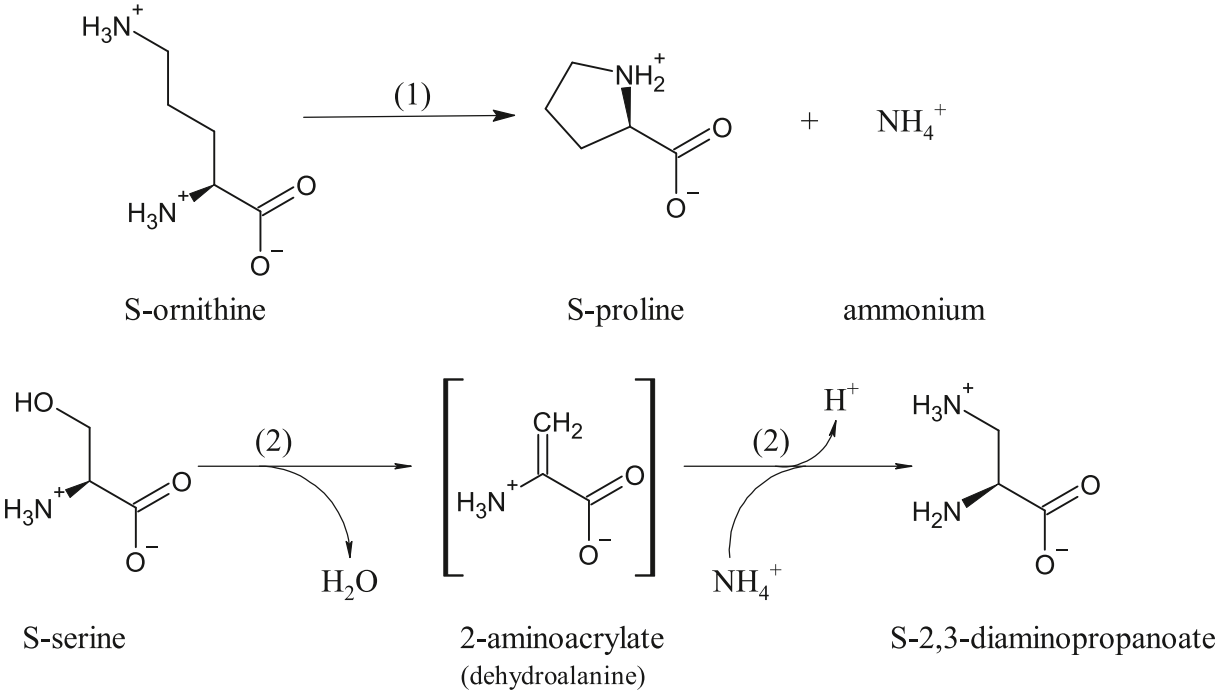

Fig. 4. Biochemical pathway to S-2,3-diaminopropionate from S-serine in *Streptomyces*, *Clostridium* and some plants. Redrawn from Thomas et al. (2003). (1) ornithine cyclodeaminase (syn: OCD, VioK, EC 4.2.1.12) catalyses the cyclisation of S-ornithine to S-proline, releasing ammonium, using NAD^+ as cofactor in *Clostridium* sp. PA 3679 (Clostridiaceae), *Nicotiana tabacum* L. (Solanaceae), *Datura stramonium* L. (Solanaceae) and in *Lupinus angustifolius* L. (Fabaceae) (Costilow and Laycock, 1971; Mestichelli et al., 1979; Muth and Costilow, 1974). (2) S-2,3-diaminopropionate synthetase (VioB, unclassified) catalyses the biosynthesis of a PLP-bound 2-aminoacrylate intermediate from S-serine or *O*-acetyl-S-serine (OAS) (Carter et al., 1974; Wang and Gould, 1993) and subsequently catalyses a β-substituent replacement reaction, similar to that observed with cysteine synthases. The enzyme nomenclature is from www.brenda-enzymes.org. The charged forms of the amino acids are those predominating at physiological pH values.

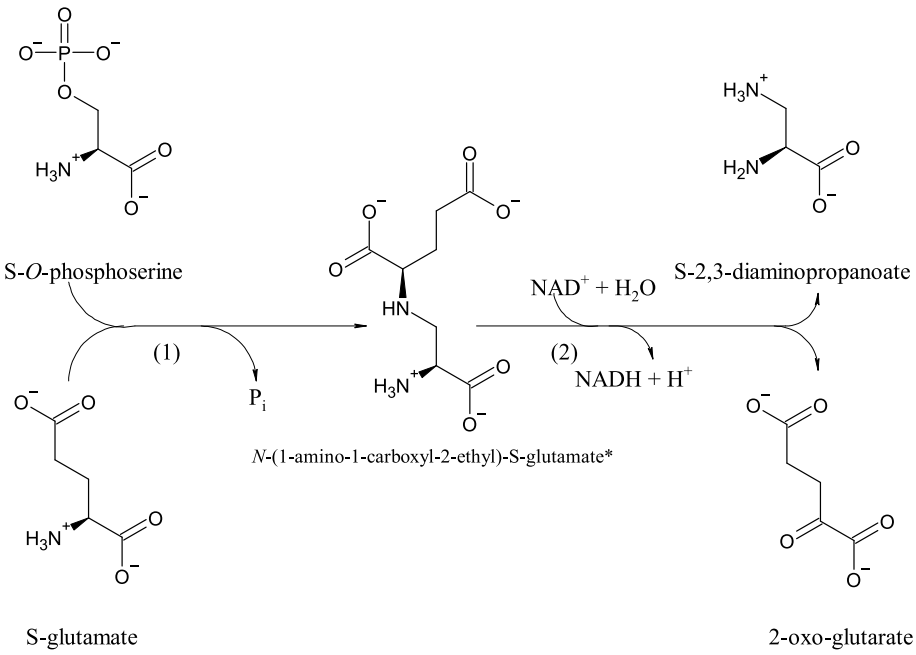

Fig. 5. Biochemical pathway to 2,3-diaminopropanoate in *Staphylococcus aureus*. Redrawn from Kobylarz et al. (2014). (1) SbnA (*N*-(2-amino-2-carboxyethyl)-S-glutamate synthase, EC 2.5.1.140) uses PLP and the substrates S–*O*-phosphoserine (OPS) and S-glutamate to form the serine-glutamate conjugate: *N*-(1-amino-1--carboxyl-2-ethyl)-S-glutamate (Kobylarz et al. (2014); syn *N*-[(2S)-2-amino-2-carboxylethyl]-S-glutamate, ACEGA*). (2) SbnB (syn: *N*-[(2S)-2-amino-2-carboxyethyl]-S-glutamate dehydrogenase, EC 1.5.1.51) oxidatively hydrolyses ACEGA, in an NAD^+-dependent reaction, to yield 2,3-diaminopropionate, 2-oxo--glutarate and NADH (Kobylarz et al., 2014). The nomenclature is from www.brenda-enzymes.org. The charged forms of the amino acids are those predominating at physiological pH values. The intermediate may or may not be released during the reaction. It has been chemically synthesised (Hsu et al., 2020).

cyanobacterial BMAA biosynthesis.

