REVIEW ARTICLE

Cyanobacterial toxins: biosynthetic routes and evolutionary roots

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Abstract

Cyanobacteria produce an unparalleled variety of toxins that can cause severe health problems or even death in humans, and wild or domestic animals. In the last decade, biosynthetic pathways have been assigned to the majority of the known toxin families. This review summarizes current knowledge about the enzymatic basis for the production of the hepatotoxins microcystin and nodularin, the cytotoxin cylindrospermopsin, the neurotoxins anatoxin and saxitoxin, and the dermatotoxin lyngbyatoxin. Elucidation of the biosynthetic pathways of the toxins has paved the way for the development of molecular techniques for the detection and quantification of the producing cyanobacteria in different environments. Phylogenetic analyses of related clusters from a large number of strains has also allowed for the reconstruction of the evolutionary scenarios that have led to the emergence, diversification, and loss of such gene clusters in different strains and genera of cyanobacteria. Advances in the understanding of toxin biosynthesis and evolution have provided new methods for drinking-water quality control and may inspire the development of techniques for the management of bloom formation in the future.

Introduction

Every year in the summer months, recurrent mass developments of microscopic algae, so-called harmful algal blooms (HABs), become a matter of public concern. Many of the HAB-causing organisms are known to produce toxins that have a variety of adverse effects, such as acute diarrhea, skin irritation, liver damage, and neurotoxicity in humans and domestic animals, and death in wild animals. Although being collectively regarded as toxic algae in the public's perception, the individual organisms causing blooms belong to different kingdoms of life: eukaryotic microalgae (HABs) and prokaryotic cyanobacteria [CyanoHABs, (Paerl *et al.*, 2011)]. The latter form a diverse group of bacteria, including single-celled picoplankton cyanobacteria as well as colonial and filamentous forms. Cyanobacteria become particularly dominant as surface scum in freshwater lakes and brackish water in the summer months, but may also form dense benthic mats in marine and freshwater habitats. A handful of cyanobacterial species belonging to diverse genera are typically involved in bloom or mat formation (Fig. 1, Table 1). Health risks from cyanobacteria are posed by swimming and other recreational activities in water bodies, by drinking water or otherwise consuming tissues or dietary supplements that have accumulated the toxins. The mass development of blooms is also connected with significant economic costs on a global scale due to the necessity of bloom management and cyanobacteria removal as well as the negative effects on farming and recreation (Steffensen, 2008).

In the past few years, biosynthetic pathways could be assigned to the majority of known cyanobacterial toxins. Knowledge about the pathways has not only revealed unique biochemical features, but also inspired the development of new techniques for the detection and differentiation of toxigenic cyanobacteria in the field. Moreover, the phylogenetic comparison of biosynthetic pathways from different producing genera allowed the reconstruction of the evolutionary history and ongoing diversification mechanisms of some of the toxins. Studying biosynthetic genes has thus strongly influenced the

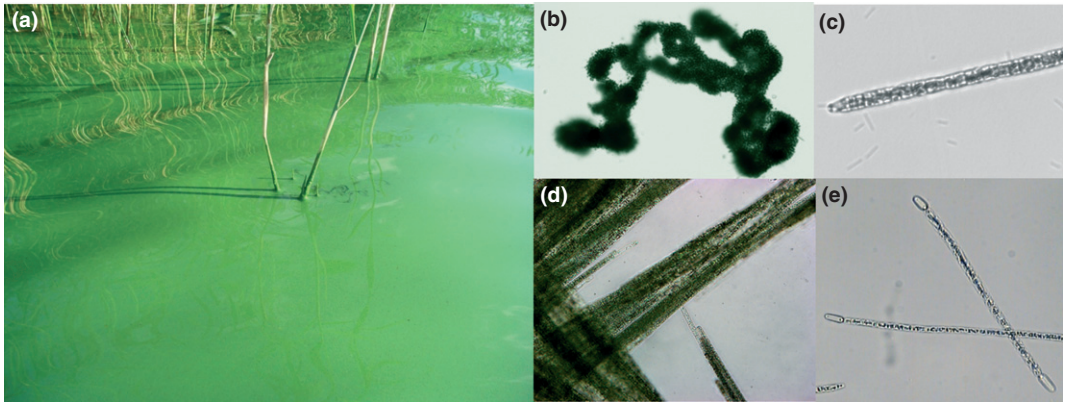

Fig. 1. Representative pictures of toxin-producing cyanobacteria. (a) Bloom of *Microcystis* at the shore of lake Wannsee, Germany. (b) Colony of *M. aeruginosa*. (c) Filament of *P. agardhii*. (d) Bundle of *Aphanizomenon flos-aquae*. (e) Filaments of *C. raciborskii*.

Table 1. Properties, distribution and biosynthetic genes of cyanobacterial toxin families

Cyanobacterial toxin	Producing genera/species	Toxic mechanism	Biosynthetic genes	Genbank accession numbers
Microcystin	*Microcystis* sp. *Planktothrix* sp. *Anabaena* sp. *Nostoc* sp. *Hapalosiphon* sp. *Phormidium* sp.	Hepatotoxic; inhibition of eukaryotic protein phosphatases of type 1 and 2A	*mcyA-J*	AF183408 AJ441056 AJ536156
Nodularin	*N. spumigena*	Hepatotoxic; inhibition of eukaryotic protein phosphatases of type 1 and 2A	*ndaA-I*	AY210783
Cylindrospermopsin	*C. raciborskii* *A. ovalisporum* *U. natans* *R. curvata* *Anabaena* sp. *Oscillatoria* sp.	Hepatotoxic, cytotoxic, neurotoxic; inhibition of glutathione synthesis, protein synthesis and cytochrome P450	*cyrA-O* *aoaA-C*	EU140798 AF395828 FJ418586
Anatoxin-a Homoanatoxin-a	*A. flos-aquae* *Oscillatoria* sp. *Aphanizomenon* sp.	Neurotoxic, mimics the neurotransmitter acetylcholine	*anaA-H*	FJ477836 JF803645
Saxitoxin	*A. circinales* *Aphanizomenon* sp. *A. grazile* *C. raciborskii* *L. wollei*	Neurotoxic, blocks voltage-gated Na+ channels	*sxtA-Z*	DQ787200
BMAA	*Many cyanobacteria*	Neurotoxic, motor neuron damage and loss	*Unknown*	–
Lyngbyatoxin	*L. majuscula* *(M. producens)*	Tumor promoting, binds to protein kinase C (PKC)	*ltxA-D*	AY588942
Aplysiatoxin	*L. majuscula* *(M. producens)*	Tumor promoting, binds to protein kinase C (PKC)	*Unknown*	–

interdisciplinary field of cyanotoxin research and provided new perspectives for bloom monitoring and management.

Here, we give an overview about known biosynthetic routes of cyanobacterial toxins. We have considered metabolites that were reportedly implicated in human health problems. Apart from giving a short summary about toxicological characteristics and the environmental distribution for each of the toxin families, we will emphasize genetic and biochemical studies that have contributed to the assignment of biosynthetic genes and elucidation of biochemical features of the pathways. A second focus of the review will be evolutionary studies on toxin bio-

synthesis genes. Finally, we will highlight how the biosynthetic and evolutionary knowledge was translated into new techniques for the detection and quantification of toxic cyanobacteria. We will not discuss studies on the environmental regulation and biological functions of cyanobacterial toxins that were covered in a recent review (Neilan *et al.*, 2012) as well as methods for extraction and analytical detection of toxins that have been summarized for example in van Apeldoorn *et al.* (2007) and McElhiney & Lawton (2005). The progress in the selected research fields strongly differs for the different toxin families. Whereas biosynthetic genes for the well-known hepatotoxin microcystin have been known for more than a decade, other toxin pathways were assigned rather recently or still await discovery. The review will thus not only sum up existing knowledge but also highlight current research gaps.

Hepatotoxins

Microcystins

The most frequently reported cyanobacterial toxin is the hepatotoxin microcystin (MCY, Fig. 2). The general structure of microcystins is cyclo(D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha), in which X and Z represent variable L amino acids. D-MeAsp is D-erythro-ß-methyl-aspartic acid, Mdha is N-methyldehydroalanine, and Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Botes *et al.*, 1984) (Fig. 2). Microcystin is predominantly produced by different genera of freshwater cyanobacteria, including *Microcystis*, *Planktothrix* and *Anabaena* (Fig. 1, Table 1) (Sivonen & Jones, 1999), although it has also been detected in terrestrial strains of the genera *Nostoc* and *Hapalosiphon* (Prinsep *et al.*, 1992; Oksanen *et al.*, 2004) or in mats of benthic *Phormidium* (Izaguirre *et al.*, 2007). Over 80 variants have been described in the scientific literature, mostly differing in amino acid positions X and Z (Sivonen & Jones, 1999). Microcystins inhibit eukaryotic protein phosphatases type 1 and 2A and have been shown to establish an irreversible covalent bond to a cysteine in the catalytic domain of these enzymes (Runnegar *et al.*, 1995a).

