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Abstract

Cyanobacteria are an ancient clade of photosynthetic prokaryotes, whose worldwide occurrence, especially in water, presents health hazards to humans and animals due to the production of a range of toxins (cyanotoxins). These include the sometimes co-occurring, non-encoded diaminoacid neurotoxins 2,4-diaminobutanoic acid (2,4-DAB) and its structural analogue β-N-methylaminoalanine (BMAA). Knowledge of the biosynthetic pathway for 2,4-DAB, and its role in cyanobacteria, is lacking. The aspartate 4-phosphate pathway is a known route of 2,4-DAB biosynthesis in other bacteria and in some plant species. Another pathway to 2,4-DAB has been described in *Lathyrus* species. Here, we use bioinformatics analyses to investigate hypotheses concerning 2,4-DAB biosynthesis in cyanobacteria. We assessed the presence or absence of each enzyme in candidate biosynthesis routes, the aspartate 4-phosphate pathway and a pathway to 2,4-DAB derived from S-adenosyl-L-methionine (SAM), in 130 cyanobacterial genomes using sequence alignment, profile hidden Markov models, substrate specificity/active site identification and the reconstruction of gene phylogenies. In the aspartate 4-phosphate pathway, for the 18 species encoding diaminobutanoate-2-oxo-glutarate transaminase, the co-localisation of genes encoding the transaminase with the downstream decarboxylase or ectoine synthase – often within hybrid non-ribosomal peptide synthetase (NRPS)-polyketide synthases (PKS) clusters, NRPS-independent siderophore (NIS) clusters and incomplete ectoine clusters – is compatible with the hypothesis that some cyanobacteria use the aspartate 4-phosphate pathway for 2,4-DAB production. Through this route, in cyanobacteria, 2,4-DAB may be functionally associated with environmental iron-scavenging, via the production of siderophores of the schizokinen/synechobactin type and of some polyamines. In the pathway to 2,4-DAB derived from SAM, eight cyanobacterial species encode homologs of SAM-dependent 3-amino-3-carboxypropyl transferases. Other enzymes in this pathway have not yet been purified or sequenced. Ultimately, the biosynthesis of 2,4-DAB appears to be either restricted to some cyanobacterial species, or there may be multiple and additional routes, and roles, for the synthesis of this neurotoxin.

Keywords

cyanobacteria, cyanotoxin, 2,4-diaminobutanoic acid, 2,4-DAB, 2,4-diaminobutyric acid, bioinformatics, diaminobutanoate-2-oxo-glutarate transaminase, diaminobutanoate decarboxylase, siderophore, ectoine.

1. Introduction

The non-encoded diaminoacid neurotoxin 2,4-diaminobutanoic acid (2,4-DAB) (syn: 2,4-diaminobutyric acid; α,γ-diaminobutyric acid; γ-aminobutyrine) (Fig. 1) was first discovered in acid hydrolysates of polymyxin antibiotics (Catch and Jones, 1948). It is widely distributed and occurs in the cell wall of Gram-positive bacteria (Perkins and Cummins, 1964), in the seeds of leguminous plants (Bell, 1962; Nigam and Ressler, 1966) and as a homopolymer in *Streptomyces celluloflavus* USE32 and *Streptoalloteichus hindustanus* NBRC15115 (Yamanaka et al., 2020). Both 2,4-DAB and its structural analogue β-N-methylaminoalanine (BMAA) are neurotoxic (Chen et al., 1972; Tan et al., 2018). These neurotoxins have also been found to be co-distributed in some cyanobacterial species (Al-Sammak et al., 2014; Rosén and Hellenäs, 2008), but may also occur alone (Krüger et al., 2010; Violi et al., 2019).

The biosynthetic pathway to 2,4-DAB is well documented in other bacteria (Yamamoto et al., 1992; Yamanaka et al., 2020) and in the legume *Lathyrus sylvestris* (Nigam and Ressler, 1966), but it has not been established in cyanobacteria. Also, the extent of 2,4-DAB biosynthesis among cyanobacteria has not been widely determined.

There are at least four factors contributing to the lack of knowledge concerning the presence of 2,4-DAB in cyanobacteria: (i) the biosynthetic pathway(s) in cyanobacteria are unknown; (ii) some early analytical techniques to analyse cyanobacterial extracts for the presence of 2,4-DAB and its analogues have lacked sensitivity and specificity (Cohen, 2012; Faassen et al., 2016; Jiang et al., 2013; Lage et al., 2016; Rosén et al., 2016); (iii) whilst adequate analytical methods can unambiguously assign 2,4-DAB biosynthesis to a monocyanobacterial axenic culture, ambiguity can arise regarding toxin origin when non-axenic cyanobacterial environmental samples and laboratory cultures, and mixtures of cyanobacterial species/strains are examined; (iv) the facultative expression of 2,4-DAB biosynthesis means that lack of evidence for this toxin is not evidence of its absence under all conditions. Some of these problems are beginning to be resolved. For example, although a consensual standard method of sample preparation and analytical techniques for the identification of 2,4-DAB and BMAA has been lacking, it is now widely agreed that tandem mass spectrometry (MS/MS) is the best currently available tool for the correct identification and quantification of these neurotoxins, as it relies on four identification criteria, whilst resolving structural isomers,

improving detection limits and reducing background interference (Faassen et al. 2016; Faassen, 2017; Metcalf et al., 2017).

The environmental and toxicological monitoring of cyanobacterial mass populations, for example by microscopy and by chemical, immunological, toxicological and remote sensing methods (Meriluoto et al., 2017), is being successfully complemented by molecular genetic methods (Kurmayer et al., 2017; Pan et al., 2002). The latter include the targeting of genes involved in the biosynthesis of specific cyanotoxins by polymerase chain reaction (PCR)-based methods (Humbert, 2017; Kurmayer et al., 2017). The contribution of molecular genetic methods to the early detection of cyanobacteria with the potential for the production of 2,4-DAB and its isomers, would similarly contribute to the overall risk management of the production of these neurotoxins. To enable this, knowledge of the biosynthetic pathway(s) for 2,4-DAB formation, with gene identification is necessary. These needs are addressed here by our bioinformatics study to test hypotheses concerning the biosynthetic pathway or pathways for 2,4-DAB in cyanobacteria.

As suggested by Nunn and Codd (2017), a possible route for the biosynthesis of 2,4-DAB is through the aspartate 4-phosphate pathway (Fig. 2). This pathway is utilised by bacteria, fungi and higher plants to produce four encoded amino acids; methionine, threonine, isoleucine and lysine, at various branchpoints (Jander and Joshi, 2010). The pathway starts at aspartate transaminase (EC 2.6.1.1), which catalyses a reversible transamination between glutamate and oxaloacetate to yield aspartate and 2-oxo-glutarate (Jansonius and Vincent, 1987). Aspartate then serves as substrate for aspartate kinase (EC 2.7.2.4), which catalyses the formation of aspartate 4-phosphate. Aspartate 4-semialdehyde is then synthesised from aspartate 4-phosphate via the action of aspartate-semialdehyde dehydrogenase (EC 1.2.1.11) (Jander and Joshi, 2010). These three enzymes are widespread in prokaryotes and are essential for their viability (Cohen, 1987; Muriana et al., 1991).

1.1. The aspartate 4-phosphate pathway and the biosynthesis of 2,4-DAB

An additional branch-point from this pathway at aspartate 4-semialdehyde begins with diaminobutanoate-2-oxo-glutarate transaminase (EC 2.6.1.76), which catalyses the biosynthesis of 2,4-DAB. 2,4-DAB has been described as a precursor for the biosynthesis of

1,3-diaminopropane (Ikai and Yamamoto, 1997; Yamamoto et al., 1992) (Fig. 3), and the compatible solutes ectoine and hydroxyectoine (Louis and Galinski, 1997; Reuter et al., 2010) (Fig. 2). In *Acinetobacter baumannii*, 2,4-DAB is also biosynthesised by the activity of diaminobutanoate-2-oxo-glutarate transaminase, utilising aspartate 4-semialdehyde as substrate (Ikai and Yamamoto, 1997). This diaminoacid is subject to decarboxylation by the activity of 2,4-DAB decarboxylase (EC 4.1.1.86) to yield 1,3-diaminopropane (Yamamoto et al., 2000; Yamamoto et al., 1992). Transaminases can exhibit multiple substrate specificity, and the substrate for the 2,4-DAB transaminase may differ. In a species of *Xanthomonas*, 2,4-DAB has been shown to be synthesised by a transaminase for 2,4-DAB, diaminobutanoate-pyruvate transaminase (EC 2.6.1.46), which uses pyruvate, instead of 2-oxo-glutarate, as the oxo-acid (Rao et al., 1969). In organisms that produce the hydrogenated pyrimidine derivative ectoine, an *ect* operon is usually found, encompassing the genes *ectB* (encoding diaminobutanoate-2-oxo-glutarate transaminase), *ectA* (encoding 2,4-diaminobutanoate acetyltransferase, EC 2.3.1.178) and *ectC* (encoding ectoine synthase, EC 4.2.1.108). EctC can be regarded as a marker enzyme for ectoine-producers (Widderich et al., 2014a). The route to ectoine biosynthesis was elucidated by Peters et al. (1990) in cell-free extracts of *Ectothiorhodospira halochloris* and *Halomonas elongata*. It begins with the transamination of aspartate 4-semialdehyde, catalysed by diaminobutanoate-2-oxo-glutarate transaminase (enzyme 4, Fig. 2), to yield 2,4-DAB, which serves as substrate for diaminobutanoate acetyltransferase (enzyme 5, Fig. 2) to produce N^4-acetyl-2,4-diaminobutanoic acid. Ectoine synthase (enzyme 6, Fig. 2) catalyses the ring closure reaction, leading to the end product, ectoine. A fraction of the organisms that encode the ectoine operon are capable of producing 5-hydroxyectoine through a position- and stereo- specific hydroxylation of ectoine, via the activity of ectoine hydroxylase (*ectD,* EC 1.14.11.55) (Bursy et al., 2007). Although compatible solutes are known in many cyanobacteria (Klähn and Hagemann, 2011), ectoine and hydroxyectoine have not been identified in these organisms (Nunn and Codd, 2017). Until this present investigation, no genetic investigations have attempted to establish whether the biosynthesis of 2,4-DAB in cyanobacteria occurs through the aspartate 4-phosphate pathway.