Peptide-bound BMAA occurs in the bacterium *Paenibacillus larvae* (Müller et al., 2014). Complex antibacterial, antifungal and cytotoxic peptide paenilamicins are biosynthesised by an elaborate hybrid NRPS/polyketide synthetase (PKS) system (*pam*), in which 2,3-DAP is methylated within the specialised cluster to form BMAA (Garcia--Gonzalez et al., 2014; Müller et al., 2014). The *pam* gene cluster has been found only in *P. larvae*. There are more than 200 characterised *Paenibacillus* species occurring naturally in the environment (Nunn and Codd, 2019). Paenilamicins are similar in structure to the antibiotic peptide galantin 1 (Shoji et al., 1975; Sakai and Ohfune, 1992), also isolated from *P. larvae* subsp. *pulvifaciens* (Nakamura 1984) Heyndrickx et al. 1996 (Sakai and Ohfune, 1990). It is hypothesised that both galantins and paenilamicins are biosynthesised by similar NRPS mechanisms, comprising a transmethylase, which produce peptide-bound BMAA (Nunn and Codd, 2017). However, the biosynthetic route to this peptide has not been investigated biochemically. Peptides larger than paenilamicins and galantins and insoluble in protein precipitants may exist in cyanobacteria, which would explain the bound BMAA found in pellet fractions (Nunn and Codd, 2017). To date, galantins and paenilamicins are the only peptides known to contain this neurotoxin.

Enzyme-bound 2,3-DAP occurs in the peptide antibiotic dapdiamides from *Pantoea agglomerans* CU0119 (Erwiniaceae/Enterobacteriaceae) (Dawlaty et al., 2010). In *P. agglomerans*, dapdiamides are biosynthesised by an NRPS module comprising two genes for the production of 2,3-DAP (*ddaA* and *ddaB*) (Hollenhorst et al., 2010).

Another possibility is that BMAA biosynthesis could occur via the dehydration or desulfydration of a serine or cysteine residue, respectively, after insertion into a peptide chain by an NRPS module (Nunn and Codd, 2017). This would generate a dehydroalanine (DHA) residue, enclosed within a protein or peptide. The addition of ammonium to the double bond of the DHA residue would generate 2,3-DAP(Eiger and Greenstein, 1948), which, if *N*-methylated, could yield peptide-bound BMAA. If hypothetical peptides were subject to metabolic turnover, free BMAA would be released intracellularly. Dehydroamino acids are abundant in cyanobacteria (Siodłak, 2015) and are chemically reactive (Humphrey and Chamberlin, 1997). Available methods for obtaining peptide-bound DHA enzymatically have not been described, and it is currently not possible to bioinformatically test the feasibility of this pathway for the production of 2,3-DAP and/or BMAA in cyanobacteria.

3. Results and discussion

3.1. Cyanobacterial species encoding enzymes in the pathway to 2,3-DAP from uracil in Albizia julibrissin

Several enzymes can catalyse the first two reactions in the pathway to 2,3-DAP from uracil (Fig. 2) and further information is needed regarding their substrate specificity in order to test the feasibility of these reactions in cyanobacteria.

Our results indicate that NADPH-dependent DUD (EC 1.3.1.2) was not found in any of the 130 cyanobacteria in our dataset. Protein sequences with some similarity to DUD are present in 125 cyanobacterial species, however they are functionally annotated to enzymes other than DUD and similarity to a sequence model of known DUD is low (Supplementary Table S1). It is currently not possible to discern if these protein sequences have the same substrate specificity and/or are able to catalyse the synthesis of 5-amino-5,6-dihydrouracil from 5-aminouracil.

DHP belongs to the cyclic amidohydrolase family of enzymes, which also include allantoinase, dihydroorotase, hydantoinase, and imidase (Holm and Sander, 1997). Although these metalloenzymes possess similar active sites and may use analogous catalysis mechanisms (Huang, 2015), they have different substrate specificities and relatively low amino acid sequence identity (Hsu et al., 2010; Peng and Huang, 2014). Site-directed mutagenesis studies on a DHP from *Pseudomonas aeruginosa* (Schroeter 1872) Migula 1900 (Pseudomonadaceae) have shown that histidine residues at positions 59, 61, 183, and 239 (H59, H61, H183, H239) and the aspartate residue at position 316 (D316) are essential for the assembly of the binuclear metal centre of the active site, whereas the tyrosine residue at position 155 (Y155), the serine residue at position 289 (S289), and the asparagine residue at position 337 (N337) are necessary for substrate-binding. These residues are conserved in all DHPs (Huang, 2015).

In our study, six cyanobacterial species appear to possess a gene putatively coding for DHP (EC 3.5.2.2): *Nodosilinea nodulosa* (Li and Brand 2007) Perkerson and Casamatta 2011 (Prochlorotrichaceae), *Lyngbya confervoides* C.Agardh 1824 (Oscillatoriaceae), *Leptolyngbya ohadii* IS1 (Leptolyngbyaceae), *Spirulina major* Kützing ex Gomont 1892 (Spirulinaceae), *Desertifilum* sp. IPPAS B-1220 (Desertifilaceae) and *Chroogloeocystis siderophila* Brown et al. 2005 (Chroococcaceae) (Fig. 6). The catalytic site described is conserved in all amino acid sequences