Toxicology and human health aspects

The acute toxicity of microcystin-LR has been tested in several studies on mice and revealed intra-peritoneal (i.p.) LD_{50} values ranging from 25 to 150 μg kg^{-1} body weight (bw). A LD_{50} value of 50–60 μg kg^{-1} bw is generally accepted (Table 2) (for a review, see (Chorus & Bartram, 1999)). Most of the structural variants of microcystin have a similar LD_{50} range as microcystin-LR (such as microcystin-LA, microcystin-YR, and microcystin-YM),

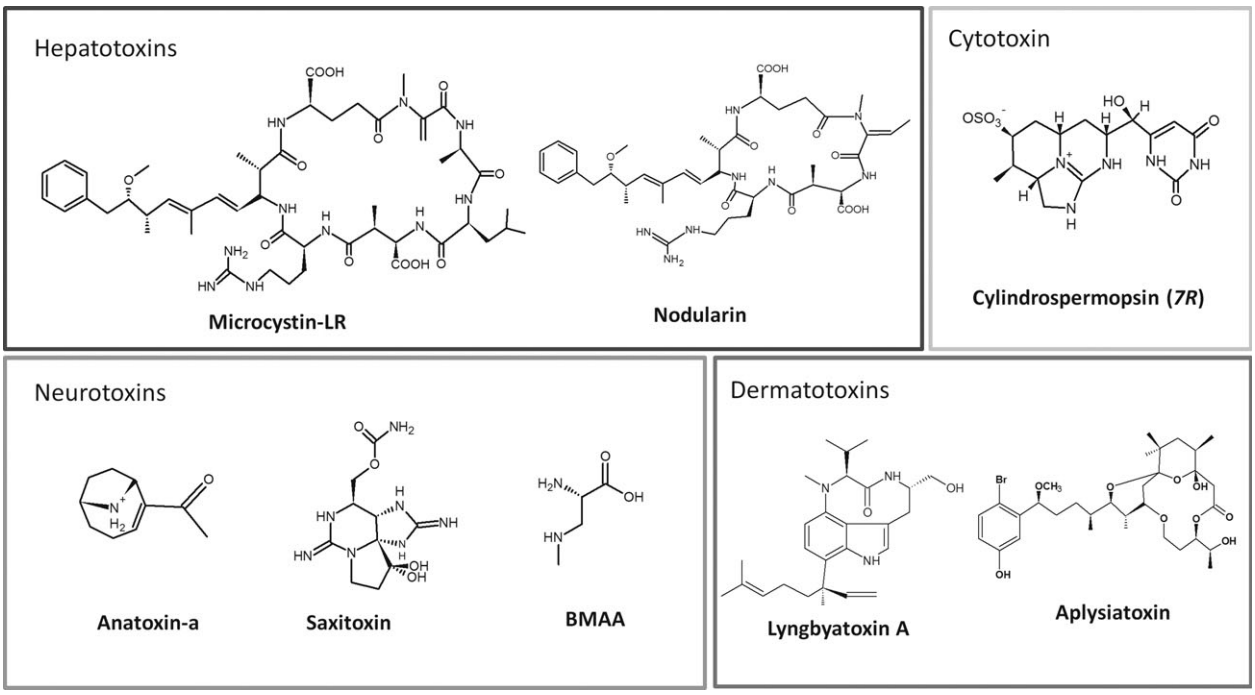

Fig. 2. Representative chemical structures for cyanobacterial toxin families.

Table 2. Selected i.p. LD_{50} values of different cyanobacterial toxins from experiments with mice

LD_{50} values i.p. in mice	μg kg^{-1} bw	Reference
Microcystin-LR	50–60	Generally accepted Chorus & Bartram (1999)
Microcystin-RR	500	Chorus & Bartram (1999)
Nodularin	30–50	Chorus *et al.* (2000)
Cylindrospermopsin	200*	Chorus & Bartram (1999)
Anatoxin-a	375	Chorus & Bartram (1999)
Homoanatoxin-a	250	Chorus & Bartram (1999)
Saxitoxin	10	Oshima (1995)
Neosaxitoxin	65	Wolf & Frank (2002)
Lyngbyatoxin	250	Ito *et al.* (2002)
Aplysiatoxin	~ 107	Ito & Nagai (1998)

*After 5–6-days.

and the i.p. LD_{50} of the abundant variant microcystin-RR, however, is about ten times higher (Table 2) (Chorus & Bartram, 1999). Both i.p. and i.v. administration of microcystins leads to disruption of liver cells, loss of sinusoidal structure, intrahepatic hemorrhage, hemodynamic shock, and finally heart failure and death of mice.

Cases of direct toxicity to humans are extremely rare; however, there are a number of human intoxications that were attributed to the toxin. The most fatal case was reported from a hemodialysis unit in Caruaru, Brazil, where at least 50 patients died with symptoms of neurotoxicity and hepatotoxicity after exposure to contaminated water from a nearby reservoir (Jochimsen *et al.*, 1998). Direct evidence for a cyanotoxicosis came from the analysis of blood samples of deceased patients that showed considerable amounts of microcystin-YR, microcystin-LR, and microcystin-AR. Although the hints for the involvement of microcystin are not always as direct as for the 'Caruaru-Syndrome', there are a number of further severe cases of gastrointestinal and hepatic illness that were attributed to microcystin. Typically, outbreaks of such diseases are linked with the breakdown of cyanobacterial blooms, such as in Armidale, Australia, in 1981 or in the area of the Itaparica Dam, Brazil, in 1988 (Chorus & Bartram, 1999).

Environmental concentrations of microcystin are mostly determined from lyophilized bloom samples. The highest concentration of microcystin measured was 7300 μg g^{-1} dry weight (dw) [(Zhang *et al.*, 1991), Table 3]. For water treatment purposes or public health management, toxin concentrations per liter of water are more relevant. In scums or other dense accumulations of cyanobacteria microcystin levels of up to 25 000 μg L^{-1} were reported [(Chorus & Bartram, 1999), Table 3]. In the environment, most of the microcystin is cell bound, and only little or no microcystin is dissolved in water. Values of dissolved microcystin typically do not exceed a value of 1 μg L^{-1} and are thus below the guideline value recommended by WHO for drinking water and not considered dangerous for humans. However, following the collapse of a bloom concentrations up to 1800 μg L^{-1} or higher were detected [(Jones & Orr, 1994), Table 3]. Bioaccumulation of microcystin in aquatic animals has been reported and may have considerable effects on aquatic food chains, in particular as biodegradation of the toxin is slow [for a review see, (van Apeldoorn *et al.*, 2007)].

Nodularin

Mass occurrences of cyanobacterial blooms dominated by *Nodularia spumigena* are reported each summer in the brackish Baltic Sea. *Nodularia* blooms can cover very large areas, around 100 000 km^2 in size. *Nodularia spumigena* invariably produces nodularin, which has been linked with the deaths of wild and domestic animals around the Baltic Sea, Australia, and New Zealand (Moffitt & Neilan, 2004). Nodularins (Table 1, Fig. 2) are closely related to microcystins and mostly possess the

Table 3. Maximum toxin concentrations detected in field samples

Toxin	Maximum toxin concentrations (μg g^{-1} dw, if not otherwise indicated)	Analysis method	Reference
Microcystin	7300	HPLC	Zhang *et al.* (1991)
	25 000*	HPLC	Chorus & Bartram (1999)
	1800†	HPLC	Jones & Orr (1994)
Nodularin	18 000	HPLC	Kononen *et al.* (1993)
Cylindospermopsin	5500	HPLC	Chorus & Bartram (1999)
Anatoxin-a	4400	GC-MS	Sivonen *et al.* (1989)
Anatoxin-a (S)	3300	ChE inhibition assay	Henriksen *et al.* (1997)
Saxitoxin	3400	HPLC	Negri *et al.* (1997)

*Value was calculated in μg L^{-1}.

†Dissolved microcystin after bloom-break-down in μg L^{-1}.

structure cyclo(D-MeAsp-L-Arg-Adda-D-Glu-Mdhb), in which Mdhb is 2-(methylamino)-2-dehydrobutyric acid (Fig. 2) (Rinehart *et al.*, 1988). Nodularin is a potent inhibitor of eukaryotic-type protein phosphatases 1 and 2A and reaches i.p. LD_{50} values of 30–50 μg g^{-1} bw of mice (Table. 2, Chorus *et al.*, 2000). Nodularins do not form a covalent bond with their target, suggesting that the overall configuration of microcystin and nodularin rather than the covalent bond is needed for effective enzyme inhibition (Annila *et al.*, 1996). Concentrations as high as 18 000 μg g^{-1} dw were reported for a bloom from the Baltic Sea (Table. 3, Kononen *et al.*, 1993). However, whereas nodularins are produced by *N. spumigena*, related benthic species of *Nodularia* typically do not produce the toxins (Lehtimäki *et al.*, 2000).

Microcystin and nodularin share a closely related assembly line

As microcystin and nodularin share closely related structures a common biosynthetic route was anticipated. Research toward elucidating the biosynthetic pathway started with two microcystin-producing strains of *Microcystis aeruginosa* (Dittmann *et al.*, 1997; Nishizawa *et al.*, 1999, 2000; Tillett *et al.*, 2000). Feeding studies and the unusual structure of the molecule suggested a nonribosomal origin of this cyclic heptapeptide (Moore *et al.*, 1991). The search for the microcystin gene cluster thus started with a general search for gene fragments encoding a nonribosomal peptide synthetase (NRPS). The correlation of these gene fragments with microcystin biosynthesis could be drawn by genetic comparison of toxic and nontoxic strains of *Microcystis* and by insertional mutagenesis of one of the genes that ultimately led to a complete loss of microcystin production in *M. aeruginosa* PCC7806 (Dittmann *et al.*, 1997). The entire 55-kb microcystin gene cluster was elucidated from genomic libraries of the strains PCC7806 and K81 and encodes ten proteins, nine of which can be assigned to direct biosynthesis of the hepatotoxin. The course of enzymatic steps underlying microcystin biosynthesis was initially predicted from bioinformatic analyses of proteins and domain specificities (Nishizawa *et al.*, 1999, 2000; Tillett *et al.*, 2000). A subset of these enzymatic reactions necessary to make microcystin have been verified or redefined by *in vitro* biochemical studies or *in vivo* mutagenesis (Fig. 3) (Christiansen *et al.*, 2003; Sielaff *et al.*, 2003; Hicks *et al.*, 2006; Pearson *et al.*, 2007).