In prokaryotes, toxicity-related genes are often found within specialised metabolite clusters (secondary metabolite clusters), which are synthesised by a process external to the default ribosomal system, via a non-ribosomal synthetic mechanism (NRPS) (Jones et al., 2010), which involves a multienzyme complex (Miller and Gulick, 2016). Compounds that, unlike

peptides, do not contain nitrogen, may be biosynthesised by similar enzyme assemblies, named polyketide synthetases (PKS) (Robbins et al., 2016), and others can be synthesised through hybrid NRPS/PKS complexes (Fisch, 2013). Many classes of cyanotoxins, including cylindrospermopsins, saxitoxins, nodularins and microcystins are known to be produced via these routes (Kellmann et al., 2008; Mbedi et al., 2005; Mihali et al., 2008; Moffitt and Neilan, 2004). A third and less widespread category of multienzyme complexes exists in cyanobacteria, namely the NRPS-independent siderophore biosynthesis systems (NIS). NIS clusters are responsible for the production of siderophores, including rhizobactin 1021, schizokinen and synechobactin (Challis, 2005; Årstøl and Hohmann-Marriott, 2019).

1.2 Siderophores

Siderophores are low molecular weight (400-1000kDa) carrier molecules that function in microbial iron uptake (Simpson and Neilands, 1976). These iron-chelators are usually Fe(III)-specific, and bind Fe(III) with an affinity of the order of 10^{30}. Of the compounds that are used as siderophores by cyanobacteria, two - schizokinen and synechobactin - have been shown to be synthesised following the decarboxylation of 2,4-DAB (Balasubramanian et al., 2006; Nicolaisen et al., 2008; Årstøl and Hohmann-Marriott, 2019).

The low concentration of iron (picomolar to nanomolar range) in the environment is typically rate-limiting for the growth of cyanobacteria and siderophores are essential to reconcile the profound insolubility of Fe(III) with the vital need of iron in cellular activities (Cunningham and John, 2017; Sunda and Huntsman, 2015). Cyanobacteria have larger iron requirements than non-photosynthetic bacteria (Shcolnick and Keren, 2006), as the processes of photosynthesis and, in some species, of N_2 fixation, require Fe-containing ferredoxin and nitrogenase, respectively (Simpson and Neilands, 1976). Cyanobacteria capable of producing siderophores under Fe-limiting conditions are probably at a selective advantage in microbial competition, protection from heavy-metal toxicity and bloom formation (Simpson and Neilands, 1976; Årstøl and Hohmann-Marriott, 2019).

Schizokinen, first discovered in *Bacillus megaterium* ATCC 19213 and isolated from *Anabaena* sp. strains PCC 7120 and PCC 6411 (Goldman et al., 1983; Simpson and Neilands, 1976), contains a derivative of 1,3-diaminopropane (Nunn and Codd, 2017). Synechobactins,

amphiphilic cyanobacterial siderophores from *Synechococcus* sp. PCC 7002 (Armstrong and Van Baalen, 1979) are identical in structure to schizokinen, except for the fully saturated fatty acid tail on one of the two α-hydroxamate groups (Ito and Butler, 2005), but also contain the same derivative of 1,3-diaminopropane (Nunn and Codd, 2017). There is currently no biochemically substantiated biosynthetic pathway established for the cyanobacterial siderophores schizokinen and synechobactin. However, it is known that citrate-based siderophores are produced by NRPS-independent synthases (NIS) in cyanobacteria (Årstøl and Hohmann-Marriott, 2019), and that the two siderophores are structurally similar to other dihydroxamate-type siderophores, such as rhizobactin 1021 (Persmark et al., 1993) and aerobactin (de Lorenzo et al., 1986). *A priori*, we hypothesise that cyanobacteria use the same metabolic route. Rhizobactin 1021 is synthesised from the hydroxylation and acetylation of two molecules of 1,3-diaminopropane, which combined with citrate by a NIS synthetase, yields schizokinen (Lynch et al., 2001) (Fig. 4).

1.3. Polyamines

Polyamines, polycationic hydrocarbon molecules with multiple amino groups, have important roles in prokaryotic protein synthesis (Igarashi and Kashiwagi, 2000; Shah and Swiatlo, 2008), also contributing to cyanobacterial bloom formation and siderophore production (Keating et al., 2002). Spermidine, a widespread polyamine in all groups of cyanobacteria (Hosoya et al., 2005), can be synthesised from putrescine via two different routes (Fig. 5). Putrescine biosynthesis is shared by both pathways and may occur via ornithine, through the action of ornithine decarboxylase (EC 4.1.1.17) or via arginine, which serves as the initial substrate for arginine decarboxylase (EC 4.1.1.19), agmatine iminohydrolase (EC 3.5.3.12) and *N*-carbamoylputrescine amidohydrolase (EC 3.5.1.53) (Zhu et al., 2015). The classical pathway begins with the decarboxylation of SAM by S-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50). The decarboxylated SAM and putrescine serve as substrates for the biosynthesis of spermidine, in a reaction catalysed by spermidine synthase (SPDS, EC 2.5.1.16) (Lee et al., 2009; Shah and Swiatlo, 2008). In the alternative pathway, carboxyspermidine dehydrogenase (CASDH, EC 1.5.1.43) catalyses the reaction between aspartate 4-semialdehyde and putrescine that leads to carboxyspermidine, which is then decarboxylated by carboxyspermidine decarboxylase (CASDC, EC 4.1.1.96) to yield spermidine (Hanfrey et al., 2011; Lee et al., 2009). In organisms encoding diaminobutanoate-

2-oxo-glutarate transaminase and diaminobutanoate decarboxylase, 1,3-diaminopropane is produced and can serve as a substrate for CASDH, yielding carboxynorspermidine, which in turn is used by CASDC to produce norspermidine (Yamamoto et al., 1986; Zhu et al., 2015). Both the putative and alternative pathways for spermidine biosynthesis have been described in cyanobacteria (Zhu et al., 2015). In these species, the route to norspermidine via the alternative pathway could represent another physiological role for the production of 2,4-DAB via the aspartate 4-phosphate pathway.

1.4. The biosynthesis of 2,4-DAB derived from SAM and other pathways to 2,4-DAB

There is another known mechanism to 2,4-DAB biosynthesis. In *Lathyrus* species, isoxazolinones are used for the biosynthesis of both 2,3-diaminopropanoic acid (2,3-DAP) and 2,4-DAB. In *Pisum sativum* and *Lathyrus sativus* seedlings, β-(isoxazolin-5-on-2-yl)-alanine (BIA) was shown to be synthesised from *O*-acetyl-L-serine (OAS) and isoxazolin-5-one by cysteine synthase (EC 2.5.1.47). 2,3-DAP can be released after enzymatic cleavage of BIA (Ikegami et al., 1991). In *Lathyrus odoratus* seedlings, 2-(3-amino-3-carboxypropyl)-isoxazolin-5-one (ACI), the higher homologue of BIA, is present and causes neurotoxic symptoms similar to 2,4-DAB (Lambein, 1981) in experimental animals. This amino acid was shown to be synthesised from SAM and isoxazolin-5-one (Callebaut and Lambein, 1977; Ikegami et al., 1993). A suitable route to 2,4-DAB would be through enzymatic transfer of the 3-amino-3-carboxypropyl moiety from SAM to an intermediate, which could be cleaved to yield 2,4-DAB (Ikegami and Murakoshi, 1994) (Fig. 6). Although the transferase from *Lathyrus odoratus* has not been purified or sequenced, SAM-dependent 3-amino-3-carboxypropyl transferases have been described that can catalyse this transfer reaction (Meyer et al., 2020; Reeve et al., 1998; Riekhof et al., 2005). However, no enzyme has been shown to be able to cause the cleavage of such a complex. Additionally, although there are no reports of isoxazolinones having been found in cyanobacteria (Nunn and Codd, 2017), the four-carbon chain of methionine has been shown to be donated from SAM to a variety of structures, other than isoxazolinones. Previously characterised naturally occurring 3-amino-3-carboxypropyl-substituted heterocycles shown to derive from SAM as the donor of the 3-amino-3-carboxypropyl side chain include discadenine in *Dictyostelium discodeideum* (Taya et al., 1978), a modified nucleotide in *Escherichia coli* tRNA (Nishimura et al., 1974), plant siderophores of the mugineic acid family (Shojima et al., 1990; Shojima et al., 1989), the

post-translationally modified histidine of elongation factor 2, diphthamide (Chen and Bodley, 1988), and the polyamine, spermidine (Zhu et al., 2015).

2,4-DAB could be also formed non-enzymatically. In the protein gliadin, the neurotoxin is formed chemically from glutamine following Hoffman degradation (Synge, 1939), and from asparagine during peptide synthesis (Ressler, 1956). There appear to be no biological equivalents to these reactions.

1.5 Our contribution

In this study, we use bioinformatics tools to investigate hypotheses concerning 2,4-DAB biosynthesis in cyanobacteria through the assessment of the presence or absence of each enzyme in the aspartate 4-phosphate pathway and a pathway to 2,4-DAB derived from SAM, across 130 cyanobacterial genomes. We propose that, in cyanobacteria, the biosynthesis of 2,4-DAB through the aspartate 4-phosphate pathway occurs but is limited to a subset of species. We highlight its potential physiological roles in siderophore and polyamine biosynthesis. We also suggest that 2,4-DAB production from SAM may be possible in some cyanobacterial species, however in-depth analyses are hindered, as candidate enzymes and intermediates participating in this pathway are lacking. Ultimately, the biosynthesis of 2,4-DAB appears to be either restricted to some cyanobacterial species, or there may be multiple and additional routes, and roles, for the synthesis of this neurotoxin.

2. Results and discussion

2.1 Cyanobacterial species encoding enzymes in the aspartate 4-phosphate pathway

The first three enzymes in the aspartate 4-phosphate pathway (Fig. 2), aspartate transaminase, aspartate kinase and aspartate-semialdehyde dehydrogenase, are widespread in cyanobacteria. These enzymes are responsible for amino acid biosynthesis and metabolism and, in the few genomes where genetic evidence for one or more of these enzymes was lacking (one instance for aspartate transaminase, five instances for aspartate kinase and four instances for aspartate-semialdehyde dehydrogenase), we propose errors in sequencing, assembly, genome annotation or functional annotation as explanations (Supplementary Tables S1-S3).

Based on knowledge from active site and substrate specificity searches (Supplementary Tables S12-S17), from the 130 cyanobacterial species present in our dataset, diaminobutanoate-2-oxo-glutarate transaminase is believed to be present in 18 species, out of which only seven also encode diaminobutanoate decarboxylase (Table 1). *Xanthomonas* sp. encodes a transaminase that uses pyruvate, instead of 2-oxo-glutarate, as the oxo-acid (Rao et al., 1969). In cyanobacteria a gene for diaminobutanoate-pyruvate transaminase (EC 2.6.1.46) was not found in the genome of any species.

No cyanobacterial genomes appeared to encode diaminobutanoate acetyltransferase or ectoine hydroxylase, and only three species appear to possess the gene coding for ectoine synthase: *Calothrix rhizosoleniae* SC01*, Hydrocoleum* sp. CS-953 and *Planktothricoides* sp. SR001.

Some species were shown to encode diaminobutanoate-2-oxo-glutarate transaminase but not diaminobutanoate decarboxylase (e.g. *Nodularia* sp. NIES-3585), and *vice-versa* (e.g. *Phormidium tenue* NIES-30). There was also variation in the range of specialised metabolite clusters in which these enzymes were co-localised (Fig. 7).