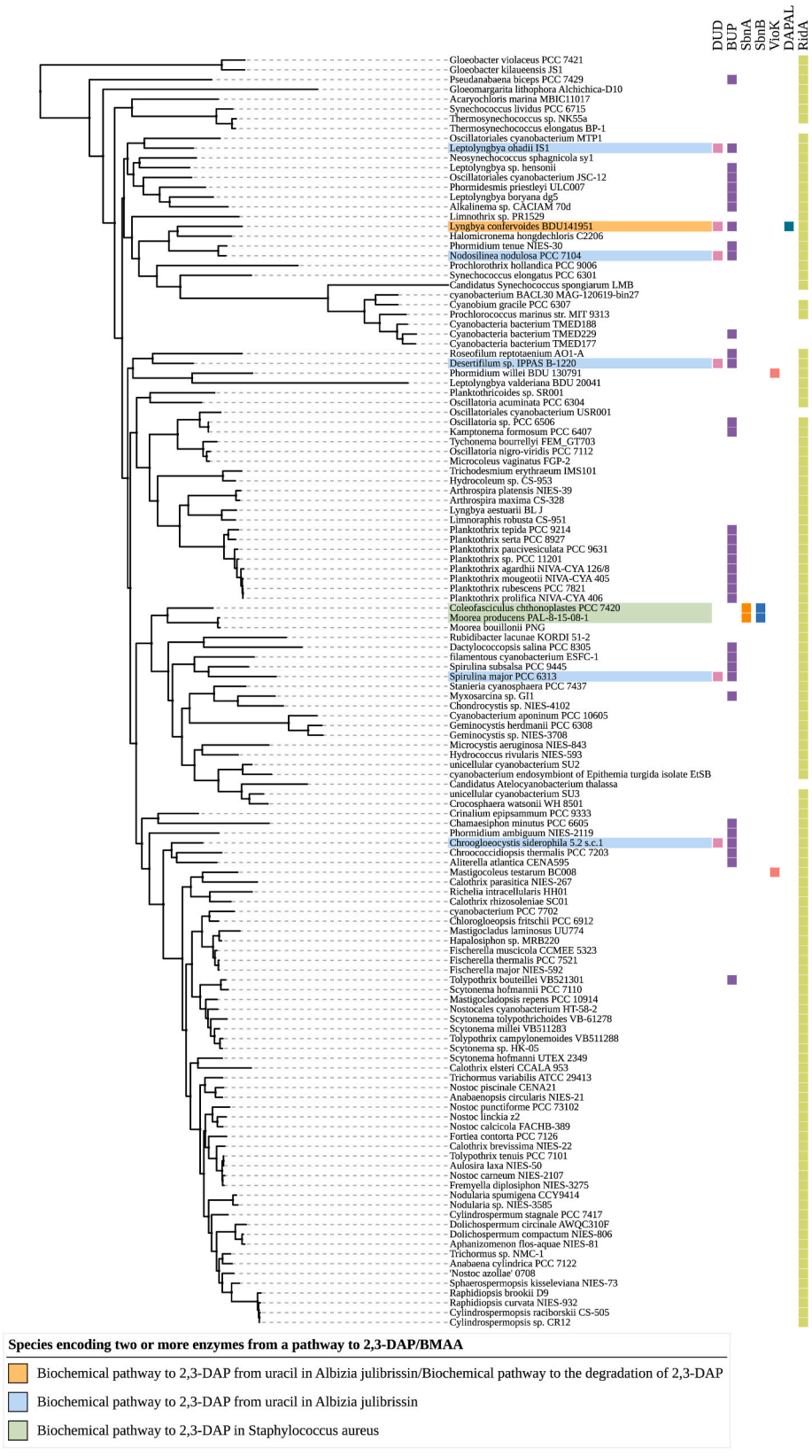

Fig. 6. Species phylogeny showing the cross-species distribution of key enzymes involved in the biosynthesis of S-2,3-diaminopropanoate and BMAA. The enzymes encoded by each species are indicated by coloured rectangles, next to the species name. DUD: dihydropyrimidine dehydrogenase; βUP: β-ureidopropionase; DAPAL: 2,3-diaminopropionate ammonia-lyase; RidA: reactive intermediate deaminase A. For the accession numbers of the enzymes, see Supplementary Tables S1-S6, S9, S15-S16. This is a re-annotation of the phylogeny of Mantas et al. (2021), who also provide bootstrap support (their Supplementary Fig. S2).

(Supplementary Table S2), except for the conserved serine residue that is substituted by threonine in five out of the six sequences functionally annotated as DHP.

Similarly to DHP, βUP (EC 3.5.1.6) belongs to a large class of amidohydrolases, including nitrilases, cyanide hydratases, aliphatic amidases and ureidohydrolases, that, although catalysing different reactions, have relatively low, but significant, amino acid sequence identity (Bork and Koonin, 1994; Novo et al., 1995). Studies on the crystal structure of *N*-carbamyl-R-amino acid amidohydrolase have indicated a conserved cysteine (C), glutamic acid (E), and lysine (K)

residue that form a catalytic triad in the active site (Novo et al., 1995; Nakai et al., 2000; Walsh et al., 2001). βUP hydrolases require a Zn^{2+} ion as a catalytic cofactor (Walsh et al., 2001).

34 cyanobacterial species appear to have a putative gene for βUP (Table 1). All amino acid sequences from these species were functionally annotated as Zn-dependent hydrolases (Supplementary Table S3).

Six cyanobacterial species appear to have putative genes for both DHP and βUP. These are the same species, listed above, that have putative genes for DHP (Fig. 6). In the apparent absence of a gene for NADP-dependent DUD, this thus appears to be an unlikely route to BMAA in cyanobacterial species. Nevertheless, given the presence of many cyanobacterial protein sequences similar to DUD, biochemical studies on the feasibility of this pathway for BMAA biosynthesis in these six cyanobacterial species are merited.

3.2. Cyanobacterial species encoding enzymes in the pathway to 2,3-DAP from β-(isoxazolin-5-on-2-yl) in Lathyrus sativus seedlings

Cysteine synthase (EC 2.5.1.47) appears to be ubiquitous in cyanobacteria with 127 of the 130 species examined having two or more copies of a putative gene coding for the enzyme. In *Hydrocoleum* sp. CS-953 (Microcoleaceae), genetic evidence for the presence of this enzyme is lacking; this may be due to errors in sequencing, assembly, genome annotation and/or functional annotation. *O*-acetyl-S-serine-dependent enzymes, such as cysteine synthase, have conserved alanine, phenylalanine and glycine residues at positions 132 (A132), 152 (F152), and 185 (G185), respectively (Kobylarz et al., 2016). These residues are conserved in most cyanobacterial cysteine synthases (Supplementary Table S4).

Although S-2,3-diaminopropionate *N*-oxalyltransferase (EC 2.3.1.58) is included in enzyme databases such as BRENDA (Chang et al., 2021), no corresponding amino acid or nucleotide sequence is available. In the protein sequence database at NCBI (https://www.ncbi.nlm.nih.gov) four sequences are associated with EC 2.3.1.58, which are all functionally annotated as cysteine synthase A. S-2,3-diaminopropionate *N*-oxalyltransferase appears to have been described once in *Lathyrus sativus* (Malathi et al., 1970), but since then has not been purified or sequenced. Whether the enzyme is specific to *L. sativus*, or was mistakenly characterised, is undetermined. Nevertheless, until further data become available on the enzyme's substrate specificity, catalytic site and taxonomic distribution, no conclusion can be advanced regarding the use of this pathway (Fig. 3) for BMAA formation in cyanobacteria.

Table 1
Cyanobacterial species with a putative gene for β-ureidopropionase (βUP, EC 3.5.1.6). *Lyngbya confervoides* C.Agardh 1824 (Oscillatoriaceae) and *Leptolyngbya ohadii* (Leptolyngbyaceae) were found to have two copies.

Species with a putative gene for β-ureidopropionase
Lyngba confervoides
Chroococcidiopsis thermalis
Phormidium ambiguum
Leptolyngbya ohadii
Desertifilum sp. IPPAS B-1220
Planktothrix tepida
Cyanobacteria bacterium TMED229
Planktothrix serta
Spirulina major
Leptolyngbya sp. "hensonii"
Chroogloeocystis siderophila
Leptolyngbya boryana
Phormidesmis priestleyi
Spirulina subsalsa
Dactylococcopsis salina
Oscillatoriales cyanobacterium JSC-12
Phormidium tenue
Myxosarcina sp. GI1
Planktothrix paucivesiculata
Chamaesiphon minutus
Planktothrix sp. PCC 11201
Kamptonema formosum
Oscillatoria sp. PCC 6506
Roseofilum reptotaenium AO1-A
Nodosilinea nodulosa
Pseudanabaena biceps
Alkalinema sp. CACIAM 70d
Planktothrix agardhii
Planktothrix prolifica
Planktothrix rubescens
Planktothrix mougeotii
filamentous cyanobacterium ESFC-1
Aliterella atlantica
Tychonema bourrellyi

3.3. Cyanobacterial species encoding genes for 2,3-DAP biosynthesis via SbnA/SbnB and VioK/VioB

SbnA has been shown to be homologous to VioB and cysteine synthase, and SbnB to be homologous to VioK (Beasley et al., 2011). Despite their structural similarity, these enzymes catalyse different reactions. Bioinformatically, in order to understand whether cyanobacteria possess either gene (i.e. *sbnA/sbnB* and/or *vioK/vioB*), it is necessary to be able to distinguish between the pairs of homologous enzymes (i.e. SbnA/-VioB/cysteine synthase and SbnB/VioK). Analysis of SbnA crystals from *Staphylococcus aureus* incubated with OPS revealed the structure of the intermediate *N*-(1-amino-1-carboxyl-2-ethyl)-S-glutamate (syn: *N*-(1-amino-1-carboxyl-2-ethyl)glutamic acid, ACEGA), allowing the identification of three conserved active site residues: arginine at position 132 (R132), tyrosine at position 152 (Y152) and serine at position (S185), essential for the highly specific OPS recognition and turnover and intermediate formation (Kobylarz et al., 2016).