The assembly starts with the activation of a phenylalanine-derived phenyl propionate starter unit at the NRPS/polyketide synthase (PKS) hybrid enzyme McyG (Hicks *et al.*, 2006) Fig. 3). As part of the elongation process a C1 unit of this starter is presumably truncated to yield phenylacetate. The remaining skeleton of the characteristic amino acid Adda is formed through the activity of four PKS modules being part of McyG, D and E. Side chain modifications of Adda are incorporated by the *O*-methyltransferase McyJ (Christiansen *et al.*, 2003) and the amino transferase domain of McyE (Tillett *et al.*, 2000) (Fig. 3). The six remaining amino acids are incorporated by the activity of six NRPS modules of McyG, A, B, and C (Tillett *et al.*, 2000). One of the moieties, D-erythro-ß-methyl-aspartic acid, requires the activity of two additional enzymes prior to incorporation in the peptide chain: the 2-hydroxy acid dehydrogenase McyI (Pearson *et al.*, 2007) and the aspartate racemase McyF (Sielaff *et al.*, 2003). The elongated peptide is finally released from the enzyme complex by the thioesterase domain of McyC (Fig. 3).

Microcystin biosynthesis genes were also characterized for the strain *Planktothrix agardhii* NIVA-CYA 126 (Christiansen *et al.*, 2003) and the strain *Anabaena* sp. 90 (Rouhiainen *et al.*, 2004). In all genera, microcystin biosynthesis follows the same course of reactions, despite some individual differences in domain specificities. The *Planktothrix* gene cluster does not encode McyF or McyI enzymes; however, it encodes an additional thioesterase, McyT, which might be responsible for proofreading during the assembly process (Christiansen *et al.*, 2008a). The organization of operons and genes also varies in the three genera. Whereas *mcy* genes in *Microcystis* and *Anabaena* are transcribed from a bidirectional promoter region between *mcyA* and *mcyD* and *mcyA* and *mcyG*, respectively, microcystin genes in *Planktothrix* are mostly unidirectional (except for *mcyT*). Only in *Anabaena* are the order of modules in agreement with the individual steps during microcystin biosynthesis and thus following the colinearity rule that is commonly found in NRPS gene clusters (Dittmann & Börner, 2005).

The nodularin biosynthesis gene cluster was characterized for a *N. spumigena* NSOR10 strain using a genome-walking method (Moffitt & Neilan, 2004). The 48-kb nodularin gene cluster consists of 9 ORFS encoding NRPS and PKS modules as well as tailoring enzymes. These *nda* genes are organized in a bidirectional operon and follow the colinearity rule. Two NRPS modules, namely the second module of McyA and the first module of McyB are deleted and the remaining modules have been fused to yield the bimodular NRPS NdaA that replaces McyA and McyB in the microcystin pathway (Moffitt & Neilan, 2004). There is also a change in the substrate specificity of NdaA resulting in the activation of L-Tyr instead of L-Ser leading to the presence of Mdhb in nodularins (Moffitt & Neilan, 2004).

All microcystin and nodularin gene clusters encode an ABC transporter protein that is composed of a membrane

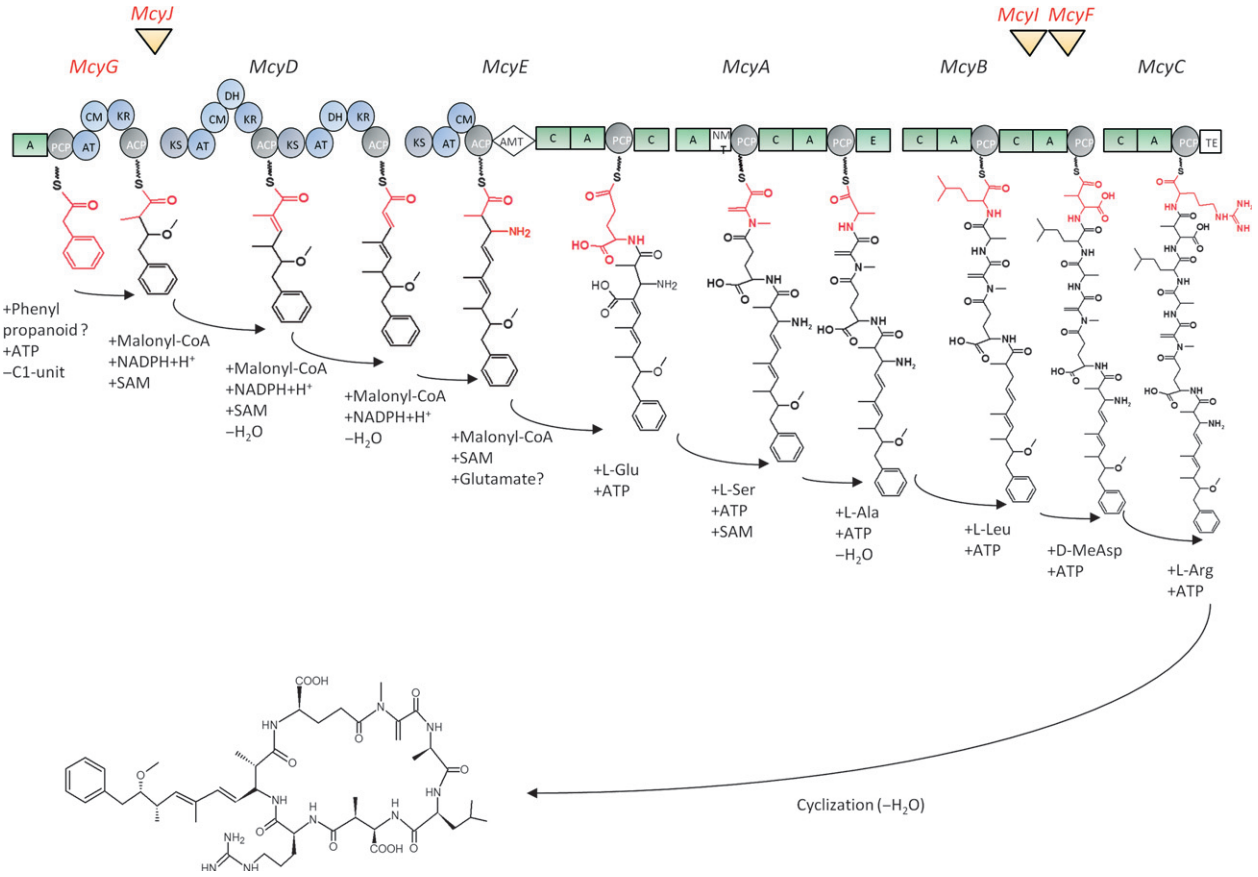

Fig. 3. Schematic representation of the biosynthetic assembly line of microcystin. Names of enzymes that were biochemically characterized are highlighted in red. Other proposed reactions are based on bioinformatic predictions. Amino acid and carboxylic acid units incorporated or modified by individual proteins are highlighted in red. PKS domains: KS: ketoacyl synthase, AT: acyltransferase, DH, dehydratase, KR, ketoreductase, ACP, acyl carrier protein, CM, C-methyltransferase; AMT: glutamate-semialdehyde aminotransferase; NRPS domains: A: adenylation domain; C: condensation domain, E: epimerization domain; NMT: N-methyltransferase; PCP: peptidyl carrier protein, TE: thioesterase domain.

domain and a cytosolic ATP binding domain. Phylogenetic analysis could assign this transporter to the exporter family (Pearson *et al.*, 2004). Direct evidence for an involvement of the transporter in microcystin or nodularin secretion, however, is currently not available. Insertional mutagenesis of the corresponding gene in *Microcystis*, McyH, leads to a complete loss of microcystin production, despite the fact that other biosynthetic genes were still transcribed (Pearson *et al.*, 2004). The transporter may therefore fulfill a scaffolding role for the biosynthesis complex in addition to its putative role as an exporter.