Single cyanobacterial species derived from axenic cultures that encode diaminobutanoate-2-oxo-glutarate transaminase (*Calothrix brevissima* NIES-22*, Chroococcidiopsis thermalis* PCC 7203*, Cyanobacterium aponinum* PCC 10605*, Cyanobacterium* PCC 7702*, Cylindrospermum stagnale* PCC 7417*, Gloeobacter violaceus* PCC 7421*, Myxosarcina* sp. GI1*, Nodularia* sp. NIES-3585*, Stanieria cyanosphaera* PCC 7437 and *Trichormus variabilis* ATCC 29413), are believed to be able to biosynthesise 2,4-DAB through the aspartate 4-phosphate pathway, since contamination by other 2,4-DAB-producing bacteria can be excluded. It is possible that other cyanobacterial species encoding the transaminase that were not cultured in axenic conditions are also 2,4-DAB-producers (namely *Acaryochloris marina* MBIC11017*, Hapalosiphon* sp. MRB220*, Hydrocoleum* sp. CS-953*, Moorea bouillonii* PNG*, Nostoc calcicola* FACHB-389*, Nostoc linckia* z2*, Phormidium ambiguum* NIES-2119 and *Tolypothrix bouteillei* VB521301). However, this would have to be verified in genomes sequenced from axenic cultures. There is no apparent correlation between species encoding genes for 2,4-DAB transaminase/decarboxylase and a specific cyanobacterial morphology, biochemistry or habitat (Fig. 7).

2.2 Where the gene coding for 2,4-DAB transaminase is located within a NIS-cluster, it probably plays a physiological role in environmental iron-scavenging

From the seven cyanobacterial species encoding both diaminobutanoate-2-oxo-glutarate transaminase and diaminobutanoate decarboxylase, three (*Cyanobacterium aponinum* PCC 10605*, Phormidium ambiguum* NIES-2119 and *Trichormus variabilis* ATCC 29413) were shown to have both enzymes, co-localised within NIS clusters, as predicted by antiSMASH (Fig. 8).

Lynch et al. (2001), proposed a biosynthetic route to rhizobactin 1021 in the symbiotic nitrogen-fixing bacterium *Sinorhizobium meliloti*, where schizokinen was indicated as the preceding compound to rhizobactin 1021. Since schizokinen is an intermediary product of the siderophore rhizobactin 1021, it is possible that these siderophores share an enzymatic pathway (Årstøl and Hohmann-Marriott, 2019). The biosynthesis of synechobactin is probably an extension of the pathway to schizokinen (Årstøl and Hohmann-Marriott, 2019). Evidence supporting these statements has been provided in cyanobacteria: Nicolaisen et al. (2008) identified a cluster of genes in *Anabaena* sp. PCC 7120 homologous to the biosynthesis-genes for rhizobactin 1021 and Balasubramanian et al. (2006) identified the siderophore characteristic IucABC-family proteins in an eight-gene operon, in *Synechococcus* sp. PCC 7002.

There are eight genes involved in the regulation, biosynthesis and transport of rhizobactin 1021 from 1,3-diaminopropane (Lynch et al., 2001). The genes *rhbA*, *rhbB*, *rhbC*, *rhbD*, *rhbE* and *rhbF* are part of a single operon and function in the biosynthesis of the siderophore. The product of *rhbA* and *rhbB* shows similarity to the proteins diaminobutanoate-2oxo-glutarate transaminase and diaminobutanoate decarboxylase (involved in 1,3-diaminopropane synthesis), whilst the products of *rhbC*, *rhbD*, *rhbF* and *rhbE* are homologous to the iron uptake chelate domains IucA, IucB, IucC and IucD, involved in the production of aerobactin (Lynch et al., 2001). These genes are characteristic of siderophore clusters (i.e. NIS biosynthesis pathways contain at least one enzyme with conserved N-terminal iron uptake chelate domains (Årstøl and Hohmann-Marriott, 2019)), and all characterised NIS clusters known to date encode either one or the two homologous synthetases IucA/IucC (de Lorenzo

et al., 1986; de Lorenzo and Neilands, 1986). These synthetases define the NIS pathway and are present in the siderophore clusters of three cyanobacterial species in the dataset (Fig. 8), which we propose are able to synthesise iron chelators of the schizokinen/synechobactin type. Additional proteins, such as (GNAT family) N-acetyltransferase, lysine 6-monooxygenase, α/β hydrolase and transport proteins (e.g. iron-siderophore ABC transporter substrate-binding protein, ABC transporter ATP-binding protein, MFS transporter, and the TonB-dependent receptor) found in the cyanobacterial NIS clusters of Fig. 8, also have similar functional annotations to the proteins in the NIS clusters of other species (Challis, 2005; Lynch et al., 2001).

The synthesis of rhizobactin 1021 is similar to that of aerobactin (Årstøl and Hohmann-Marriott, 2019). Both iron-chelators are citrate-based hydroxamate siderophores with similar structures, differing only in the presence of a fatty acid moiety in rhizobactin 1021 and an additional carboxylic acid moiety and two saturated carbon atoms in each of the symmetrical arms of aerobactin (Lynch et al., 2001). It has been argued that, if aerobactin was derived from the same substrate as rhizobactin 1021 (i.e. 1,3-diaminopropane instead of lysine), the resulting product would have the same structure as rhizobactin 1021 (Lynch et al., 2001). Given this, Lynch et al. (2001) proposed that 1,3-diaminopropane is incorporated into rhizobactin 1021 by steps similar to those involved in the biosynthesis of aerobactin. However, since there is no evidence in the literature for the production of these siderophores in cyanobacteria, we hypothesise that the enzymes used for the biosynthesis of schizokinen/synechobactin in cyanobacteria also correspond to those used for the biosynthesis of aerobactin and rhizobactin 1021.

The co-localisation of the 2,4-DAB transaminase and decarboxylase with the IucA/IucC synthetase genes, inside an NIS cluster, suggests that these enzymes provide the catalysis to form siderophores of the schizokinen/synechobactin type (Årstøl and Hohmann-Marriott, 2019). Hence, we propose that, in some cyanobacteria, 2,4-DAB can be biosynthesised through the aspartate 4-phosphate pathway, which is likely to play a physiological role in environmental iron-scavenging.

Since hydroxamate siderophores have the capability of complexing with metals in addition to iron (i.e. copper, uranium) (McKnight and Morel, 1980; Rashmi et al., 2013), additional

physiological roles of 2,4-DAB as an antimicrobial agent (Matz et al., 2004) or in the detoxification of heavy metals (Årstøl and Hohmann-Marriott, 2019), respectively, may exist.

The genomes of *Cyanobacterium aponinum* PCC 10605 and *Trichormus variabilis* ATCC 29413 were both derived from axenic cultures, and therefore, based on our genomic analyses, it seems probable that these species are able to synthesise 2,4-DAB through the aspartate 4-phosphate pathway, possibly as a by-product of the production of siderophores of the schizokinen/synechobactin type. Although *Cyanobacterium aponinum* PCC 10605*, Trichormus variabilis* ATCC 29413 and *Phormidium ambiguum* NIES-2119 were all isolated from freshwater environments, siderophores are also known to be produced in terrestrial and marine environments (Årstøl and Hohmann-Marriott, 2019), possibly via the same pathways.

Since no cyanobacterial species in the dataset encode a gene for diaminobutanoate-pyruvate transaminase, the synthesis of 2,4-DAB, where present in cyanobacteria, appears to occur via a 2,4-DAB transaminase that uses 2-oxo-glutarate, and not pyruvate, as the oxo-acid. However, the possibility of uncharacterised diaminobutanoate-pyruvate transaminase enzymes, non-homologous to those that are known, cannot be disregarded. Without further knowledge of the relevant metabolite pool sizes in cyanobacteria, particularly of pyruvate, and the kinetic properties of the transaminase, the possibility of 2,4-DAB biosynthesis in cyanobacteria via diaminobutanoate-pyruvate transaminase cannot be excluded.

2.3 Co-localisation of 2,4-DAB transaminase within hybrid NRPS/PKS and unanswered questions

Many siderophores are polypeptides that are biosynthesised by members of the NRPS multienzyme family (Challis, 2005). These siderophores are different in structure from schizokinen (Jeanjean et al., 2008). Eleven species of cyanobacteria were found to encode 2,4-DAB transaminase within hybrid NRPS/PKS clusters. The product of these specialised metabolite clusters and the role played by 2,4-DAB transaminase is unknown. However, some similarity to pyoverdine clusters was found (Supplementary Table S4), possibly suggesting still unknown/uncharacterised siderophore pathways in cyanobacteria.

Although siderophores of the schizokinen type have been described in *Anabaena* sp. (Armstrong and Van Baalen, 1979; Simpson and Neilands, 1976), we did not find the genes for diaminobutanoate-2-oxo-glutarate transaminase or diaminobutanoate decarboxylase in the genome of *Anabaena cylindrica* PCC 7122. This is suggestive of a different pathway to siderophore production (i.e. not via the aspartate 4-phosphate pathway) and/or a yet-uncharacterised siderophore in this species. Our results are compatible with those of Goldman et al. (1983), who showed that *Anabaena cylindrica* PCC 7122 is able to produce hydroxamate siderophores with different characteristics compared to schizokinen.

Gloeobacter violaceus PCC 7421 was shown to encode both 2,4-DAB transaminase and decarboxylase, via adjacent genes on the genome, but not within a specialised metabolite cluster. Given that in cyanobacteria, siderophores were found to be produced in all but the most early-branching clades (Årstøl and Hohmann-Marriott, 2019), this finding may point to potentially additional and unknown physiological roles for the production of 2,4-DAB; for example, to enable this species to synthesise siderophores from 1,3-diaminopropane via a yet uncharacterised route; or to circumvent the loss of key genes essential for the production of iron chelators of the schizokinen/synechobactin type in this species.

2.4 An incomplete ectoine cluster as supporting evidence for the use of the aspartate 4-phosphate pathway for the production of 2,4-DAB in cyanobacteria

From the three cyanobacterial species that encode ectoine synthase (Fig. 7), *Calothrix rhizosoleniae* SC01 is the only one that also possesses diaminobutanoate-2-oxo-glutarate transaminase. The genes coding for these enzymes appear adjacent on the genome and are predicted to be co-localised within an ectoine cluster (Fig. 8).

The fact that the genes for diaminobutanoate-2-oxo-glutarate transaminase and ectoine synthase appear adjacent on the genome of this species is compatible with our hypothesis that cyanobacteria use the aspartate 4-phosphate pathway for the production of 2,4-DAB from aspartate-4-semialdehyde.