Site-directed mutagenesis studies by Kobylarz et al. (2016) showed that, in addition to R132, Y152 and S185, substrate specificity also required the positively-charged residues lysine at position 100 (K100), and arginine at position 224 (R224), thought to be responsible for S-glutamate binding. A four amino acid insertion: glycine at position 126 and 127 (G126, G127), tyrosine at position 128 (Y128), and leucine at position 129 (L129) was also shown to be moderately conserved in SbnA and in homologs that biosynthesise 2,3-DAP (Kobylarz et al., 2016). The substrate specificity of SbnB is still uncertain (Beasley et al., 2011). Nevertheless, it was shown to differ from ornithine cyclodeaminases (including VioK) and alanine dehydrogenases as its active site has been expanded to accommodate a larger substrate than ornithine or alanine (Kobylarz et al., 2014). This is evident by the presence of an arginine at position 94 (R94), forming a salt bridge to the ACEGA terminal carboxylate (Kobylarz et al., 2014).

Two cyanobacterial species appear to encode both SbnA and SbnB: *Coleofasciculus chthonoplastes* (Gomont 1892) Siegesmund et al. (2008) (Coleofasciculaceae) and *Moorea producens* Engene and Tronholm, 2019 (Oscillatoriaceae) (Fig. 6). In these species, the corresponding catalytic residues characteristic of SbnA (i.e. K100, G126, L129, R132, Y152, S185, and R224) and SbnB (i.e. R94) are conserved (Supplementary Table S5-S6). Other protein sequences, functionally annotated as cysteine synthase family proteins, include the corresponding residues K100, G126, R132, and S185 (Supplementary Table S5). The possibility exists that these enzymes perform the same catalytic functions as SbnA. No species appear to have orphan copies of *sbnA* or *sbnB* (i.e. encoding SbnA but not SbnB, or *vice-versa*).

In the cyanobacterium *Coleofasciculus chthonoplastes,* putative *sbnAB* appear together and are co-localised within an NRPS/PKS1 cluster (Fig. 7). The product of this cluster is unknown, but the complex resembles the NPRS assembly responsible for synthesising the siderophore amonabactin P 750 in *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966 (Aeromonadaceae) (Barghouthi et al., 1989). Given that *sbnAB* are involved in the biosynthesis of staphyloferrin B in *Staphylococcus aureus*, and that the NRPS cluster containing these genes in cyanobacteria is similar to that of a siderophore-producing cluster in *Aeromonas hydrophila*, we hypothesise that, in some cyanobacteria, 2,3-DAP is probably

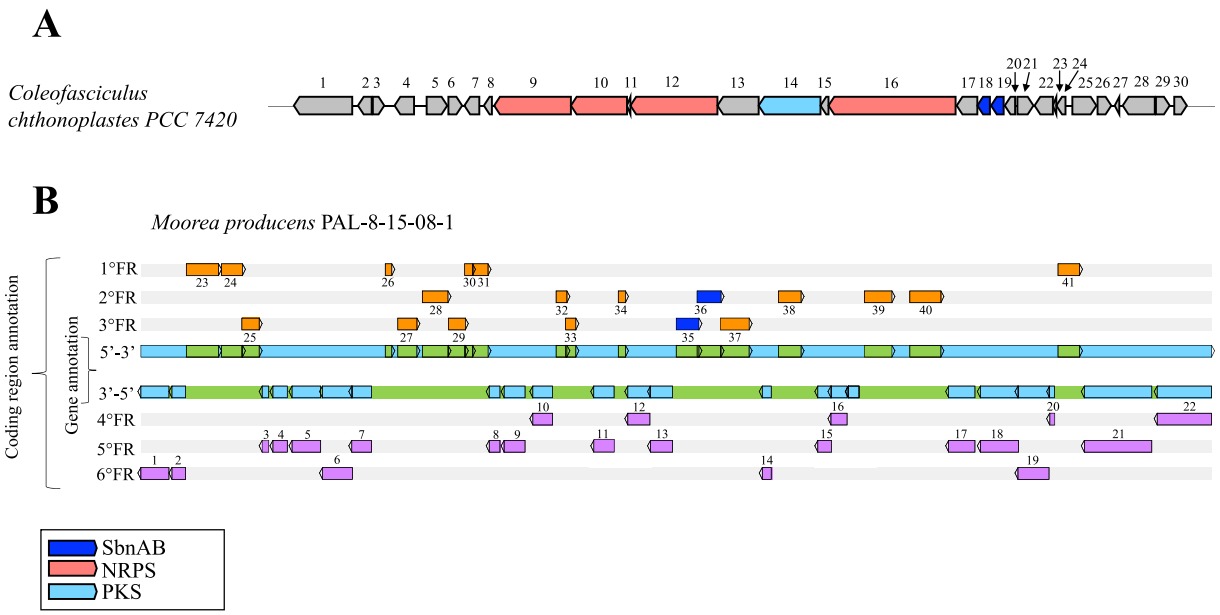

Fig. 7. Localisation and characterisation of the genomic neighbourhood surrounding the enzymes SbnA and SbnB in two cyanobacterial species. (A) In *Coleofasciculus chthonoplastes* PCC 7420 (Coleofasciculaceae), SbnAB (EC 2.5.1.140, 1.5.1.51) (in dark blue) are co-localised within a hybrid NRPS/PKS cluster, the product of which is currently unknown. The gene cluster was adapted from antiSMASH (v.5.1.2). (B) In *Moorea producens* PAL-8-15-08-1 (Oscillatoriaceae), SbnAB are found adjacent to each other on the genome, but are not co-localised within a specialised metabolite cluster. SbnA and SbnB appear on the third and second frames of translation (FR), respectively. The genomic neighbourhood was adapted from Artemis Comparison Tool (ACT; Carver et al., 2005). For protein accession numbers and functional annotations, see Supplementary Table S22. Arrows indicate the orientation of transcription. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

involved in the production of iron-chelators. Our previous study indicated the same physiological role for 2,4-DAB, a structural isomer of BMAA (Mantas et al., 2021). Nevertheless, in the absence of a nearby methyltransferase, a feasible route to BMAA is lacking in *Coleofasciculus chthonoplastes*. In the cyanobacterium *Moorea producens,* putative *sbnAB* appear adjacent on the genome, but are not co-localised within a specialised metabolite cluster, and do not have an adjoining methyltransferase (Fig. 7). Nearby genes are not suggestive of any specific biosynthetic pathway, indicating multiple and different roles of 2,3-DAP in cyanobacteria.