Evolution and diversification of microcystin and nodularin biosynthetic gene clusters

Microcystins are predominantly produced by strains of genera *Microcystis*, *Anabaena*, and *Planktothrix* (Prinsep *et al.*, 1992; Sivonen & Jones, 1999; Izaguirre *et al.*, 2007). Not all strains of these genera can produce microcystins, and toxic and nontoxic strains coexist in water bodies around the world. Horizontal gene transfer was anticipated to play a role in the distribution of the microcystin biosynthetic genes with plasmid-mediated transfer hypotheses (Schwabe *et al.*, 1988; Bolch *et al.*, 1997). However, phylogenetic evidence pointed toward an ancient origin of the toxin (Moffitt & Neilan, 2004; Rantala *et al.*, 2004) (Fig. 4). Comparison of phylogenetic trees based on microcystin synthetase genes and genes involved in primary metabolism were congruent (Rantala *et al.*, 2004). This phylogenetic congruency and the large genetic differences between different genera of microcystin producers were inconsistent with models based on horizontal gene transfer (Rantala *et al.*, 2004). The presence of the microcystin gene cluster in different cyanobacterial genera is now thought to reflect the loss of the gene cluster in other genera that do not produce

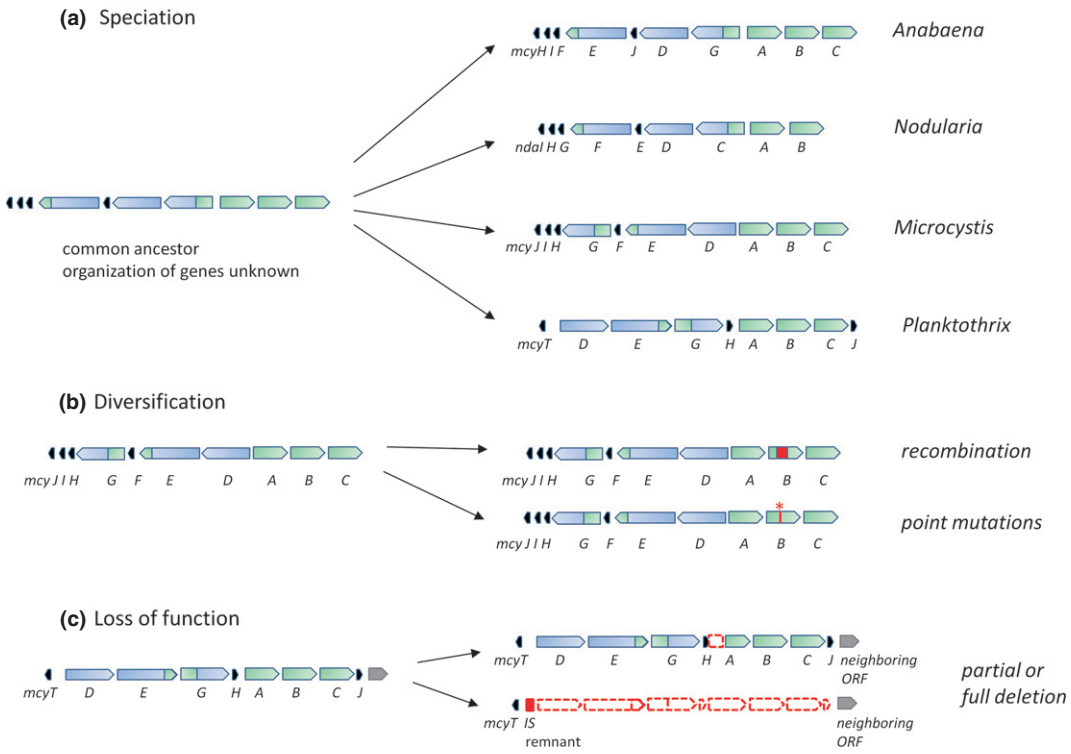

Fig. 4. Evolutionary forces acting on microcystin synthetase gene clusters. NRPS and PKS genes are shown in green and blue, respectively. (a) Diverse clusters of microcystin and nodularin synthetases are monophyletic and have evolved from a common ancestor. The genetic organization, domain specificities and the presence of absence of tailoring genes vary between different microcystin-producing genera. (b) Ongoing diversification of microcystin synthetases. Microcystin synthetase genes are the subject of frequent recombination events and are further diversified by point mutations. The diversification process is reflected by the more than 80 microcystin variants that have been described in the literature. Examples are shown for microcystin synthetase of *Microcystis*. (c) Loss of the capability of producing microcystin by deletion of partial or entire clusters in individual strains through the activity of transposases. Examples are shown for microcystin synthetase of *Planktothrix*.

microcystin (Moffitt & Neilan, 2004; Rantala *et al.*, 2004). The authors also came to the conclusion that additional cyanobacterial genera may have retained the ability to produce microcystin. This phylogeny-based hypothesis could indeed be confirmed by experimental studies as additional microcystin producers were recently found in the genera *Fischerella*, *Phormidium*, *Nostoc,* and *Hapalosiphon* (Fewer *et al.*, 2007; Izaguirre *et al.*, 2007; Fiore *et al.*, 2009; Kaasalainen *et al.*, 2009).

The co-occurrence of toxic and nontoxic strains of the same genus is most easily attributed to the presence or absence of the microcystin gene cluster (Dittmann *et al.*, 1997). However, a number of recent studies demonstrate that inactivation and loss of microcystin gene clusters are ongoing processes (Kurmayer *et al.*, 2004; Christiansen *et al.*, 2008a, ,b; Fewer *et al.*, 2011) (Fig. 4). Particularly in *Planktothrix rubescens,* inactivation of the *mcy* gene cluster by insertion sequences appears to be common place (Kurmayer *et al.*, 2004; Christiansen *et al.*, 2008a, b). Horizontal gene transfer of entire microcystin gene clusters between strains of the same genus is also thought to play a role in the occurrence of toxic and nontoxic strains (Tooming-Klunderud *et al.*, 2008). Horizontal gene transfer and homologous recombination replacing parts of the microcystin gene cluster has been reported in *Microcystis* (Tanabe, *et al.*, 2004, Tanabe, *et al.*, 2009).

Microcystins are a chemically diverse family of peptides and well over 80 structural variants have been reported to date. The bulk of this chemical variation has a genetic basis and a number of genetic diversification mechanisms have been linked with the chemical variety of microcystins (Fig. 4). The most frequently reported mechanism is replacement of adenylation domains through homologous recombination events (Fewer *et al.*, 2007; Christiansen *et al.*, 2008b). Recurrent adenylation domain replacement in the McyB and McyC modules that are responsible for the incorporation of L-Leu and L-Arg into microcystins has been reported in two studies assessing the impact of recombination events on the evolution of microcystin biosynthesis gene clusters (Mikalsen, *et al.*, 2003, Fewer *et al.*, 2007). Similar replacement of adenylation domains has been reported from the *mcyA* gene of some *Plankto-*

thrix strains resulting in the incorporation of Dhb instead of Mdha (Christiansen *et al.*, 2008a, ,b). The source of the adenylation domains is unclear but may be the result of horizontal gene transfers or intragenomic reshuffling of adenylation domains.

Positive selection has also been shown to act on microcystin synthetase genes resulting in changes in substrate specificity of the enzymes assembling microcystins (Tooming-Klunderud *et al.*, 2008). Point mutations have been linked with changes in substrate specificity in other cases too, but it is unclear whether these changes are adaptive or not (Fewer *et al.*, 2007; Christiansen *et al.*, 2008a, ,b) (Fig. 4). Point mutations in substrate-binding sequence motifs of the *N*-methyltransferase domain of the *mcyA* gene have been linked with the production of demethylated variants of microcystin by some strains of the genus *Microcystis* (Tooming-Klunderud *et al.*, 2008).

Deletion events are reported more rarely from microcystin synthetase gene clusters. Deletions were described for *Planktothrix* and *Microcystis* strains and associated with the inactivation of microcystin gene cluster (Christiansen *et al.*, 2008a, ,b; Tooming-Klunderud *et al.*, 2008; Noguchi *et al.*, 2009) (Fig. 4). However, microcystin synthetase gene clusters in some strains of *Anabaena* have also undergone a deletion event that removed *N*-methyltransferase domains but yielding functional peptide synthetases (Fewer *et al.*, 2008). This mutation results in the production of microcystins that lack the *N*-methyl group.

The nodularin pathway shows stronger deviation from the closely related microcystin pathway. Elucidation of the *nda* gene cluster demonstrated that the nodularin synthetase evolved from a microcystin synthetase through a deletion event and a change in substrate specificity (Moffitt & Neilan, 2004). To date nontoxic strains of the *N. spumigena* have not been reported, and there is no evidence for inactivation of the gene cluster. Likewise, there is very little chemical variation in nodularins with just six variants reported from the literature.

Detection and quantification of microcystin-producing cyanobacteria

Environmental analysis of microcystin is mostly carried out using HPLC or LC-MS based on quantitative standards, using ELISAs based on sensitive antibodies against microcystin or through bioassays such as protein phosphatase inhibition assays (for a review, see (McElhiney & Lawton, 2005 and van Apeldoorn *et al.*, 2007).

The discovery of the microcystin synthetase gene cluster promoted the development of culture-independent methods to detect microcystin-producing cyanobacteria directly in the field. The genetic difference between different genera of microcystin-producing cyanobacteria meant that is was possible to develop PCR-based techniques to discriminate microcystin producers without the need to culture those (Hisbergues *et al.*, 2003). This type of analysis can complement the detection of microcystin by chemical and immunological analyses, in particular in field samples containing different potential microcystin-producing cyanobacteria. Molecular techniques allow for an assignment of the actual producer of microcystin and to provide insights into the dynamics of toxin-producing cyanobacteria and environmental regulation of toxins in the field; however, they cannot substitute methods that directly quantify toxins under any circumstances. The initial PCR techniques were later advanced to allow direct PCR from individual colonies or filaments (Kurmayer *et al.*, 2002; Via-Ordorika *et al.*, 2004). Furthermore, several research groups have established and applied real-time PCR protocols for the quantification of microcystin-producing cyanobacteria (Kurmayer & Kutzenberger, 2003; Vaitomaa *et al.*, 2003; Al-Tebrineh *et al.*, 2011). Chip-based assays have also been developed to sensitively detect microcystin or nodularin genes from five different cyanobacterial genera (Rantala *et al.*, 2008; Sipari *et al.*, 2010). Although the advancement of these molecular techniques for the detection of microcystin has been an environmental monitoring success story, it has also uncovered difficulties in the interpretation of the results. Bias in each of these different approaches can result from the occurrence of nontoxic mutants in the field that can give rise to false-positive data (Kurmayer *et al.*, 2004; Fewer *et al.*, 2008) and from variations in the microcystin synthetase sequence that can result in false-negative data (Via-Ordorika *et al.*, 2004; Mbedi *et al.*, 2005). The latter problem can be avoided by understanding how microcystin synthetase gene clusters have diversified and targeting conserved regions of the *mcy* gene cluster (Jungblut & Neilan, 2006).