Our results are supported by the findings of Czech et al. (2018) and Widderich et al. (2014a), who showed that, when present in cyanobacteria, ectoine synthase genes appear alone (i.e. as

orphan genes), and not as part of complete ectoine clusters. It is currently not clear whether the solitary ectoine synthases are remnants of previously intact ectoine biosynthetic routes, whether they were recruited by existent genes in the ectoine pathway, whether they rely on an environmental provision of substrate to ectoine synthesis, or whether they have evolved new enzymatic functions, while possibly still retaining the capability of converting N^4-acetyl-2,4-diaminobutanoic acid to ectoine (Czech et al., 2018; Widderich et al., 2014a).

The cyanobacterium *Calothrix rhizosoleniae* SC01 was isolated from a symbiotic diatom of the genus *Chaetoceros*. In resemblance to what occurs in *Pseudomonas syringae* pv. syringae B728a (Kurz et al., 2010), we speculate that the diatom could provide the cyanobacterial symbiont with the substrate for the synthesis of ectoine. Genome-driven investigations of compatible solutes in the plant pathogen *Pseudomonas syringae* pv. syringae B728a indicated that this bacterium does not produce ectoine naturally under laboratory conditions (i.e. as it lacks the *ectAB* genes), but when surface-sterilised leaves of its host plant *Syringa vulgaris* were subjected to osmotic stress, ectoine production was observed (Kurz et al., 2010). The clustering of cyanobacteria in the phylogenomic studies of Czech et al. (2018) suggest that, when present, ectoine synthases in these species are probably catalytically competent.

2.5 Polyamine biosynthesis in cyanobacteria and additional potential physiological roles of 2,4-DAB

The co-localisation of the 2,4-DAB transaminase with CASDH and CASDC could indicate another physiological role for the biosynthesis of 2,4-DAB in cyanobacteria: that of polyamine (e.g. spermidine and norspermidine) biosynthesis (Fig. 5).

The gene pair CASDH/CASDC is present in nine cyanobacterial species in the dataset: *Scytonema* sp. HK-05, *Tolypothrix bouteillei* VB521301*, Chamaesiphon minutus* PCC 6605*, Scytonema hofmannii* PCC 7110*, Nostocales cyanobacterium* HT-58-2*, Nostoc linckia* z2*, Leptolyngbya boryana* dg5*, Scytonema tolypothrichoides* VB-61278 and *Myxosarcina* sp. GI1 (Fig. 7), and are only adjacent in the genome of the latter.

The presence, in a species, of diaminobutanoate-2-oxo-glutarate transaminase and diaminobutanoate decarboxylase with CASDH and CASDC is indicative of the ability to

produce norspermidine through the alternative pathway for polyamine biosynthesis (Lee et al., 2009) (Fig. 5). However, the gene pair CASDH/CASDC was not found to be adjacent to, nor included in the same specialised metabolite cluster of diaminobutanoate-2-oxo-glutarate transaminase in any cyanobacterial species in the dataset. This suggests that, in cyanobacteria, the alternative pathway for polyamine biosynthesis is largely used for spermidine biosynthesis rather than norspermidine production, consistent with the findings of Lee et al. (2009).

However, in *Tolypothrix bouteillei* VB521301*,* the enzymes participating in the synthesis of spermidine via the classical pathway, SAMDC and SPDS, appear co-localised with diaminobutanoate-2-oxo-glutarate transaminase, within the same hybrid NRPS/PKS cluster (Fig. 9). We suggest that, in some cyanobacteria, spermidine synthesis may be linked to 2,4-DAB production, which is itself associated with the biosynthesis of yet unknown specialised metabolites, probably siderophores (i.e. the product of the NRPS/PKS cluster is currently undetermined). This would not be unprecedented as, in addition to their core physiological roles (e.g. in cell proliferation, growth and development), polyamines have been shown to be involved in microbial swarming (Sturgill and Rather, 2004), biofilm formation (Lee et al., 2009; McGinnis et al., 2009) and siderophore production (Brickman and Armstrong, 1996; Griffiths et al., 1984; Pfleger et al., 2007).

2.6 The route to 2,4-DAB derived from SAM: cyanobacterial species encoding homologs of SAM-dependent 3-amino-3-carboxypropyl transferase and unanswered questions

In *Lathyrus odoratus*, ACI was shown to be synthesised from isoxazolin-5-one and SAM (Ikegami et al., 1993). The enzymatic cleavage of ACI is a possible route to 2,4-DAB (Ikegami and Murakoshi, 1994) (Fig. 6). Bioinformatics analysis of the feasibility of this pathway in cyanobacteria is hindered as the transferase catalysing the donation of the four-carbon chain of methionine from SAM to isoxazolin-5-one has not been purified, and an enzyme capable of cleaving this complex is currently unknown. Additionally, there are no reports of isoxazolinones having been found in cyanobacteria (Nunn and Codd, 2017), and so other candidate acceptors would be needed to test this hypothesis.

However, SAM-dependent 3-amino-3-carboxypropyl transferases, shown to catalyse the transfer reaction from SAM, have been described (Meyer et al., 2020; Reeve et al., 1998; Riekhof et al., 2005), which enabled the search for homologous enzymes in cyanobacterial genomes. Homologs of SAM-dependent 3-amino-3-carboxypropyl transferases proteins, encompassing a methyltransferase fold (Fauman et al., 1999; Martin and McMillan, 2002) with a conserved SAM-binding pocket, were found in eight cyanobacterial species in the dataset: *Oscillatoriales cyanobacterium* MTP1, *Roseofilum reptotaenium* AO1-A, *Spirulina subsalsa*, *Spirulina major*, *Trichormus* sp. NMC-1, *Desertifilum* sp. IPPAS B-1220 and *Oscillatoria acuminata*. In all but one case, the homolog was functionally annotated as Domain of Unknown Function (DUF) 3419 domain-containing protein (Supplementary Table S17). In *Roseofilum reptotaenium* AO1-A, the protein found was annotated as S-adenosylmethionine—diacylglycerol (DAG) 3-amino-3-carboxypropyl transferase, suggesting in this species, a reaction with a diacylglycerol acceptor. Complexes resulting from the transfer of the four-carbon chain of methionine from SAM to DAG have been described in bacteria (Riekhof et al., 2005), plants and fungi (Künzler and Eichenberger, 1997), and are involved in the replacement of membrane phospholipids with non-phosphorus lipids, when phosphate becomes limited in the environment (Benning et al., 1995; Geiger et al., 1999).

Cyanobacterial species encoding SAM-dependent transferases are candidates for 2,4-DAB production derived from SAM. The purification and characterisation of the enzymes in this pathway and their specificity, as well as knowledge regarding possible acceptors for the transfer reaction are needed to further analyse the possibility of 2,4-DAB production in cyanobacteria through this route.

2.7 Comparison between bioinformatics results and the analytical chemistry data from the literature

In an attempt to support our bioinformatics findings with analytical data from the literature, we compared our results with those of 13 studies where evidence for 2,4-DAB- or 2,4-DAB- and BMAA-producing cyanobacteria was provided (Supplementary Table S5). A scenario where a particular species is shown to encode the necessary genes needed for the biosynthesis of 2,4-DAB, and is proven to produce the same compound via analytical studies, serves as a

strong indicator that this species is a 2,4-DAB-producer. On the other hand, a species that lacks the genes responsible for 2,4-DAB production, but is shown by analytical studies to produce the neurotoxin could be indicative of a false-positive result derived from a failed analytical method (especially probable if non-axenic cultures and/or low-specificity methods were employed), or suggests the existence of other, still undescribed routes to the biosynthesis of 2,4-DAB. All studies used variations of MS/MS for 2,4-DAB/BMAA identification and quantification, however only four analyses were derived from axenic cultures. Our 130 cyanobacterial species dataset and the compilation from the literature overlapped in six species: *Aphanizomenon flos-aquae*, *Cylindrospermopsis raciborskii*, *Microcystis aeruginosa*, *Nodularia spumigena*, *Planktothrix agardhii* and *Synechococcus elongatus.* All species, apart from the latter, do not appear to have genes coding for 2,4-DAB transaminase, 2,4-DAB decarboxylase, and ectoine genes. *Synechococcus elongatus*, derived from an axenic culture, has a gene coding for 2,4-DAB decarboxylase, which is not included in a specialised metabolite cluster, and is lacking a gene for 2,4-DAB transaminase. This observation may suggest different routes for 2,4-DAB biosynthesis, or that there are unknown 2,4-DAB transaminase enzymes, non-homologous to those that have been characterised. The scarce amount of analytical studies using axenic cultures of clearly defined cyanobacterial species (most studies only include the cyanobacterial genus) does not allow for further comparisons.

3. Summary and conclusions

We have provided an initial bioinformatics insight into potential pathways of 2,4-DAB biosynthesis in cyanobacteria, which can lead to future experimental investigations.

The bioinformatics results point towards 2,4-DAB biosynthesis in some cyanobacterial species via the aspartate 4-phosphate pathway. In some cyanobacteria, the enzymes 2,4-DAB transaminase/decarboxylase are co-localised within NIS clusters, suggesting a functional association with siderophore biosynthesis. The fact that 2,4-DAB transaminase is found adjacent to an *ectC* gene in one cyanobacterial species serves as supporting evidence that cyanobacteria can produce 2,4-DAB via the aspartate 4-phosphate pathway, where it can serve as a substrate for either 1,3-diaminopropane or ectoine synthesis.

Due to the occurrence of 2,4-DAB transaminase/decarboxylase within hybrid NRPS/PKS clusters, we suggest that 2,4-DAB production may also be associated with polyamine synthesis, which itself is associated with biofilm formation and siderophore production. Species in which these enzymes occur outside specialised metabolite clusters may have developed new and, as yet, unknown physiological roles for 2,4-DAB biosynthesis, or may have lost genes needed for the production of siderophores, ectoine or polyamines.

In some cyanobacterial species, 2,4-DAB may be derived from SAM. Further information regarding the enzymes in this pathway, their specificity and possible acceptors is needed to further test this hypothesis.

Ultimately, the ability to synthesise 2,4-DAB via the aspartate 4-phosphate pathway and the pathway derived from SAM does not appear to be universal among cyanobacteria. This means that either 2,4-DAB is not a widespread neurotoxin in these species, or that there are additional and yet unknown pathways for the synthesis of 2,4-DAB. The presence of NIS clusters is also limited, appearing scattered in the species phylogeny. 2,4-DAB production and the presence of NIS clusters is not linked to specific environments, biochemistry or morphology.

4. Experimental

4.1 Phylogenetic reconstruction

130 high-quality cyanobacterial genomes and three outgroup genomes (one Proteobacteria, one Actinobacteria, and one Chloroflexi) were selected from RefSeq (Supplementary Material Section S4).

The genomes used were sequenced from species cultured under different conditions. The majority (53-54%) of genomes were extracted from species grown in axenic culture, whilst the remaining genomes were derived from species in non-axenic monocyanobacterial cultures (21-22%), or consisted of metagenome-assembled genomes (15-16%). The culture types of the organisms yielding the remaining 8-9% of the genomes are unknown (Supplementary Table S6).