Moorea producens and *Coleofasciculus chthonoplastes* are in the same clade of the cyanobacterial species phylogeny (Fig. 6). Both are marine species, prone to forming thick mats and blooms (Siegesmund et al., 2008; Engene et al., 2012). Our results suggest that, although never detected/quantified, 2,3-DAP can potentially be biosynthesised in cyanobacteria through a pathway similar to that used by *Staphylococcus aureus* for the synthesis of SB. This pathway to 2,3-DAP does not appear to be widespread in cyanobacteria.

Homologs of SbnA and SbnB have also been described in a novel NRPS cluster responsible for poly(S-diaminopropanoic acid) synthesis in *Streptomyces albulus* PD-1 (Streptomycetaceae) (NjxA and NjxB) (Xu et al., 2015), and in the gene cluster for sulfazecin in *Pseudomonas acidophila* Imada et al. 1980 (Burkholderiaceae) (SulG and SulH) (Li et al., 2017) (note, diaminopropionic acid is a valid synonym of diaminopropanoic acid). In *Streptomyces albulus* PD-1, NjxA and NjxB, homologous to cysteine synthase/serine dehydratase and OCD respectively, use S-serine and S-ornithine as substrates and lack the conserved residues indicated in SbnA and SbnB (Supplementary Table S7-S8). From the description of Xu et al. (2015), it appears that NjxA and NjxB are more structurally similar to VioK and VioB, than to SbnA and SbnB. The substrate specificities of SulG, homologous to OCD, and SulH, homologous to cysteine synthase, have not been characterised. However, they too lack the conserved residues encoded in SbnA and SbnB (Supplementary Table S7-S8). It is possible that cyanobacterial homologs of SbnA and SbnB, with distinct amino acid residues at the catalytic site, are still capable of synthesising 2,3-DAP.

In *S. albulus* PD-1, the genes responsible for 2,3-DAP biosynthesis (NjxA nd NjxB) were found to be adjacent on the genome, but not co-localised within poly(S-diaminopropionic acid) synthetase gene clusters (Xu et al., 2015). The possibility that putative SbnA and SbnB in the cyanobacterium *Moorea producens* are responsible for the biosynthesis of 2,3-DAP for use in an unidentified, specialised metabolite cluster cannot be disregarded.

In contrast to SbnA and SbnB, to date, the active sites of VioK and VioB have not been characterised. Provisionally, VioB and VioK may be distinguished from their homologs SbnA and SbnB by functional annotation in bioinformatics databases. VioK can also be differentiated from SbnB by excluding homologous sequences encompassing active site residues characteristic of SbnB (Kobylarz et al., 2014). Given that VioB uses nitrogen atoms from ammonium as the nucleophile, while cysteine synthase uses sulfur atoms from hydrogen sulfide (Thomas et al., 2003), and that VioB uses OAS as substrate whilst SbnA uses OPS, it is not expected that these enzymes (i.e. VioB/cysteine synthase and VioB/SbnA) share identical catalytic sites (i.e. VioB sequences are not expected to contain residues A132, F152 and G185, characteristic of cysteine synthases, nor residues R132, Y152, and S185, characteristic of SbnA). The gene product of *vioB*, S-2,3-diaminopropionate synthetase, does not have an assigned enzyme commission (EC) number and searches in protein sequence databases yield no results with the same name. Until the active sites of VioK and VioB are described, the possibility that these enzymes share the same catalytic residues as SbnB and SbnA/cysteine synthase, respectively, cannot be excluded.

Two cyanobacterial species appear to have *vioK* genes, coding for ornithine cyclodeaminase: *Phormidium willei* (Gardner, 1927) Anagnostidis and Komarek 1988 (Oscillatoriaceae) and *Mastigocoleus testarum* Lagerheim ex Bornet et Flahault 1887 (Hapalosiphonaceae) (Supplementary Table S9). The putative gene was not included in a specialised metabolite cluster, and no genes functionally annotated to a methyltransferase were found in its genetic neighbourhood (Fig. 8). Although *vioK* was found in two cyanobacteria, it is not accompanied by

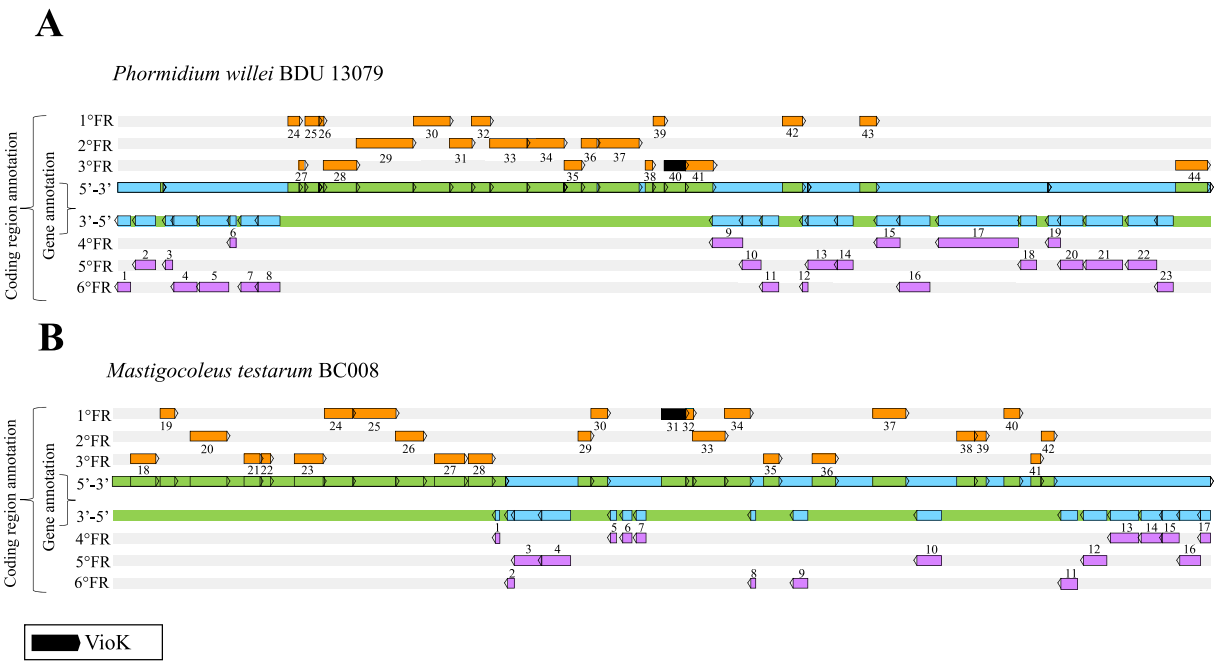

Fig. 8. Localisation and characterisation of the genomic neighbourhood surrounding the enzyme VioK in two cyanobacterial species. VioK is present in both *Phormidium willei* BDU 13079 (Oscillatoriaceae) (A) and *Mastigocoleus testarum* BC008 (Hapalosiphonaceae) (B), but is not co-localised within a specialised metabolite cluster. (A) VioK appears on the third frame of translation (FR), adjacent to a protein functionally annotated as FAD-dependent oxidoreductase. (B) VioK appears on the first frame of translation (FR), adjacent to a protein functionally annotated as (2Fe–2S)-binding protein. The genomic neighbourhood was adapted from Artemis Comparison Tool (ACT). For protein accession numbers and functional annotations, see Supplementary Table S23. Arrows indicate the orientation of transcription.

vioB. It is unclear whether the species encoding a gene for OCD have developed new physiological roles for the protein, or if a gene coding for S-2,3-diaminopropionate synthetase was lost from these species. In the apparent absence of VioB and given the seeming restricted distribution of OCD, the production of 2,3-DAP, and hence, of BMAA in cyanobacteria is unlikely to occur via a pathway analogous to that present in *Streptomyces* and *Clostridium*.