Cytotoxins

Cylindrospermopsin

Cylindrospermopsin (CYN) is a cyclic sulfated guanidine alkaloid (Fig. 2) causing general cytotoxic, hepatotoxic, and neurotoxic effects (Ohtani *et al.*, 1992). Structural variants include 7-epi-CYN and 7-deoxy-CYN (Ohtani *et al.*, 1992; Banker *et al.*, 2001; Li *et al.*, 2001). CYN production has been associated with various species such as *Cylindrospermopsis raciborskii* (Fig. 1e), *Umezakia natans*, *Aphanizomenon* sp., *Raphidiopsis curvata*, *Lyngbya wollei,* and *Anabaena bergii* (Table 1) CYN has been predominantly reported from tropical and subtropical waters where it occurs in different environments such as lakes, reservoirs, rivers, ponds, and dams (Pearson *et al.*, 2010).

Cylindrospermopsis raciborskii is also increasingly detected in lakes of temperate climates in Germany and France and considered as an invasive species due to global warming (Fastner *et al.*, 2007). However, this species does not typically produce cylindrospermopsin in temperate lakes, whereas the toxin is frequently detected in *Aphanizomenon* spp. in these countries (Fastner *et al.*, 2007).

Toxicology and human health aspects

The toxicity of cylindrospermopsin is due to the inhibition of glutathione and protein synthesis, the inhibition of cytochrome P450, and direct interaction with DNA (Table 1) (Runnegar *et al.*, 1995b, 2002; Humpage *et al.*, 2000; Kiss *et al.*, 2002). Administration of pure cylindrospermopsin in mice leads to a delayed toxicity with a decrease in i.p. LD_{50} values of 2100 μg kg^{-1} bw after 24 h to 200 μg kg^{-1} bw after 5–6 days [(Chorus & Bartram, 1999), Table 2]. The most prominent case of human poisoning attributed to cylindrospermopsin was the so-called Palm Island Mystery Disease in 1979, where a major outbreak of hepato-enteritis among the Aboriginal community on a tropical island in Queensland, Australia, could not be related to any infectious organism but to the consumption of drinking water from one particular source, Solomon Dam. The dam contained a bloom of *C. raciborskii*. Cultivation of the organisms from the dam and administration to mice lead to similar tissue injuries as found in the Aboriginal community (Hawkins *et al.*, 1985). Subsequent research on this cyanobacterium led to the discovery of cylindrospermopsins (Ohtani *et al.*, 1992). Research on environmental concentrations and bioaccumulation of cylindrospermopsin is lagging behind those studies for microcystin; however, concentrations of up to 5500 μg g^{-1} dw of bloom material were reported from Australia [(Chorus & Bartram, 1999), Table. 3].

Cylindrospermopsin is an NRPS-/PKS-derived alkaloid

Putative cylindrospermopsin biosynthesis genes (*cyr*) were first identified in *C. raciborskii* (Schembri *et al.*, 2001) based on the conserved nature of the NRPS and PKS sequences in cyanobacteria (Neilan *et al.*, 1999). Shalev–Alon *et al.*, have later identified an amidinotransferase gene (*aoaA*) in *Aphanizomenon ovalisporum* likely involved in formation of the guanidinoacetic acid precursor of cylindrospermopsin. (Shalev-Alon *et al.*, 2002). Genome walking subsequently revealed the complete gene cluster (*cyr*) in *C. raciborskii* (Mihali *et al.*, 2008). The *cyr* locus spans 42-kb encoding 15 open-reading frames (Fig. 5a). Screening of cylindrospermopsin-producing and nonproducing cyanobacteria for the presence of genes encoding the sulfotransferase CyrJ (Mihali *et al.*, 2008), the NRPS/PKS hybrid CyrB, and the PKS CyrC (Schembri *et al.*, 2001) showed that these genes are exclusively present in cylindrospermopsin producers.

Bioinformatic analysis of the gene cluster together with isotope-labeled precursor feeding experiments (Burgoyne *et al.*, 2000) predicted that a number of genes encoding unusual catalytic domains and enzymes were present in cylindrospermopsin biosynthesis, and a pathway was proposed (Mihali *et al.*, 2008). Biochemical characterization of some of the enzymes involved has confirmed this pathway (Muenchhoff *et al.*, 2010; Mazmouz *et al.*, 2011) (Fig. 5b).

Initially, guanidinoacetate is formed via the transamidination of L-arginine to glycine by CyrA (Muenchhoff *et al.*, 2010) which is then activated by the adenylation domain of a nonribosomal peptide synthetase/polyketide synthase hybrid (NRPS/PKS), CyrB (Fig. 5b). This is followed by five polyketide extensions to form the carbon skeleton. Carbonyl groups later become the site of uracil ring formation. This constitutes a novel pathway for the biosynthesis of a pyrimidine and is supported by the lack of a thioesterase domain in any of the cylindrospermopsin PKSs, which are usually responsible for the release of product from a PKS (Mihali *et al.*, 2008). It also corresponds with the isotope-labeled precursor feeding study that indicated the uracil moiety does not originate from primary metabolism (Burgoyne *et al.*, 2000).

Spontaneous, nonenzymatic formation of the three rings of the carbon backbone of cylindrospermopsin is predicted by the lack of candidate genes in the *cyr* cluster, as well as by molecular modeling of the intermediate's conformation that is favorable for ring formation (Burgoyne *et al.*, 2000). Biosynthesis is then completed with two tailoring reactions: sulfation and hydroxylation, with the latter resulting in different stereoisomers (Mazmouz *et al.*, 2011) (Fig. 5b).

Besides genes for the biosynthesis of cylindrospermopsin, the cluster also encodes genes for transport of the toxin and regulatory proteins. Bioinformatic analysis of *cyrK* demonstrated that its product is most similar to sodium ion-driven multidrug and toxic compound extrusion proteins of the NorM family. As most cyanobacterial toxins seem to be exported out of the cell, CyrK is also thought to be a transporter for cylindrospermopsin (Mihali *et al.*, 2008). The gene product CyrO has low sequence similarity to any other proteins but shows homology to WD repeat proteins, which have diverse regulatory and signal transduction roles, as well as to AAA family proteins, which often perform chaperone-like functions in the assembly, operation, or disassembly of protein complexes (Mihali *et al.*, 2008).

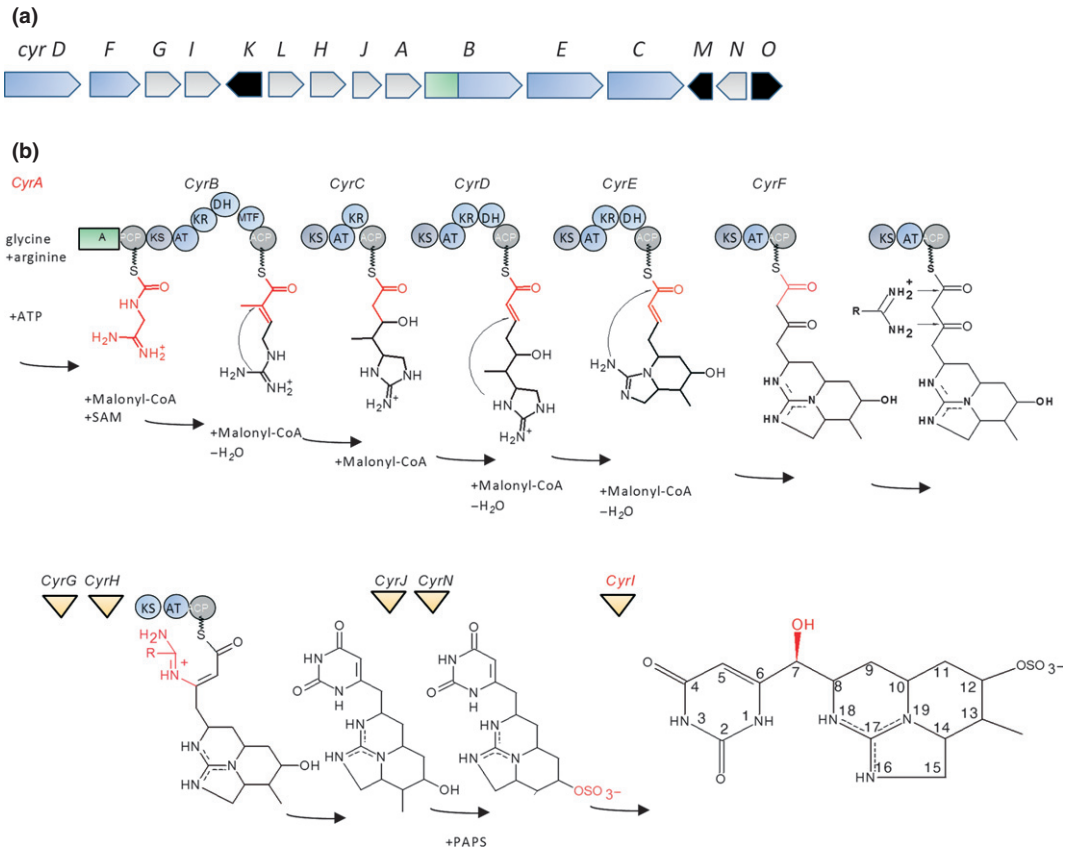

Fig. 5. (a) Schematic representation of cylindrospermopsin gene cluster of *C. raciborskii* AWT205. PKS and NRPS genes are highlighted in blue and green, respectively. Precursor biosynthesis genes, tailoring genes and genes putatively involved in uracil ring formation are shown in gray; genes putatively implicated in regulation and transport and gene transposition are indicated in black. (b) Schematic representation of the biosynthetic assembly line of cylindrospermopsin. Names of enzymes that were biochemically characterized are highlighted in red. Other proposed reactions are based on bioinformatic predictions. Carboxylic acid units or amino acid units incorporated or modified by individual proteins are highlighted in red. Abbreviations of PKS and NRPS domains are as in Fig. 3, MTF: methyltransferase.