Genome-wide protein sets were compared with BLAST (Altschul et al., 1997) and clustered into orthogroups using OrthoFinder (v.2.1.2) (Emms and Kelly, 2015) with a permissive MCL (Van Dongen, 2000) inflation value of 1.1 for species phylogeny reconstruction. This resulted in 9591 orthogroups of two or more sequences each (Supplementary Material Section S5). Multiple sequence alignments were generated for all orthogroups using MAFFT (v.7.307) (Katoh and Standley, 2013). Orthogroups were mapped to pre-defined reliable AMPHORA2 markers for bacteria (Wu and Eisen, 2008). 29 orthogroups showed no strong evidence of horizontal gene transfer, or systematic deviation from the single maximum likelihood species tree, which was reconstructed from the concatenation of these 29 gene families using the LG+C60+F+R substitution model in IQ-TREE (v.1.6.10) (Nguyen et al., 2015) (Supplementary Material Section S6). Branch support was estimated using ultrafast bootstrap with 1000 replicates (Hoang et al., 2017), the tree was rooted between the ingroup and outgroup, and the outgroup was removed. The tree was annotated using iTOL software (Letunic and Bork, 2006).

4.2 Profile hidden Markov models

From the protein sequence database at the NCBI (https://www.ncbi.nlm.nih.gov/), amino acid sequences with the same enzyme nomenclature as given in the BRENDA database (Chang et al., 2021) were selected for each enzyme in the aspartate 4-phosphate pathway (Fig. 2) and for SAM-dependent 3-amino-3-carboxypropyl transferase (Fig. 6). Sequence choice prioritised cyanobacterial proteins and, where absent/scarce, bacterial proteins. The selection of multiple sequences from the same species was avoided. The number of sequences selected for the construction of each profile hidden Markov model (pHMM) ranged from 15 to 62 (Supplementary Table S8), comparable with an average of 22 sequences used for seed alignments in the Pfam database (Sonnhammer et al., 1998). For each enzyme, multiple sequence alignments were generated using MAFFT (v.7.307), with the E-ins-I algorithm, and pHMMs were generated using HMMER (v. 3.1b2) (hmmer.org).

Quality assessments were performed to analyse how accurately the generated pHMMs were at finding homologues, using cytochrome C oxidase subunit III, phycobilisome protein,

photosystem II protein and photosystem II reaction centre M protein as positive controls (Supplementary Material Section S7.1).

The pHMMs for enzymes in the aspartate 4-phosphate pathway were used to search proteins of the 130 cyanobacterial genomes for homologues using HMM search in the HMMER package, with the default threshold (E-value ≤ 0.01).

4.3 Online database searches

Where necessary to confirm absence, enzymes in the aspartate 4-phosphate pathway (Fig. 2) and for SAM-dependent 3-amino-3-carboxypropyl transferase (Fig. 6) were also used as queries in BLAST searches against all cyanobacterial proteomes available at NCBI.

4.4 Substrate specificity and active site identification

In order to differentiate between the enzymes present in the aspartate 4-phosphate pathway (Fig. 2) and the pathway to 2,4-DAB from SAM (Fig. 6) from homologous enzymes that carry out different functions, active site and substrate specificity searches were conducted for enzymes downstream of- and including diaminobutanoate-2-oxo-glutarate transaminase, and the SAM-dependent 3-amino-3-carboxypropyl transferase, based on knowledge from the literature, indicated below.

Sequence alignments, including information about strictly conserved sites, residues involved in ligand-binding, pyridoxal 5'-phosphate (PLP)-binding, iron-binding, SAM-binding, and stabilisation of loop-architectures were available for diaminobutanoate-2-oxo-glutarate transaminase (Richter et al., 2019), diaminobutanoate acetyltransferase (Richter et al., 2020), ectoine synthase (Widderich et al., 2016), ectoine hydroxylase (Reuter et al., 2010; Widderich et al., 2014a; Widderich et al., 2014b), SAM-dependent 3-amino-3-carboxypropyl transferase (Reeve et al., 1998; Riekhof et al., 2005), and to some extent for diaminobutanoate decarboxylase (Yamamoto et al., 2000). No information is currently available regarding the active site of diaminobutanoate-pyruvate transaminase. Where data were available, multiple sequence alignments were constructed for each of these enzymes using MAFFT, which comprised the significant pHMM search hits from the cyanobacterial

species in the dataset, as well as sequences with known active sites and substrate specificity from the literature, listed above. Multiple alignments were visualised using Jalview (v. 2.11.1.0) (Waterhouse et al., 2009). Amino acid differences between sequences with known active sites and the significant pHMM search hits in cyanobacteria were counted, giving a Hamming distance (Supplementary Tables S12-S17). Low Hamming distance indicates well-conserved sequence at the active site. This serves as supporting evidence that the pHMM search hit in question corresponds to a true enzyme in the aspartate 4-phosphate pathway or in the pathway to 2,4-DAB derived from SAM.

4.5 Gene family phylogeny reconstruction

To further aid in the differentiation between enzymes belonging to the aspartate 4-phosphate pathway (Fig. 2) and the pathway to 2,4-DAB from SAM (Fig. 6) from homologs with different functions, gene trees were reconstructed for enzymes downstream of- and including diaminobutanoate-2-oxo-glutarate transaminase, and the SAM-dependent 3-amino-3-carboxypropyl transferase, using IQ-TREE with the substitution model chosen according to BIC at the ModelFinder step (Kalyaanamoorthy et al., 2017). Branch support was estimated with ultrafast bootstrap for 1000 replicates (Supplementary Fig. S3-S9). The grouping of sequences with different functional annotations was assessed. For example, in a gene phylogeny, if one sequence is included within a group of sequences with a different and consistent functional annotation, it is plausible to assume that the first sequence may have been mis-annotated and should adopt the annotation of the neighbouring branches.

4.6 Analysis of specialised metabolite clusters

AntiSMASH (v.5.1.2) (Blin et al., 2019) was used for the identification and annotation of specialised metabolite clusters in the cyanobacterial species in the dataset. Diaminobutanoate-2-oxo-glutarate transaminase and diaminobutanoate decarboxylase were checked for their co-localisation within specialised metabolite clusters and their proximity to each other. In species where these enzymes were not inserted in specialised metabolite clusters, the Artemis Comparison Tool (ACT) (Carver et al., 2005) was used for the localisation of the proteins in the genome, their juxtaposition, and assessment of the neighbouring genes.

4.7 Compilation of evidence of 2,4-DAB-producing cyanobacteria from the literature

Supplementary Table S5 presents a compilation of 13 studies which have reported evidence from analytical chemistry of cyanobacterial species capable of producing 2,4-DAB or 2,4-DAB and BMAA. Information regarding the analytical method used to identify and quantify the neurotoxins, as well as the culture type of the cyanobacterial samples are included, if available.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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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/3057

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Table

	Proximity on genome	**Inclusion in specialised metabolite cluster**	**Specialised cluster type**
Chroococcidiopsis thermalis PCC 7203	Adjacent	No	Not applicable
Cyanobacterium aponinum PCC 10605	Adjacent	Yes	Siderophore/NIS cluster
Gloeobacter violaceus PCC 7421	Adjacent	No	Not applicable
Hapalosiphon sp. MRB220	Not adjacent	Yes (transaminase), No (decarboxylase)	NRPS/PKS cluster (transaminase)
Moorea bouillonii PNG	Not adjacent	No	Not applicable
Phormidium ambiguum NIES-2119	Adjacent	Yes	Siderophore/NIS cluster
Trichormus variabilis ATCC 29413	Adjacent	Yes	Siderophore/NIS cluster

Table 1. Localisation in the genome of the enzymes diaminobutanoate-2-oxo-glutarate transaminase and diaminobutanoate decarboxylase. On the left are the cyanobacterial species encoding both enzymes. AntiSMASH was used to identify and annotate specialised metabolite clusters containing both 2,4-DAB transaminase (EC 2.6.1.76) and decarboxylase (EC 4.1.1.86). When these enzymes where not included in specialised metabolite clusters, the Artemis Comparison Tool (ACT) was used to locate the proteins and characterise the neighbouring genes.

Figure captions

Figure 1. Structure of 2,4-diaminobutanoic acid in the ionised form (2,4-diaminobutanoate). This form is present at physiological pH values. The carboxyl group is then completely ionised and positive charge is shared between the two amino groups.

Figure 2. The aspartate 4-phosphate pathway. Established routes to 2,4-diaminobutanoate (2,4-diaminobutanoic acid) and derivatives. The nomenclature is from www.brenda-enzymes.org; the Enzyme Commission numbers are as follows: (1) aspartate transaminase: EC 2.6.1.1; (2) aspartate kinase: EC 2.7.2.4; (3) aspartate-semialdehyde dehydrogenase: EC 1.2.1.11; (4) diaminobutanoate-2-oxo-glutarate transaminase: EC 2.6.1.76; (5) diaminobutanoate acetyltransferase: EC 2.3.1.178; (6) ectoine synthase: EC 4.2.1.108; and (7) ectoine hydroxylase: EC 1.14.11.55. Multiple-step pathways are indicated by *.

Figure 3. The synthesis of 1,3-diaminopropane from 2,4-DAB. Established route to 1,3-diaminopropane. The nomenclature is from www.brenda-enzymes.org; the Enzyme Commission numbers are as follows: (1) diaminobutanoate-2-oxo-glutarate transaminase: EC 2.6.1.76; (2) diaminobutanoate decarboxylase: EC 4.1.1.86.

Figure 4. The biochemical pathway to schizokinen. The established route to rhizobactin 1021 in *Sinorhizobium meliloti* (Lynch et al., 2001) includes schizokinen as its immediate precursor. (The enzyme responsible for the biosynthesis of rhizobactin 1021 from schizokinen is currently unknown). Nomenclature is from www.brenda-enzymes.org. The genes *rhbA* and *rhbB* (Lynch et al., 2001), correspond to the enzymes diaminobutanoate-2-oxo-glutarate transaminase (**RhbA**, EC 2.6.1.76) and diaminobutanoate decarboxylase **RhbB**, EC 4.1.1.86). The enzymes **RhbD** (an acetylase), **RhbE** (which catalyses the oxidation of a single amino group of 1,3-diaminopropane), **RhbC** and **RhbF** (which catalyse condensation reactions) are unclassified. Redrawn from Lynch et al., (2001). The molecule in square brackets* represents 2,4-diaminobutanoate (2,4-diaminobutanoic acid) after rotation of the amino and carboxylate functions about carbon-2 to allow visual alignment of the amino groups in 1,3-diaminopropane and subsequent derivatives.