From the cyanobacterial species that appear to have either *sbnAB* or *vioK* (Fig. 6), only *Coleofasciculus chthonoplastes* was sequenced from an axenic monocyanobacterial culture (Mantas et al., 2021, their Supplementary Table S6). It is believed that this species can biosynthesise 2, 3-DAP through an analogous pathway to that of *Staphylococcus aureus*, since contamination by other potential 2,3-DAP-producing bacteria can be excluded. Results from the remaining species would have to be verified in genomes sequenced from axenic cultures.

3.4. The search for the pam gene cluster in cyanobacterial, Paenibacillus, and diatom species

The *pam* gene cluster, encoding a bound form of BMAA, has been found in *Paenibacillus larvae*. The gene products of *pamS* and *pamR* are responsible for the biosynthesis of 2,3-DAP, the methylation of which within the metabolite cluster could form BMAA (Müller et al., 2014). Since in the similar complex peptide galantin 1, only one of the two 2, 3-DAP residues is methylated, and in the paenilamicins, both 2,3-DAP residues are methylated, it is possible that the *pam* gene cluster first assembles 2,3-DAP and then methylates it within the complex.

Although it was not specified by Kobylarz et al. (2014), our results show that the 2,3-DAP constituent of paenilamicin is probably derived from the concerted action of genes homologous to *sbnAB*, as the active sites of both SbnA and SbnB are conserved in PamR and PamS (Supplementary Table S7-S8). In the peptide antibiotic dapdiamide from *Pantoea agglomerans*, the genes responsible for 2,3-DAP production (*ddaA* and *ddaB*) are also homologs of *sbnA/sbnB*, with conservation of the enzyme's catalytic site (Supplementary Table S7-8). It appears that, when present, 2,3-DAP, and possibly BMAA, are most likely derived from the action of *sbnAB* rather than of *vioB/vioK*.

Our results confirm that the paenilamicin gene cluster occurs in 10 *Paenibacillus larvae* genomes (Supplementary Table S10). However, evidence for the presence of the hybrid *pam* gene cluster was not found in any of the 130 cyanobacterial genomes included in our dataset (Supplementary Table S11), or in an additional 93 *Paenibacillus* genomes other than *P. larvae* (Supplementary Table S12), or in the genomes of two BMAA-producing diatom species, *Thalassiosira pseudonana* CCMP1335 (Thalassiosiraceae) and *Phaeodactylum tricornutum* CCAP 1055/1 (Phaeodactylaceae) (Jiang et al., 2014; Réveillon et al., 2016) (Supplementary Tables S13-S14).

In the cyanobacterial species examined, the *pam* gene cluster does not appear to be a viable route of BMAA biosynthesis. So far, this specialised cluster appears to be restricted to *Paenibacillus larvae*, suggesting that paenilamicin production is likely not a widespread environmental source of the neurotoxin BMAA. Given the limited number of published diatom genomes, it cannot be concluded that the *pam* gene cluster is absent in all such species. However, our results indicate that, when BMAA is present in diatoms, it is probably not derived from paenilamicin-like peptides. The reduced number of specialised secondary clusters in the diatom species investigated (Supplementary Table S13-S14), and the absence of genes homologous to those implicated in 2,3-DAP synthesis suggest that, in these species, BMAA is probably either acquired from other sources (i.e. not by direct biosynthesis by the diatoms themselves), or that additional unknown routes to BMAA biosynthesis exist.

It is intriguing that a metabolically expensive and complex peptide such as paenilamicin can apparently only be found in *P. larvae*. It may be that the *pam* gene cluster represents a specialised version of a more general mechanism. However, although *Paenibacillus* spp. other than *P. larvae* do not appear to synthesise BMAA via the *pam* gene cluster, many species, including those isolated from human cerebrospinal fluid (Hehnly et al., 2020), appear to be able to synthesise other compounds, such as ectoines, derived from the BMAA structural isomer 2,4-DAB

(Nunn and Codd, 2017; Mantas et al., 2021; Supplementary Table S12). The neurological significance of the widespread occurrence of *Paenibacillus* spp. in milk and prepared foods merits investigation (Nunn and Codd, 2019).

3.5. A potential explanation for the apparent absence of 2,3-DAP in cyanobacteria

2,3-DAP expresses severe cytotoxicity in some cell types, and many organisms have developed strategies that prevent endogenous accumulation of specific amino acids (Ernst et al., 2016). DAPAL (EC 4.3.1.15) is a prokaryotic type II PLP-dependent enzyme that catalyses the degradation of R- and S-forms of 2,3-DAP to 2-aminoacrylate and ammonium (Bisht et al., 2012) (Fig. 9). 2-aminoacrylate is a three-carbon reactive enamine intermediate synthesised by several PLP-dependent enzymes (Downs and Ernst, 2015). This compound is formed in the reaction catalysed by VioK/VioB; however, there it is contained, bound to a specific position within the enzyme's catalytic site. 2-aminoacrylate is susceptible to tautomerisation and spontaneous hydrolysis in solvent water, releasing ammonium and pyruvate (Datta and Bhadra, 1978). It can also react with other compounds, leading to covalent modification and the inactivation of enzymes involved in essential metabolic processes (Flynn and Downs, 2013; Downs and Ernst, 2015) (Fig. 9).

DAPAL catalyses analogous reactions and is known to share significant amino acid sequence identity with type II PLP-dependent enzymes (Bisht et al., 2012), especially with the biosynthetic serine/threonine dehydratase/deaminase (TDH, EC 4.3.1.19), where sequence similarity can be up to 55% (Khan et al., 2003). The active site of DAPAL has been described in *Escherichia coli* K-12 (Enterobacteriaceae) (*Ec*DAPAL) (Bisht et al., 2012) and *Salmonella enterica* serovar Typhimurium LT2 (Enterobacteriaceae) (*St*DAPAL) (Nagasawa et al., 1988). In *Escherichia coli*, the residues lysine at position 77 (K77), aspartic acid at positions 120 and 189 (D120, D189), and tyrosine at position 168 (Y168) are important for catalytic function (Bisht et al., 2012). K77 and D120 are potentially responsible for the abstraction of protons from the R- and S-isoforms of 2,3-DAP, respectively (Bisht et al., 2012). All residues contained within the catalytic domain of TDH are conserved in *Ec*DAPAL (Uo et al., 2002).