The genome of the cylindrospermopsin-producing *C. raciborskii* CS-505, also isolated from Australia, was sequenced (Stucken *et al.*, 2010) revealing a *cyr* locus identical to that of *C. raciborskii* AWT205, except for the presence of an additional transposase. Recently, *cyr* clusters from cyanobacterial species other than *C. raciborskii*, namely *Aphanizmoenon* sp. 10E6 and *Oscillatoria* PCC 6506, have also been identified (Mazmouz *et al.*, 2010; Stuken & Jakobsen, 2010) revealing a variety of genetic rearrangements across different genera.

Evolution of cylindrospermopsin gene clusters

Two main scenarios are purported to explain the known distribution and organization of *cyr* gene clusters. The first involves an ancient origin of the *cyr* genes and implies that they were present in a common ancestor of all CYN-producing genera (Kellmann *et al.*, 2006; Yilmaz & Phlips, 2011, Jiang, *et al.*, 2012). The phylogeny of *cyr* biosynthesis genes mirrors that of the producer organisms (Kellmann *et al.*, 2006; Jiang, *et al.*, 2012). The present-day distribution of cylindrospermopsin-producing and nonproducing strains would therefore be due to loss or inactivation of the *cyr* gene cluster in the nonproducing species. In the second scenario, horizontal gene transfer is taken to play a defining role in the sporadic distribution of the *cyr* gene clusters. The high sequence conservation of *cyr* genes from *C. raciborskii* and *Aphanizomenon* spp. compared with genes involved in primary metabolism was taken to indicate horizontal gene transfer as a mechanism to explain the sporadic distribution of *cyr* gene clusters (Kellmann *et al.*, 2006; Stuken & Jakobsen, 2010). Atypical GC content and synteny are interpreted as supporting the proposal that *cyr* gene clusters have been horizontally transferred as a genomic island (Stuken & Jakobsen, 2010). However, cylindrospermopsin genes were recently reported from *Oscillatoria* sp. PCC6506 with sequence similarity to other cylindrospermopsin gene

clusters between 84% and 91% (Mazmouz *et al.*, 2010). This sequence divergence is comparable to that of genes involved in primary metabolism and does not support the horizontal gene transfer model (Jiang, *et al.*, 2012). Therefore, the second scenario has been amended, and it is suggested that recent horizontal gene transfer may explain the sequence similarity between the *cyr* gene clusters of some of the cylindrospermopsin producers (Jiang, *et al.*, 2012). The *cyr* genes appear to be under neutral or purifying selection, and it is suggested that the *cyr* genes do not have a single evolutionary pathway (Yilmaz & Phlips, 2011, Jiang, *et al.*, 2012). The resolution of these two evolutionary scenarios may await increased taxon sampling or the discovery of new producers of cylindrospermopsin.

Neurotoxins

Anatoxins

The cyanobacterial alkaloid anatoxin-a (ATX) (Fig. 2) was first being described in the 1970s (Devlin *et al.*, 1977). The neurotoxin and its methylene homolog homoanatoxin-a are found in strains of *Anabaena* and *Oscillatoria* (Sivonen & Jones, 1999; Cadel-Six *et al.*, 2009). Although neurotoxic blooms are less common than hepatotoxic blooms, they have been frequently implicated in mammal and bird poisoning (Stewart *et al.*, 2008). As for the hepatotoxins, the ability to produce anatoxins has a sporadic distribution even among closely related strains, precluding discrimination of toxic and nontoxic strains by traditional light microscopy techniques. Anatoxin-a(s) (ATS) is a phosphate ester of a cyclic N-hydroxyguanidine and thus unrelated to anatoxin-a despite its similar name.

Toxicity and human health aspects

Anatoxin-a is a nicotinic agonist that binds to neuronal nicotinic acetylcholine receptors (Devlin *et al.*, 1977). The toxin affects signal transmission between neurons and muscles, eventually leading to respiratory arrest and death within a few minutes. In mice, the i.p. LD_{50} value is 375 μg kg^{-1} [Table 2, (Chorus & Bartram, 1999)]; however, oral administration of up to 510 μg kg^{-1} in drinking water of rats did not lead to any signs of toxicity (van Apeldoorn *et al.*, 2007). Homoanatoxin-a has with 250 μg kg^{-1} bw a slightly lower i.p. LD_{50} value in mice (Chorus & Bartram, 1999). Anatoxins show only acute toxicity and published information on human toxicity is completely lacking.

A number of studies have assessed environmental concentrations of anatoxins in blooms with analytical techniques such as LC/MS, GC/MS, and GC/ECD and reported concentrations ranging from 0.02 to 4400 μg g^{-1} dw of bloom [(Sivonen *et al.*, 1989), Table 3]. It should be noted that the highest concentrations were from scums or very dense accumulations of cyanobacteria.

The unrelated toxin anatoxin-a(S) irreversibly blocks acetylcholinesterase (AChE), thereby affecting signal transduction in neurons (Matsunaga *et al.*, 1989). Typical signs of poisoning include salivation thus explaining the 's' in the naming of the toxin. The i.p. LD_{50} values of anatoxin-a (S) for mice and rats are 31 and 20 μg kg^{-1} bw (van Apeldoorn *et al.*, 2007). Although the toxin is only occasionally reported, it has also been implicated in animal poisonings (Mahmood *et al.*, 1988). Available literature on anatoxin-a(S) is only sparse. Using an AChE inhibition test environmental concentration of up to 3300 μg kg^{-1} dw have been reported from bloom samples [(Henriksen *et al.*, 1997), Table 3].

Anatoxin-a synthesis occurs on a PKS assembly line starting with proline

A gene cluster for the anatoxin-a biosynthesis (*ana*) was first described for the strain *Oscillatoria* sp. PCC6506 (Cadel-Six *et al.*, 2009). Feedings studies suggested that the carbon skeleton of the alkaloids is derived from acetate and glutamate (Hemscheidt *et al.*, 1995). The biosynthetic scheme was thus expected to involve polyketide synthases. Based on this assumption, the identification of anatoxin genes started with a general screen for polyketide synthase genes in the anatoxin-a producing strain PCC6506 (Cadel-Six *et al.*, 2009). One of the polyketide synthase gene fragments could be correlated with anatoxin production by screening fifty strains of the Pasteur Culture Collection (Cadel-Six *et al.*, 2009). The entire anatoxin biosynthesis gene cluster was subsequently elucidated and shown to encode seven biosynthetic proteins: AnaA-G [(Mejean *et al.*, 2009), Fig. 6].

A detailed biochemical analysis of the NRPS type adenylation domain protein AnaC revealed the activation of proline and not glutamate as starter (Fig. 6). The activated precursor is then tethered to the acyl carrier protein AnaD and modified by prolyl-AnaD oxidase AnaB to yield (S)-pyrroline-5-carboxyl-AnaD (Mejean *et al.*, 2010; Mann *et al.*, 2011). Polyketide chain assembly then proceeds with the polyketide synthases AnaE and AnaF. The predicted protein ORF1 that is encoded in direct proximity of the *ana* cluster is expected to catalyze a cyclization step (Fig. 6, Cy) to form the characteristic bicyclic ring structure of anatoxin while the growing chain is tethered to the AnaF acyl carrier protein (ACP) domain. Finally, the bicyclic thioester is suggested to be transferred for chain extension to the polyketide synthase AnaG followed by chain release expected to be catalyzed by the thioester-

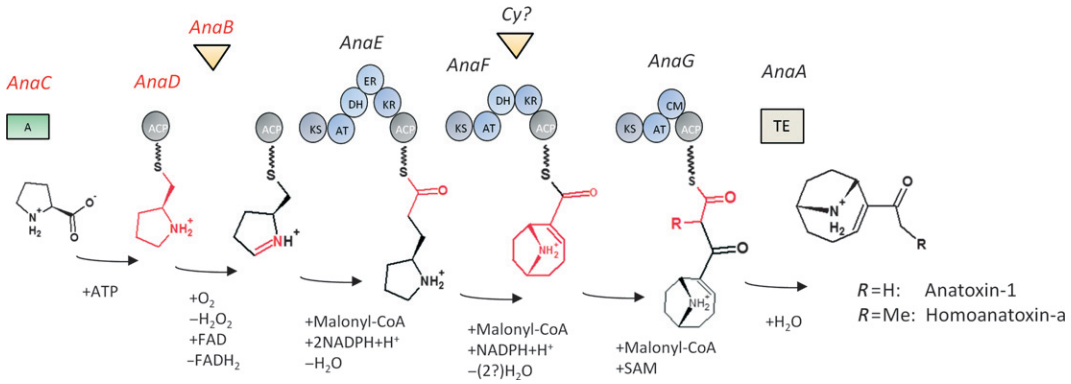

Fig. 6. Schematic representation of the biosynthetic assembly line of anatoxins. Names of enzymes that were biochemically characterized are highlighted in red. Other proposed reactions are based on bioinformatic predictions. Carboxylic acid units or amino acid units incorporated or modified by individual proteins are highlighted in red. Abbreviations of PKS and NRPS domains are as in Fig. 3.

ase AnaA (Fig. 6). The reaction scheme as proposed would yield 11-carboxyanatoxin-a and 11-carboxyhomoanatoxin-a. Either a spontaneous or an enzymatically catalyzed decarboxylation step is thus necessary to finally yield anatoxin-a and homoanatoxin-a [(Mejean *et al.*, 2010), Fig. 6].