Figure 5. The biochemical pathways to spermidine and norspermidine in cyanobacteria. The established route to spermidine and norspermidine is adapted from Zhu et al. (2015) and the figure of Lee et al. (2009) has been redrawn and updated. The nomenclature is from www.brenda-enzymes.org; the Enzyme Commission numbers are as follows: (1) arginase: EC 3.5.3.1; (2) arginine decarboxylase: EC 4.1.1.19; (3) agmatine ureohydrolase: EC 3.5.3.11; (4) agmatine iminohydrolase: EC 3.5.3.12; (5) *N*-carbamoylputrescine amidohydrolase: EC 3.5.1.53; (6) ornithine decarboxylase: EC 4.1.1.17; (7) S-adenosylmethionine decarboxylase: EC 4.1.1.50; (8) spermidine synthase: EC 2.5.1.16; (9) diaminobutanoate-2-oxo-glutarate transaminase: EC 2.6.1.76; (10) diaminobutanoate decarboxylase: EC 4.1.1.86; (11) carboxyspermidine dehydrogenase: EC 1.5.1.43; (12) carboxyspermidine decarboxylase: EC 4.1.1.96. Reactions (7) and (8) are involved on the biosynthesis of spermidine from putrescine *via* the classical pathway. Reactions (11) and (12) are involved in the biosynthesis of spermidine (in species without 1,3-diaminopropane) or norspermidine *via* the alternative pathway (where 1,3-diaminopropane is available).

Figure 6. The pathway to 2,4-DAB derived from S-adenosylmethionine (SAM). The formation of 2,4-diaminobutanoate (2,4-diaminobutanoic acid) from S-adenosylmethionine (SAM) and isoxazolin-5-one in *Lathyrus sylvestris* (Callebaut and Lambein, 1977; Ikegami and Murakoshi, 1994). ACI: 2-(3-amino-3-carboxypropyl)-isoxazolin-5-one (Kuo et al., 1982). The enzymes catalysing the two reactions are unclassified.

Figure 7. Species phylogeny showing the cross-species distribution of specialised metabolite clusters containing a gene coding for diaminobutanoate-2-oxo-glutarate transaminase (EC 2.6.1.76). The enzymes encoded by each species are indicated by coloured circles, next to the species name. For enzymes' accession numbers, see Supplementary Tables S12-S17. See Supplementary Fig. S2 for bootstrap support of each branch.

Fig 8. The localisation of the enzymes diaminobutanoate-2-oxo-glutarate transaminase, the downstream decarboxylase, and ectoine synthase within siderophore clusters and incomplete ectoine clusters. The top three clusters correspond to NIS clusters and the bottom cluster corresponds to an incomplete ectoine cluster. The organisation of NIS clusters follows the same pattern: diaminobutanoate-2-oxo-glutarate transaminase (EC 2.6.1.76),

followed by diaminobutanoate decarboxylase (EC 4.1.1.86) and IucA/IucC (EC 6.3.2.38, EC 6.3.2.39) family siderophore biosynthesis protein. Arrows point to the orientation of transcription. AntiSMASH was used to identify and annotate specialised metabolite clusters containing both 2,4-DAB transaminase and decarboxylase or 2,4-DAB transaminase and ectoine synthase (EC 4.2.1.108).

Fig 9. The localisation of the enzymes diaminobutanoate-2-oxo-glutarate transaminase, S-adenosylmethionine decarboxylase and spermidine synthase within a hybrid NRPS/PKS cluster. The product of the NRPS/PKS cluster is unknown. Arrows point to the orientation of transcription. The red arrow indicates the location of the gene pair SPDS/SAMDC (EC 2.5.1.16, EC 4.1.1.50), which is transcribed in the same direction as diaminobutanoate-2-oxo-glutarate transaminase (EC 2.6.1.76).

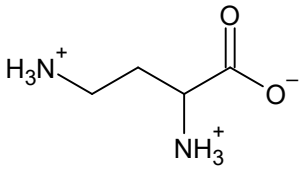
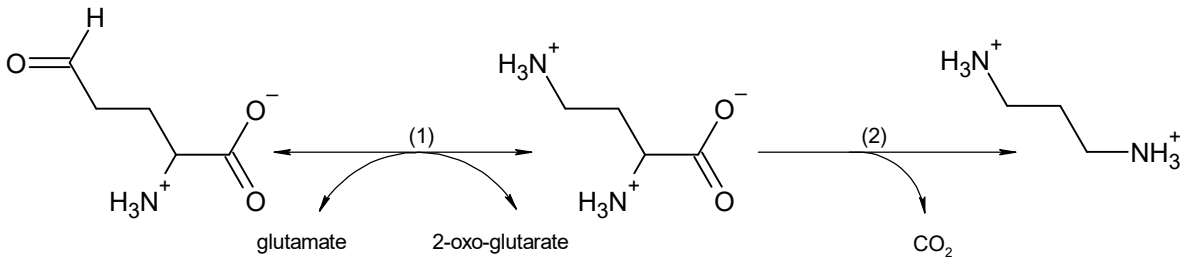


Figure 1



aspartate 4-semialdehyde

2,4-diaminobutanoate
(2,4-diaminobutanoic acid)

1,3-diaminopropane

Figure 2

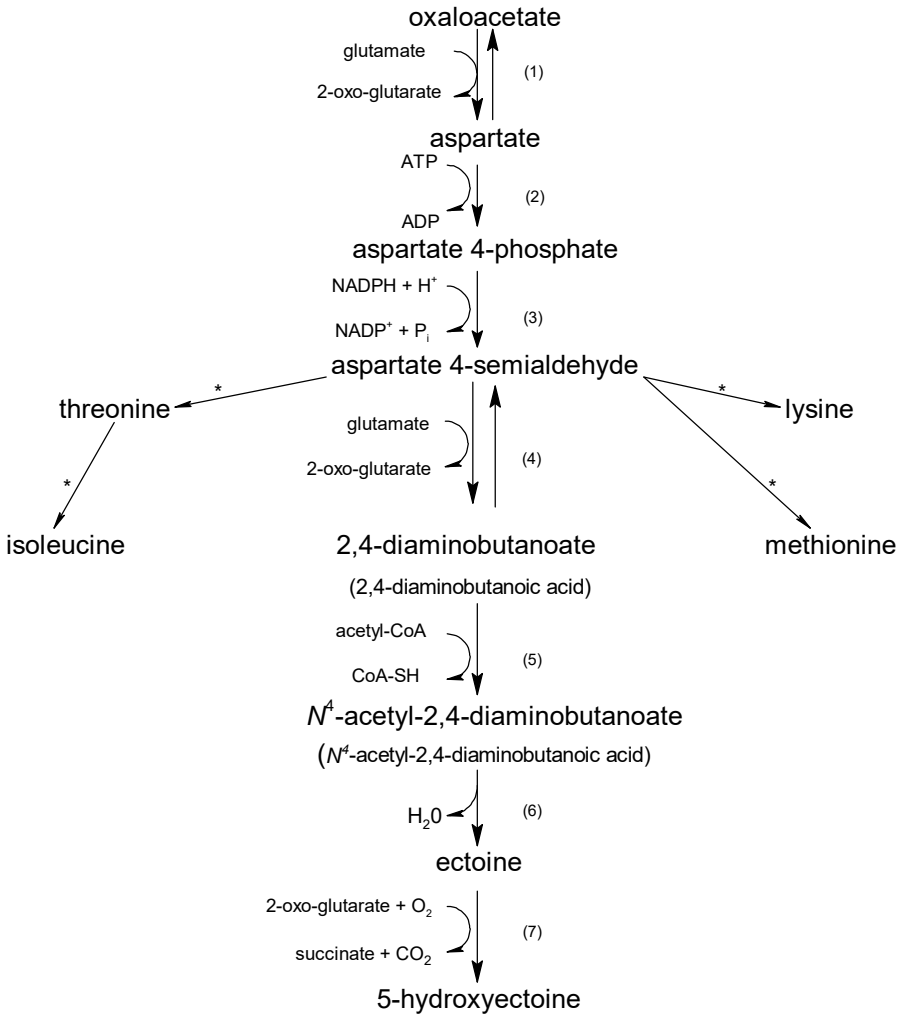

oxaloacetate
glutamate
2-oxo-glutarate
(1)
aspartate
ATP
ADP
(2)
aspartate 4-phosphate
NADPH + H+
NADP+ + Pi
(3)
aspartate 4-semialdehyde
threonine
lysine
glutamate
2-oxo-glutarate
(4)
isoleucine
2,4-diaminobutanoate
(2,4-diaminobutanoic acid)
methionine
acetyl-CoA
CoA-SH
(5)
N4-acetyl-2,4-diaminobutanoate
(N4-acetyl-2,4-diaminobutanoic acid)
H20
(6)
ectoine
2-oxo-glutarate + O2
succinate + CO2
(7)
5-hydroxyectoine