The similarities between DAPAL and other PLP-dependent enzymes, the fact that DAPAL exhibits amino acid sequence dissimilarity in closely related species (Khan et al., 2003; Kalyani et al., 2012; Deka et al., 2018), and that the structure of DAPAL has not been characterised in cyanobacteria, hinder the search and distinction of DAPAL homologs in the 130 cyanobacterial genomes analysed. However, an enzyme functionally annotated as DAPAL and exhibiting amino acid residues similar to *Ec*DAPAL is present in the cyanobacterium *Lyngbya confervoides* (Fig. 6). Additionally, 108 cyanobacterial species were found to encode enzymes functionally annotated to biosynthetic threonine ammonia-lyases (Supplementary Table S15). Residue D120 is not conserved in any of the cyanobacterial homologs evaluated, including in *L. confervoides,* suggesting a different mode of abstraction of C1 protons from the S-DAP isoform, or loss of function. Given the observed similarities between the catalytic cleft of *Ec/St*DAPAL and cyanobacterial biosynthetic threonine ammonia-lyases (Supplementary Table S15), it is possible that the ability to degrade 2,3-DAP into 2-aminoacrylate and ammonium is widespread in cyanobacteria. Purification and molecular characterisation studies of cyanobacterial DAPAL are needed to clarify the prevalence and action of the enzyme in these species.

In this pathway (Fig. 9), RidA (EC 3.5.99.10) is required to quench 2-aminoacrylate, preventing enamine stress (Downs and Ernst, 2015). RidA is apparently present across all life, with the vast majority of free-living organisms encoding at least one RidA homolog (Irons et al., 2020). It has a defined role as a 2-aminoacrylate-stress modulator, responding to endogenously generated reactive metabolites, by facilitating and enhancing the rate of hydrolysis of the enamine or its tautomer to pyruvate and ammonium, thereby detoxifying 2-aminoacrylate and averting metabolic imbalance (Lambrecht et al., 2012, 2013; Downs and Ernst, 2015). RidA proteins appear to play diverse, but important, molecular functions, many of which are still poorly understood (Oka et al., 1995; Goupil-Feuillerat et al., 1997; Asagi et al., 1998). Crystal structure studies in *Escherichia coli* identified a signature of five conserved amino acids (Liu et al., 2016), although only a highly conserved arginine at position 105 (R105) was shown to be strictly necessary and sufficient for biochemical activity (Burman et al., 2007; Lambrecht et al., 2012). The residues glutamic acid at position 120 (E120), tyrosine at position 17 (Y17), serine at position 30 (S30), and asparagine at position 88 (N88) were also shown to be important for RidA catalytic activity (Burman et al., 2007; Lambrecht et al., 2012; Degani et al., 2018).

Putative RidA appears to be omnipresent in cyanobacteria. 4275 homologs of Rid family proteins were found, 611 of which have a conserved arginine residue. Only seven cyanobacterial species appear to lack a RidA homolog with a conserved arginine at the catalytic site (Supplementary Table S16) (Fig. 6). There are several species encoding homologs with full amino acid sequence conservation at the active site. Many of these homologs are annotated as hypothetical proteins, and less frequently as endoribonuclease L-PSP (liver-perchloric acid-soluble protein). Apart from the active site, amino acid sequence identity was low between cyanobacterial homologs and those from other bacteria, yeast and mammals. This is consistent with the data from Mistiniene et al. (2003) and Parsons et al. (2003) who found amino acid sequence identity to vary between RidA homologs from different origins, with some members sharing <8% sequence similarity. The observed

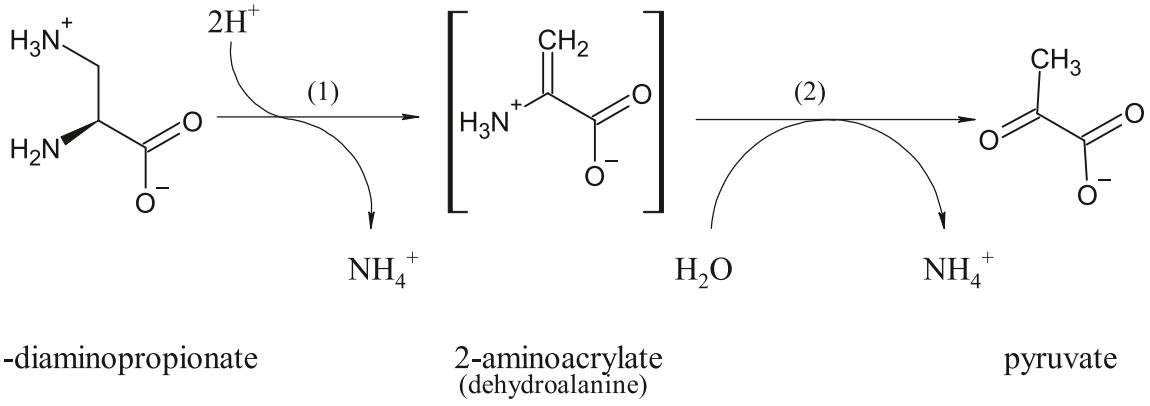

Fig. 9. Pathway of the degradation of 2,3-diaminopropanoate to pyruvate and ammonium. Redrawn from Bisht et al. (2012) and Lambrecht et al. (2012). The nomenclature is from www.brenda-enzymes.org; Enzyme Commission numbers are as follows: (1) 2,3-diaminopropionate ammonia-lyase (DAPAL): EC 4.3.1.15; (2) reactive intermediate deaminase A (RidA): EC 3.5.99.10. The charged forms of the amino acids are those predominating at physiological pH values.

sequence diversity and variation in functional annotation corroborates the premise that RidA may execute myriad different roles (Niehaus et al., 2015).

Our results show that the failure to detect free 2,3-DAP in cyanobacteria may be explained by the presence of DAPAL and RidA. DAPAL would catalyse the synthesis of 2-aminoacrylate and ammonium from 2,3-DAP (Fig. 9). If free 2,3-DAP and the ammonia-lyase were present in cyanobacteria, RidA would be required to quench 2-aminoacrylate, preventing disruption of cellular functions (Downs and Ernst, 2015). The expression of RidA has been shown to be inversely correlated with iron availability (Irons et al., 2020). In conditions of iron limitation, cyanobacteria produce siderophores (Whitton, 2012). If cyanobacteria can synthesise 2,3-DAP-based iron-chelators, this would corroborate our hypothesis that, in some species, 2,3-DAP and hence BMAA, may be involved in siderophore formation.