A closely related anatoxin-a biosynthesis gene cluster encoding AnaA-G as well as ORF1 was recently identified in *Anabaena* sp. strain 37, suggesting a common route for anatoxin biosynthesis in two different genera (Rantala-Ylinen *et al.*, 2011). Major differences, however, were seen in the genetic organization of the gene clusters in *Oscillatoria* and *Anabaena*. Whereas the clustering of the *anaB-G* genes was similar, the orientation of the *anaA* and *orf1* genes differed in the two strains examined. A putative multidrug exporter was identified in the vicinity of the *ana* gene cluster in *Anabaena* but not in *Oscillatoria*. However, there is currently no direct evidence for the active transport of anatoxin-a.

Evolution of anatoxin biosynthesis gene clusters

The sporadic distribution of anatoxin-a has long been anticipated to be the result of plasmid-mediated horizontal gene transfer (Gallon, *et al.*, 1990; Gallon, *et al.*, 1994). Anatoxin-a and homoanatoxin-a biosynthetic gene clusters are known from strains of the genera *Oscillatoria* (Cadel-Six *et al.*, 2009) and *Anabaena* (Rantala-Ylinen *et al.*, 2011). However, the evolutionary origins of *ana* biosynthetic gene cluster have not been subject to phylogenetic studies. The *ana* gene clusters in these two genera display similar levels of sequence divergence as genes involved in primary metabolism (Rantala-Ylinen *et al.*, 2011). There is no clear evidence suggesting that horizontal gene transfer alone would explain their sporadic distribution. Information of the evolutionary origins and distribution of *ana* gene clusters awaits an increase in taxon sampling or the discovery of new producers of anatoxins.

Saxitoxins

Saxitoxins (STX) are a group of carbamate alkaloid toxins consisting of a tetrahydropurine group and two guanidinium moieties (Schantz *et al.*, 1975). They are classified into nonsulfated (STX), singly sulfated (gonyautoxins-GTX), doubly sulfated (C-toxins), and decarbamylated analogs (Fig. 2). STX represent the principle toxins responsible for paralytic shellfish poisoning (PSP) (Adelman *et al.*, 1982; Ikawa *et al.*, 1982). The naming relates to the frequent production of the toxins by marine algal blooms formed by eukaryotic dinoflagellates, such as *Alexandrium*, *Gymnodinium,* and *Pyrodinium*. Paralytic shellfish toxins (PSTs) accumulate in the marine food web via filter-feeding invertebrates. In freshwater environments, STX production has been reported for cyanobacterial strains of the species *Anabaena circinales, Aphanizomenon* sp.(Fig. 1d), *Aphanizomenon grazile, C. raciborskii* (Fig. 1e), and *L. wollei* (Table 1). Saxitoxin thus represents a remarkable example of a toxin that is produced by organisms belonging to two different kingdoms of life.

Toxicology and human health aspects

Saxitoxins can reversibly bind to voltage-gated Na^+ channels in an equimolar ratio. Animals treated with saxitoxin show typical neurological effects including nervousness, jumping, convulsions, and paralysis (van Apeldoorn *et al.*, 2007). STX is the most toxic variant of the saxitoxin family with an i.p. LD_{50} value of 10 μg kg^{-1} bw of mice

(Chorus & Bartram, 1999). For neoSTX, a LD_{50} value of 65 μg kg^{-1} bw of mice has been indicated (Wolf & Frank, 2002; Table 2). Despite these clear toxic effects on mice, no reported case of human intoxication related to STX from freshwater environments is known (van Apeldoorn *et al.*, 2007). PSP toxins from marine origins that are accumulating in aquatic marine filter feeders, however, were associated with severe cases of food poisonings (Ikawa *et al.*, 1982).

Several studies have assessed environmental concentrations of saxitoxin in freshwater blooms or water samples and detected values ranging 5–2040 μg g^{-1} dw for STX or STX equivalents (Negri *et al.*, 1997). Dissolved STX concentrations have not been reported (Chorus & Bartram, 1999).

Saxitoxin: biosynthesis of a nonterpene bacterial alkaloid

The molecular structure of the neurotoxic alkaloid, saxitoxin has been known since the mid-1970s (Schantz *et al.*, 1975); however, its unusual chemical pathway and corresponding biosynthesis genes were only recently elucidated (Kellmann *et al.*, 2008b; Stuken *et al.*, 2011). Radioisotope feeding experiments performed by Shimizu and coworkers in the 1980s suggested that the toxin was derived from arginine, acetate (via acetyl-coenzyme A), and methionine methyl (via S-adenosylmethionine). The pattern of incorporation of these precursors led Shimizu to propose a biosynthesis pathway for saxitoxin involving a rare Claisen condensation between arginine and acetate (Shimizu *et al.*, 1984).

In 2008, Kellmann *et al.* used a reverse genetics approach to locate the saxitoxin biosynthesis gene cluster in the cyanobacterium, *C. raciborskii,* T3 (Kellmann *et al.*, 2008b). The same group had previously conducted *in vitro* biosynthesis studies that confirmed arginine, acetyl-CoA, SAM, and carbamoylphosphate as precursors for saxitoxin biosynthesis (Kellmann & Neilan, 2007). Degenerate PCR screening revealed a putative saxitoxin *O*-carbamoyltransferase gene, *sxtI* that was exclusively present in paralytic shellfish poison (PSP)-producing cyanobacteria (Kellmann *et al.*, 2008a). Genome walking from *sxtI* in *C. raciborskii* T3 revealed a 35-kb putative saxitoxin biosynthesis gene locus.

The *C. raciborskii* T3 *sxt* cluster was the first nonterpene alkaloid gene cluster described for a bacterium (Kellmann *et al.*, 2008a). Homologous saxitoxin biosynthesis gene clusters have as been identified in *A. circinalis* AWQC131C, *Aphanizomenon* sp. NH-5 (Mihali *et al.*, 2009), *Raphidiopsis brookii* D9 (Soto-Liebe *et al.*, 2010), and *L. wollei* (Mihali *et al.*, 2011). These *sxt* clusters range in size from 25.7 kb (in *R. brookii*) to 36 kb (in *L. wollei*) and the presence or absence of *sxt* genes as well as their organization reflects the specific toxin profile expressed by each strain.

Using comparative sequence analysis as well as mass spectrometric analysis of saxitoxin intermediates, Kellmann *et al.* (2008b) assigned 30 catalytic functions to 26 of the *sxt*-encoded proteins in *C. raciborskii* T3. An unusual polyketide synthase-like enzyme, SxtA, is predicted to perform an initial methylation of acetate and a Claisen condensation reaction between propionate and arginine to produce 4-amino-3-oxo-guanidinoheptane, designated compound A'. Further transamidination, cyclization, epoxidation, aldehyde reduction, and finally carbamoylation and hydroxylation reactions may result in the saxitoxin parent molecule. Following synthesis of the parent molecule, several tailoring enzymes produce decarbamoylated, N-1-hydroxylated (e.g. neosaxitoxin), or sulfated analogs of saxitoxin (Kellmann *et al.*, 2008b). Some of the tailoring genes described for the biosynthesis pathway differ or are absent in some saxitoxin-producing species. It was postulated that these genes may be complemented by another locus in the genome of the producer organisms or are not essential for PST biosynthesis (Mihali *et al.*, 2009).

Several studies have provided evidence for an active transport mechanism for PSP toxins in cyanobacteria (Castro *et al.*, 2004; Pomati *et al.*, 2004). It is predicted that sodium-driven multidrug and toxic compound extrusion (MATE) proteins, SxtF and SxtM comprise the saxitoxin export pathway in *C. raciborskii* (Kellmann *et al.*, 2008b). However, these genes are absent from the *A. circinalis* and *Aph.* sp. *sxt* clusters, and a protein belonging to the drug and metabolite transport family, SxtPER, may constitute an alternative route for saxitoxin export in these species (Mihali *et al.*, 2009). The *sxt* gene cluster in *C. raciborskii* T3 encodes two transcriptional factors, suggesting that saxitoxin biosynthesis in this organism may be regulated at the transcriptional level in response to phosphate and other environmental factors. The *sxt* cluster in *A. circinalis* lacks these genes and therefore might be regulated in a different manner.