Figure 3

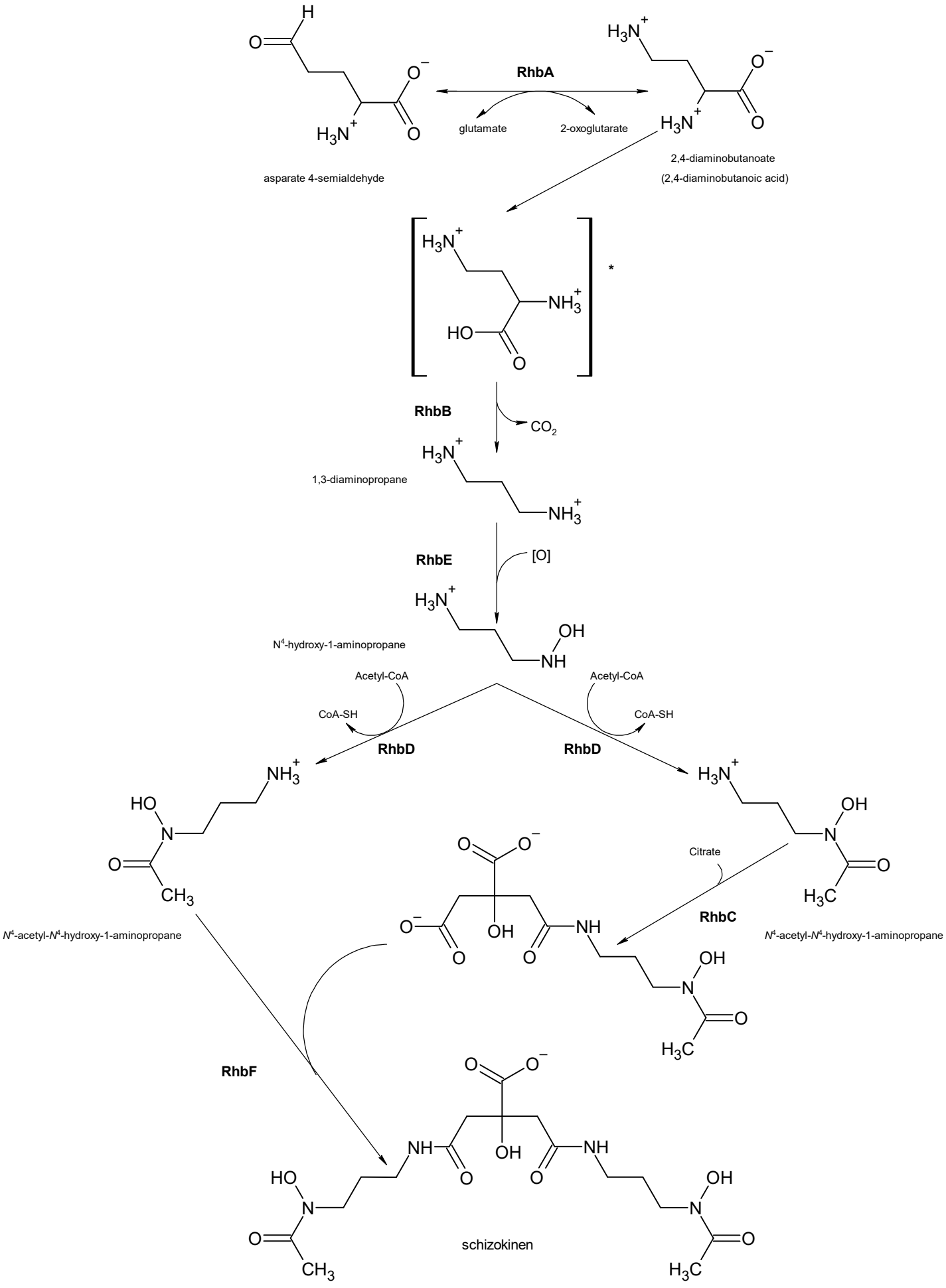

RhbA
glutamate
2-oxoglutarate
asparate 4-semialdehyde
2,4-diaminobutanoate
(2,4-diaminobutanoic acid)
RhbB
CO2
1,3-diaminopropane
RhbE
[O]
N4-hydroxy-1-aminopropane
Acetyl-CoA
CoA-SH
RhbD
RhbD
Citrate
RhbC
N4-acetyl-N4-hydroxy-1-aminopropane
N4-acetyl-N4-hydroxy-1-aminopropane
RhbF
schizokinen

Figure 4

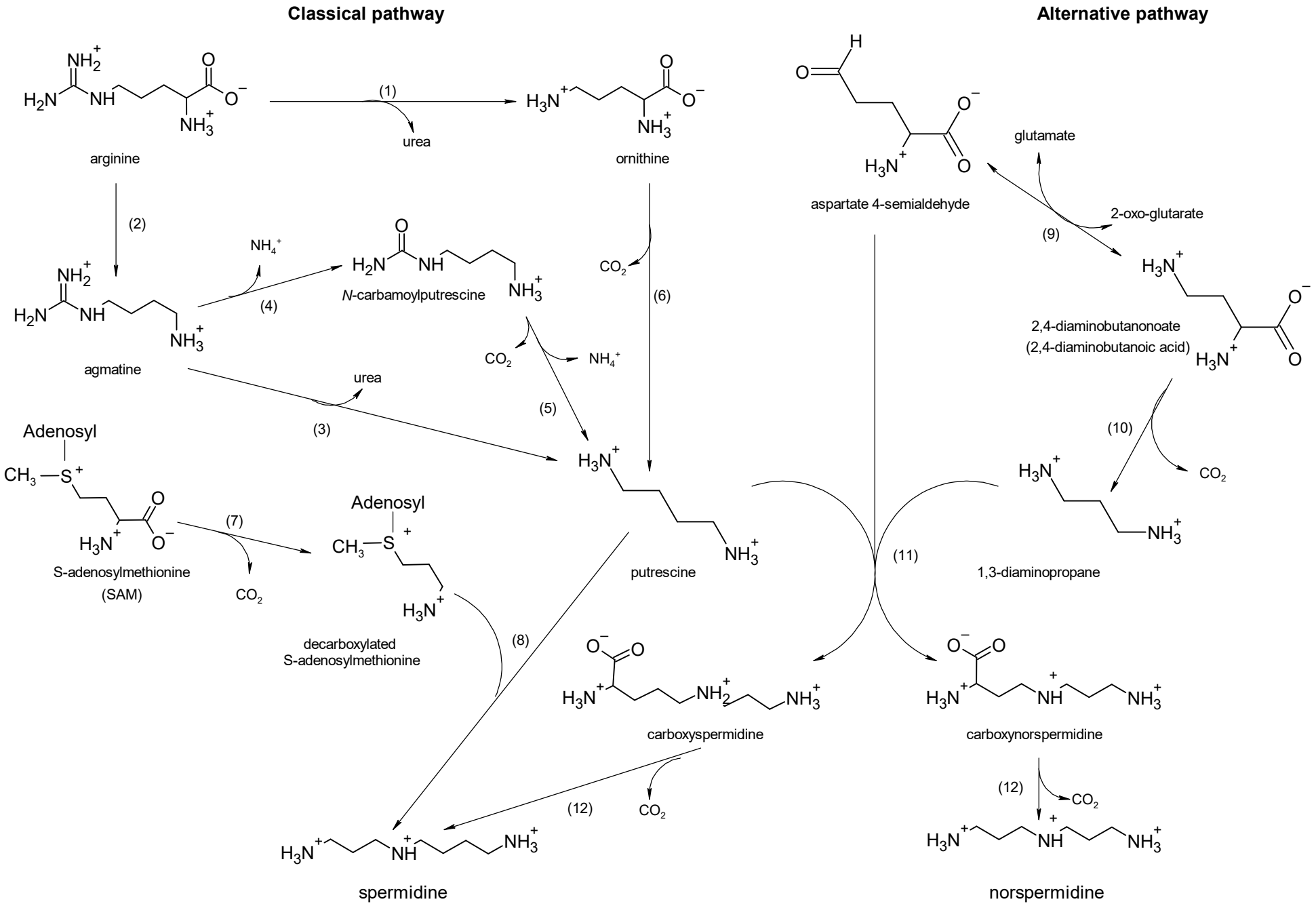

Classical pathway
Alternative pathway
arginine
urea
(1)
ornithine
(2)
agmatine
(4)
N-carbamoylputrescine
(6)
(3)
urea
(5)
S-adenosylmethionine
(SAM)
(7)
decarboxylated
S-adenosylmethionine
(8)
putrescine
spermidine
aspartate 4-semialdehyde
glutamate
2-oxo-glutarate
(9)
2,4-diaminobutanonoate
(2,4-diaminobutanoic acid)
(10)
1,3-diaminopropane
(11)
carboxyspermidine
carboxynorspermidine
(12)
(12)
norspermidine

Figure 5

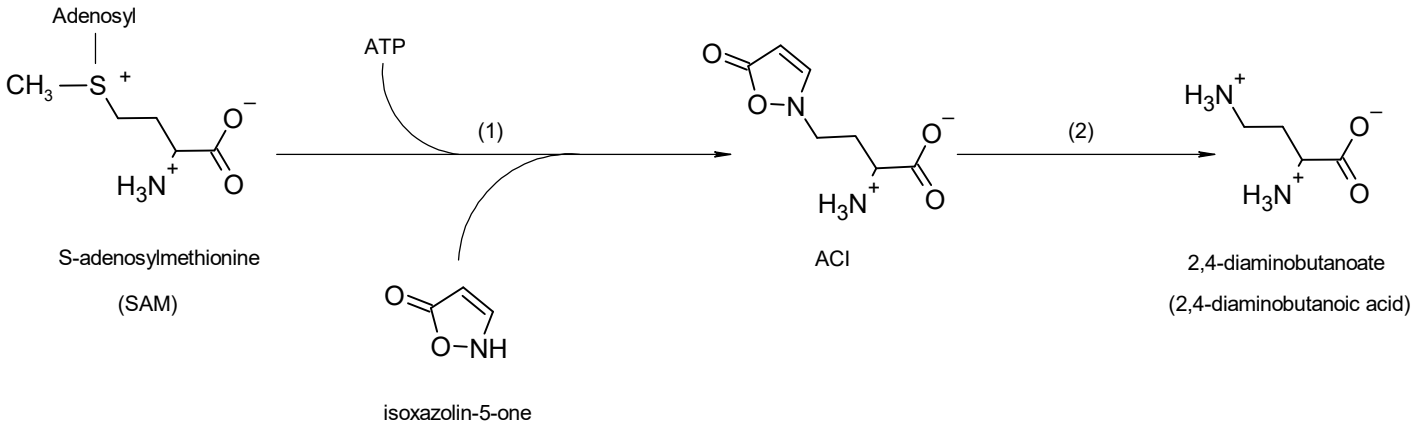
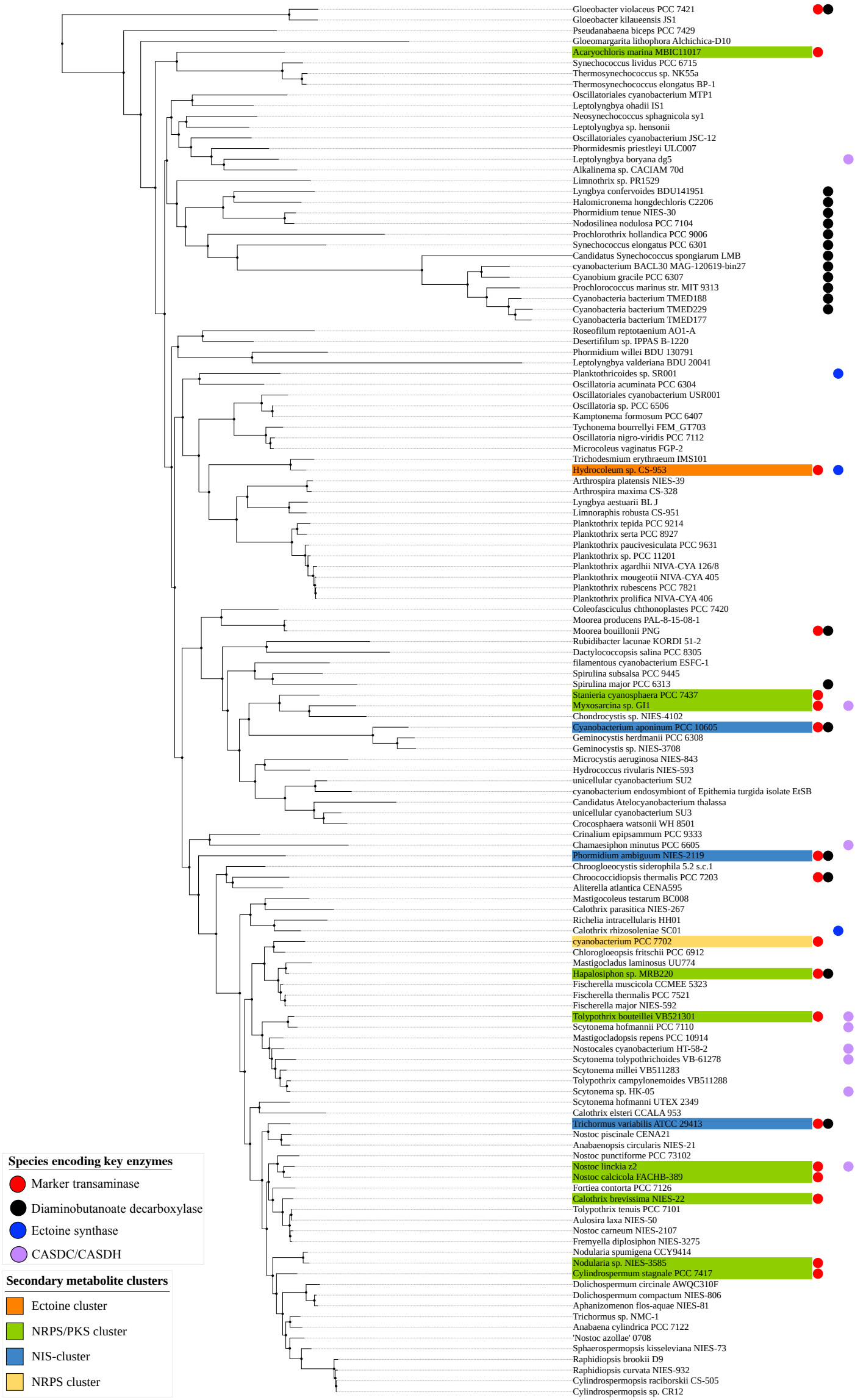