3.6. Comparison between bioinformatics results and the analytical chemistry data from the literature

Following the methodology of Mantas et al. (2021), our bioinformatics findings are compared with those of 28 biochemical studies where evidence for BMAA-, or BMAA- and 2-4-DAB-producing cyanobacteria has been provided (Supplementary Table S17). A scenario where a particular species is shown to possess the necessary genes for BMAA biosynthesis, and is proven to produce the same compound via analytical studies, serves as a strong indicator that this species is a BMAA-producer. However, a species that lacks known genes required for BMAA formation, but is shown by analytical studies to produce the neurotoxin could be indicative of a false-positive result derived from an ambiguous analytical approach (especially probable if non-axenic cyanobacterial cultures and/or low-specificity analytical methods were employed), or suggests the existence of other, still undescribed routes for BMAA biosynthesis. 24 of the 28 studies used variations of MS/MS for BMAA/2,4-DAB identification and quantification, and more than one analytical method was commonly used (either to test the accuracy and specificity of one analytical method compared to others, or to cross-check results). Only seven analyses for the neurotoxin itself were derived from all-axenic cultures, and some surveys involved a combination of axenic cultures, non-axenic monocyanobacterial cultures, and environmental samples. Others did not report on the culture type of the isolates studied. Our 130 cyanobacterial species genomic dataset and the compilation of BMAA production from the literature overlapped in seven species: *Prochlorococcus marinus* Chisholm et al. 2001 (Prochlorococcaceae), *Planktothrix agardhii* (Gomont 1892) Anagnostidis and Komarek 1988 (Microcoleaceae), *Aphanizomenon flos-aquae* (L.) Ralfs (Aphanizomenonaceae), *Cylindrospermopsis raciborskii* (Wołosz.) Seenayya & Subbaraju (Aphanizomenonaceae), *Nodularia spumigena* Mertens ex Bornet and Flahault, 1888 (Aphanizomenonaceae), *Microcystis aeruginosa* (Kutzing) Kutzing 1846 (Microcystaceae) and *Microcoleus vaginatus* Gomont ex Gomont 1892 (Microcoleaceae). None of these common bloom-forming species (Whitton, 2012) appear to have genes coding for relevant enzymes in the pathways to 2,3-DAP/BMAA described above (Figs. 2–5), or specialised gene clusters analogous to those of dapdiamides, galatins, or paenilamicin peptides. Apart from the *Microcystis aeruginosa* used by Downing et al. (2011), the studies that have used these species for the identification and quantification of BMAA employed analytical techniques other than MS/MS, and/or used non-axenic cyanobacterial cultures (i.e. non-axenic monocyanobacterial cultures and/or environmental samples) in their analyses. It cannot be excluded that these correspond to false-positive results as a consequence of the use of low-specificity analytical techniques and non-axenic cultures. However, it is also possible that cyanobacteria synthesise BMAA through a yet-undiscovered route or routes.

4. Summary and conclusions

Our bioinformatics survey has provided insight into potential pathways for the biosynthesis of 2,3-DAP/BMAA in cyanobacteria, which can lead to future experimental investigations. The bioinformatics results indicate potential 2,3-DAP biosynthesis in some cyanobacterial species via the staphyloferrin B pathway. The fact that, in *Staphylococcus aureus*, 2,3-DAP is synthesised via *sbnAB* for inclusion in siderophores, and that in one cyanobacterial species (*Coleofasciculus chthonoplastes*) putative *sbnA* and *sbnB* were found to be co-localised within an NRPS/PKS1 cluster similar to amonabactin P 750 from *Aeromonas hydrophila*, suggests a functional association with siderophore biosynthesis. 2,3-DAP also appears to be synthesised by homologs of *sbnAB* in the chemoheterotrophic bacteria *Paenibacillus larvae* (*pamRS*) and *Paenibacillus agglomerans* (*ddaAB*), indicating that this is a more common route to free and bound 2,3-DAP, and potentially to BMAA, in prokaryotes (compared to the homologous *vioKB*).

A homolog of the *pam* gene cluster from *P. larvae* was not detected in any of the 130 cyanobacterial genomes in our analysis, two diatom species or *Paenibacillus* other than *P. larvae*. Other mechanisms may exist that remain to be discovered.

2,3-DAP has not been reported in cyanobacteria. We have shown that, in some species, the presence of putative DAPAL and RidA could explain the failure to detect this compound from cyanobacterial analytical results. RidA expression is upregulated during periods of iron scarcity, which corroborates our hypothesis that, in some cyanobacterial species, 2,3-DAP may be implicated in environmental iron-scavenging.

The existence of many homologs of enzymes potentially leading to 2,3-DAP and BMAA biosynthesis, and the presence of uncharacterised enzymes, with amino acid sequences and structural characterisation not yet available, hinder the search for possible pathways to 2,3-DAP and BMAA in cyanobacteria. Further information regarding the enzymes in the described pathways and their specificity is needed to further test our hypotheses. Also, analytical studies using MS/MS techniques and axenic cyanobacterial cultures would be helpful to ensure accurate and consistent results concerning the identification, origin(s) and quantification of BMAA. Ultimately, the ability to synthesise 2,3-DAP and BMAA does not appear to be universal among cyanobacteria. This suggests that either this compound is not widespread in cyanobacteria, or that there are additional and as yet unknown pathways for the synthesis of 2,3-DAP and BMAA.

5. Experimental

Methods are based on those of Mantas et al. (2021), summarised here. For further details, see Supplementary Section S7.

iTOL (Letunic and Bork, 2016) was used to re-annotate a previously published phylogeny of 130 high-quality cyanobacterial genomes of various culture types (Mantas et al., 2021).

From the protein sequence database at the NCBI (https://www.ncbi.nlm.nih.gov), amino acid sequences with the same enzyme nomenclature as in the BRENDA database were selected for each enzyme in the known pathways of 2,3-DAP and/or BMAA biosynthesis (Figs. 2–5) to build profile hidden Markov models (pHMMs) (Supplementary Section 7.1). The pHMMs for each enzyme were used to search proteins of the 130 cyanobacterial genomes for homologs using HMM search (HMMER package v.3.1b2, hmmer.org), with a default threshold (E-value ≤ 0.01). Where necessary to confirm absence, protein sequences for enzymes in the known pathways for 2,3-DAP and/or BMAA biosynthesis were also used as queries in BLAST searches (Altschul et al., 1997) against all cyanobacterial proteomes available at the NCBI.

To differentiate between enzymes present in the known pathways for 2,3-DAP and/or BMAA biosynthesis and homologous enzymes carrying out different functions, active site and substrate specificity searches were conducted for DUD, DHP, βUP, cysteine synthase, SbnA, SbnB, VioK, DAPAL, and RidA based on knowledge from the literature

(Supplementary Section 7.2). To further aid in differentiation, gene trees were reconstructed for DUD, DHP, βUP, cysteine synthase, SbnA, SbnB, VioK, and DAPAL using IQ-TREE (Nguyen et al., 2014) (Supplementary Fig. S1-S8).

AntiSMASH (bacterial version, v.5.1.2) (Blin et al., 2019) was used to search for the *pam* gene cluster of *P. larvae* (Supplementary Table 19) in the 130 cyanobacterial species in the dataset and in an additional 93 *Paenibacillus* spp. genomes (Supplementary Table S20). In two diatom genomes (Supplementary Table S21), sequenced from species previously shown to produce BMAA (Jiang et al., 2014; Réveillon et al., 2016), a combination of antiSMASH bacterial and plant (plantiSMASH) options were used to search for the *pam* gene cluster (Oliver et al., 2021).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at the University of Edinburgh's DataShare repository, https://doi.org/10.7488/ds/3270

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