Adenosyl
CH3—S+
O−
H3N+
O
S-adenosylmethionine
(SAM)
ATP
(1)
O
O−NH
isoxazolin-5-one
O
O−N
O−
H3N+
O
ACI
(2)
H3N+
O−
H3N+
O
2,4-diaminobutanoate
(2,4-diaminobutanoic acid)

Figure 6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Gloeobacter kilaueensis JS1
Pseudanabaena biceps PCC 7429
Gloeomargarita lithophora Alchichica-D10
Acaryochloris marina MBIC11017
Synechococcus lividus PCC 6715
Thermosynechococcus sp. NK55a
Thermosynechococcus elongatus BP-1
Oscillatoriales cyanobacterium MTP1
Leptolyngbya ohadii IS1
Neosynechococcus sphagnicola sy1
Leptolyngbya sp. hensonii
Oscillatoriales cyanobacterium JSC-12
Phormidesmis priestleyi ULC007
Leptolyngbya boryana dg5
Alkalinema sp. CACIAM 70d
Limnothrix sp. PR1529
Lyngbya confervoides BDU141951
Halomicronema hongdechloris C2206
Phormidium tenue NIES-30
Nodosilinea nodulosa PCC 7104
Prochlorothrix hollandica PCC 9006
Synechococcus elongatus PCC 6301
Candidatus Synechococcus spongiarum LMB
cyanobacterium BACL30 MAG-120619-bin27
Cyanobium gracile PCC 6307
Prochlorococcus marinus str. MIT 9313
Cyanobacteria bacterium TMED188
Cyanobacteria bacterium TMED229
Cyanobacteria bacterium TMED177
Roseofilum reptotaenium AO1-A
Desertifilum sp. IPPAS B-1220
Phormidium willei BDU 130791
Leptolyngbya valderiana BDU 20041
Planktothricoides sp. SR001
Oscillatoria acuminata PCC 6304
Oscillatoriales cyanobacterium USR001
Oscillatoria sp. PCC 6506
Kamptonema formosum PCC 6407
Tychonema bourrellyi FEM_GT703
Oscillatoria nigro-viridis PCC 7112
Microcoleus vaginatus FGP-2
Trichodesmium erythraeum IMS101
Hydrocoleum sp. CS-953
Arthrospira platensis NIES-39
Arthrospira maxima CS-328
Lyngbya aestuarii BL J
Limnoraphis robusta CS-951
Planktothrix tepida PCC 9214
Planktothrix serta PCC 8927
Planktothrix paucivesiculata PCC 9631
Planktothrix sp. PCC 11201
Planktothrix agardhii NIVA-CYA 126/8
Planktothrix mougeotii NIVA-CYA 405
Planktothrix rubescens PCC 7821
Planktothrix prolifica NIVA-CYA 406
Coleofasciculus chthonoplastes PCC 7420
Moorea producens PAL-8-15-08-1
Moorea bouillonii PNG
Rubidibacter lacunae KORDI 51-2
Dactylococcopsis salina PCC 8305
filamentous cyanobacterium ESFC-1
Spirulina subsalsa PCC 9445
Spirulina major PCC 6313
Stanieria cyanosphaera PCC 7437
Myxosarcina sp. GI1
Chondrocystis sp. NIES-4102
Cyanobacterium aponinum PCC 10605
Geminocystis herdmanii PCC 6308
Geminocystis sp. NIES-3708
Microcystis aeruginosa NIES-843
Hydrococcus rivularis NIES-593
unicellular cyanobacterium SU2
cyanobacterium endosymbiont of Epithemia turgida isolate EtSB
Candidatus Atelocyanobacterium thalassa
unicellular cyanobacterium SU3
Crocosphaera watsonii WH 8501
Crinalium epipsammum PCC 9333
Chamaesiphon minutus PCC 6605
Phormidium ambiguum NIES-2119
Chroogloeocystis siderophila 5.2 s.c.1
Chroococcidiopsis thermalis PCC 7203
Aliterella atlantica CENA595
Mastigocoleus testarum BC008
Calothrix parasitica NIES-267
Richelia intracellularis HH01
Calothrix rhizosoleniae SC01
cyanobacterium PCC 7702
Chlorogloeopsis fritschii PCC 6912
Mastigocladus laminosus UU774
Hapalosiphon sp. MRB220
Fischerella muscicola CCMEE 5323
Fischerella thermalis PCC 7521
Fischerella major NIES-592
Tolypothrix bouteillei VB521301
Scytonema hofmannii PCC 7110
Mastigocladopsis repens PCC 10914
Nostocales cyanobacterium HT-58-2
Scytonema tolypothrichoides VB-61278
Scytonema millei VB511283
Tolypothrix campylonemoides VB511288
Scytonema sp. HK-05
Scytonema hofmanni UTEX 2349
Calothrix elsteri CCALA 953
Trichormus variabilis ATCC 29413
Nostoc piscinale CENA21
Anabaenopsis circularis NIES-21
Nostoc punctiforme PCC 73102
Nostoc linckia z2
Nostoc calcicola FACHB-389
Fortiea contorta PCC 7126
Calothrix brevissima NIES-22
Tolypothrix tenuis PCC 7101
Aulosira laxa NIES-50
Nostoc carneum NIES-2107
Fremyella diplosiphon NIES-3275
Nodularia spumigena CCY9414
Nodularia sp. NIES-3585
Cylindrospermum stagnale PCC 7417
Dolichospermum circinale AWQC310F
Dolichospermum compactum NIES-806
Aphanizomenon flos-aquae NIES-81
Trichormus sp. NMC-1
Anabaena cylindrica PCC 7122
'Nostoc azollae' 0708
Sphaerospermopsis kisseleviana NIES-73
Raphidiopsis brookii D9
Raphidiopsis curvata NIES-932
Cylindrospermopsis raciborskii CS-505
Cylindrospermopsis sp. CR12
Species encoding key enzymes
Marker transaminase
Diaminobutanoate decarboxylase
Ectoine synthase
CASDC/CASDH
Secondary metabolite clusters
Ectoine cluster
NRPS/PKS cluster
NIS-cluster
NRPS cluster

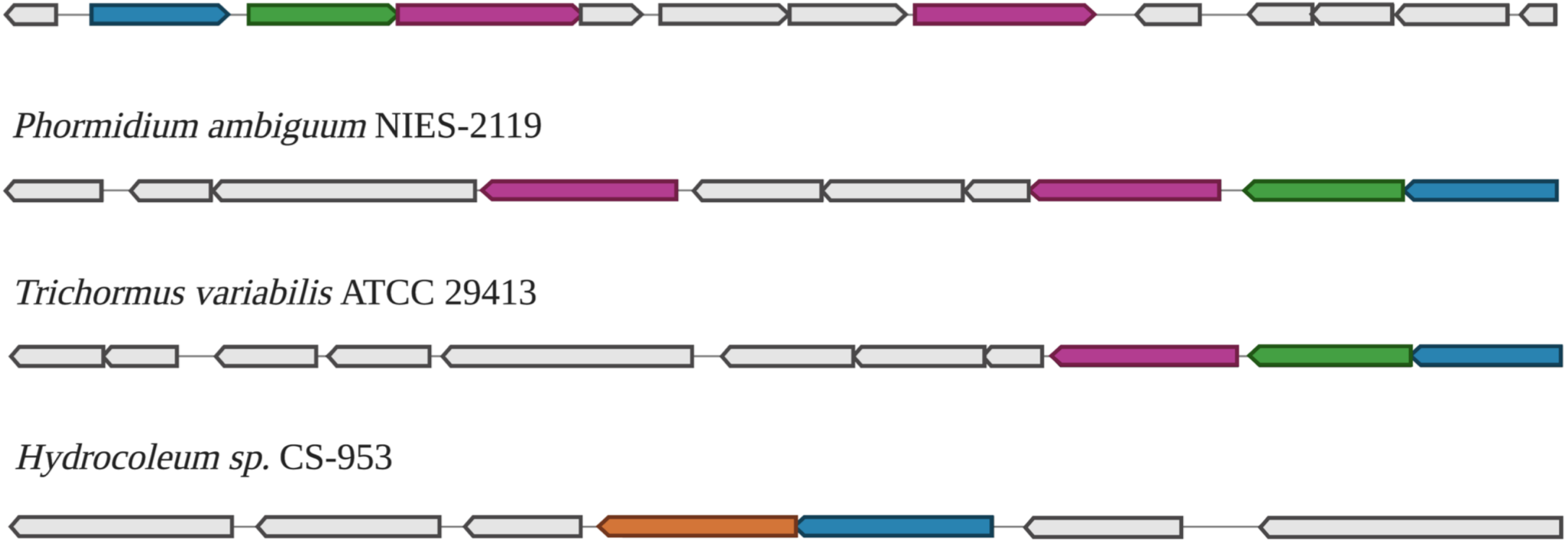
Figure 7

Cyanobacterium aponinum PCC 10605

Phormidium ambiguum NIES-2119

Trichormus variabilis ATCC 29413

Hydrocoleum sp. CS-953



diaminobutanoate 2-oxoglutarate transaminase

diaminobutanoate decarboxylase

IucA/IucC family siderophore biosynthesis protein

ectoine synthase

Figure 8

Tolypothrix bouteillei

diaminobutanoate 2-oxoglutarate transaminase

SPDS/SAMDC

Figure 9