Evolutionary origin of saxitoxin biosynthesis

A detailed analysis of saxitoxin biosynthesis gene clusters strains from the genera *Anabaena*, *Lyngbya*, *Aphanizomenon*, *Cylindrospermopsis,* and *Raphidiopsis* identified a set of core genes, common to all *sxt* clusters, and a set of genes that vary between different clusters (Kellmann *et al.*, 2008a; Moustafa *et al.*, 2009; Murray *et al.*, 2011). The organization of *sxt* genes in each of the saxitoxin producers mirrors the species phylogeny with the most similar *sxt* gene organization found in the most closely

related producers (Murray *et al.*, 2011). However, just a subset of the *sxt* genes displayed phylogenies that mirror the species phylogeny (Moustafa *et al.*, 2009; Murray *et al.*, 2011). The remainder is proposed to have been recruited through horizontal gene transfer events from proteobacteria or actinobacteria (Moustafa *et al.*, 2009). Transposase genes were identified within or proximity to the *sxt* clusters of *C. raciborskii, A. circinalis,* and *L. wollei* (Kellmann *et al.*, 2008a; Mihali *et al.*, 2009, 2011). The presence of these transposases and the mosaic structure of the *sxt* clusters have led to speculation that small cassettes of *sxt* genes are mobile and that saxitoxin biosynthesis evolved in an ancestral cyanobacterium that successively acquired genes from other bacteria via horizontal gene transfer (Mihali *et al.*, 2009). Multiple horizontal gene transfer events were proposed to explain the origin of part of the saxitoxin biosynthetic machinery (Moustafa *et al.*, 2009; Murray *et al.*, 2011). However, further evidence suggests that the saxitoxin cluster has evolved through gene loss and rearrangements, as well as recombination, and positive selection resulting in extraordinarily conserved biosynthetic machinery through stabilizing selection (Murray *et al.*, 2011). This finding implies that the sporadic distribution of saxitoxin producers in cyanobacteria is the result of a series of gene losses.

Saxitoxins are produced by different genera of marine dinoflagellates and cyanobacteria (Moustafa *et al.*, 2009; Murray *et al.*, 2011). The genetic basis for saxitoxin production in dinoflagellates was subject of speculation but proved elusive (Stuken *et al.*, 2011). The synthesis of saxitoxins by autonomous bacteria associated with the dinoflagellate cell has been ruled out (Stuken *et al.*, 2011). A saxitoxin biosynthetic gene *SaxA* has been recently reported from the dinoflagellates *Alexandrium fundyense* and *Alexandrium minutum* (Stuken *et al.*, 2011). The genes encoding SaxA contain eukaryotic polyA- tails and typical dinoflagellate spliced-leader sequences (Stuken *et al.*, 2011). The authors explain the production of saxitoxins by dinoflagellates and cyanobacteria as the result of an ancient horizontal gene transfer event (Stuken *et al.*, 2011). Genome projects of saxitoxin-producing dinoflagellates will shed light upon this fascinating example of a secondary metabolites produced by organisms belonging to two different kingdoms of light.

β-methylamino-L-alanine

β-methylamino-L-alanine (BMAA, Fig. 2) is a cyanobacterial nonproteinogenic amino acid that has been connected with high incidents of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and dementia, on the island of Guam (Cox *et al.*, 2003) (Table 1). BMAA is concentrated in the symbiotic cyanobacteria-inhabiting reproductive seeds of the cycad *Cycas micronesia* that are part of the diet of indigenous Chamorro people. Furthermore, animals such as flying foxes, pigs, and deer forage on the seeds leading to a biomagnifications in the food chain. It should be noted that the BMAA hypothesis is strongly criticized by part of the neurological science community with the argument that people would have to eat kilograms of cycad flour to ingest an effective dose (Duncan *et al.*, 1990). BMAA has nevertheless been shown to elicit motor neuron losses in dissociated mixed spinal cord cultures. The toxin has been proposed to have a long latency period and is reportedly synthesized by strains representing all five cyanobacterial sections (Cox *et al.*, 2005). BMAA production has also recently been shown in cyanobacteria of the genera *Nodularia* and *Aphanizomenon* that dominate massive blooms in the Baltic Sea and in the food chain of this brackish water environment (Jonasson *et al.*, 2010). Bioaccumulation of the toxin and possible human consumption has only been scarcely investigated and may require larger attention in the future. A biosynthetic pathway toward BMAA has not yet been elucidated (Table 1).

Dermatotoxins

Lyngbyatoxin

Lyngbyatoxins (LTX) are produced by *Lyngbya majuscula*, a cyanobacterium found in estuarine and coastal waters in tropical and subtropical climates (Fig. 2). The original assignment to the genus *Lyngbya* was based on morphological criteria; however, recent phylogenetic analyses led to the introduction of a new genus and species name, namely *Moorea producens* (Engene *et al.*, 2012). The different congeners are characterized by an indolactam ring and contain prenyl side chains closely resembling teleocidin, a toxin frequently found in *Streptomyces* sp. (Cardellina *et al.*, 1979). This benthic cyanobacterium grows loosely attached to seagrass, sand, and rocky outcrops (Osborne *et al.*, 2001). *Lyngbya majuscula* has also been reported from other tropical and subtropical countries including Australia, Curacao, Fiji, Madagascar, Guam, Palau, Papua New Guinea, and Okinawa (Osborne *et al.*, 2001).

Toxicity and human health aspects

Lyngbyatoxins are the causative agent of a blistering dermatitis called 'swimmers itch' that was frequently reported by surfers in Hawaii (Cardellina *et al.*, 1979). Lyngbyatoxins are potent tumor promoters that operate by competitively binding to protein kinase C (PKC) (Jeffrey & Liskamp, 1986). The i.p. LD_{50} value for 3-week-

old mice for lyngbyatoxin-a has been reported as 250 μg kg^{-1} bw (Table 2). Administration of 300 μg kg^{-1} bw to mature mice led to inactivity for 2 h with subsequent recovery (van Apeldoorn *et al.*, 2007). It should be noted that *M. producens* frequently produces several yet unidentified toxins that may lead to additive effects. Environmental concentrations of lyngbyatoxin have not yet been explored (Chorus & Bartram, 1999).

Lyngbyatoxin is assembled on a small NRPS complex involving a novel aromatic prenyl transferase

The identification of the biosynthesis pathway for lyngbyatoxin is based on feeding studies of the closely related toxin teleocidin in *Streptomyces* sp. that revealed the incorporation of L-valine and L-tryptophan into the characteristic indolactam ring (Irie *et al.*, 1990). Edwards & Gerwick (2004) proposed that this core ring is assembled on a bimodular NRPS that likely contains a domain for the reductive release of the resulting dipeptide (Edwards & Gerwick, 2004). Using a specifically designed probe, they could identify a gene cluster in *L. majuscula* encoding a bimodular NRPS with adenylation domain specificities for L-valine and L-tryptophane (Edwards & Gerwick, 2004). Subsequently, the entire gene cluster was identified that consists of the four genes *ltxA-D*. The lyngbyatoxin gene cluster is thus the smallest gene cluster assigned to known cyanotoxin biosyntheses. The scheme of reactions was proposed and has been partially verified. The dipeptide assembled at the NRPS LtxA is tethered to a PCP domain and reductively released by the terminal NADPH-dependent reductase domain of LtxA. The indolactam ring is formed through the activity of the P450-dependent monooxygenase/cyclase LtxB (Huynh *et al.*, 2010). Finally, the aromatic prenyltransferase, LtxC, performs a prenylation step adding geranyl pyrophosphate (GPP) to the indolactam core. LtxD is predicted to be involved in the conversion of lyngbyatoxin-a into the minor variant isoforms lyngbyatoxin-b and lyngbyatoxin-c (Edwards & Gerwick, 2004).

Aplysiatoxins

Aplysiatoxin (APX, Fig. 2) and debromoaplysiatoxin are phenolic bislactones (Kato & Scheuer, 1974). As with lyngbyatoxins, they are considered the causative agents of severe contact dermatitis (Moore *et al.*, 1984). The toxins have high inflammatory potential and activate PKC, thereby promoting the development of tumors (Jeffrey & Liskamp, 1986). Aplysiatoxins were detected in strains of *L. majuscula* isolated off Hawaii (now renamed as *M. producens*), in the sea hare *Stylocheilus longicauda* (Kato & Scheuer, 1974) and the edible red alga *Gracularia coronopifolia* (Ito & Nagai, 1998). Whereas *S. longicauda* is known to preferentially feed on *L. majuscula,* the association of the toxins with eukaryotic algae could be due to epiphytic colonization by cyanobacteria (Ito & Nagai, 1998). The i.p. LD_{50} value for mice is *c.* 107 and 117 μg kg^{-1} bw for 3- and 5-week-old mice, respectively (Ito & Nagai, 1998). Environmental concentrations of aplysiatoxin have not yet been assessed and a biosynthetic gene cluster has not yet been assigned.

Outlook

There is no doubt that the assignment of biosynthetic pathways to major cyanobacterial toxin families has opened a new era of cyanotoxin research. Elucidation and comparison of the gene sequences provided a basis for the reconstruction of the evolutionary history of the toxin families and the development of new methodologies for the detection of toxic cyanobacteria directly in the field. Moreover, the genes provided a basis for the analysis of the impact of environmental stimuli on toxin production and allowed for the construction of nontoxic mutants.

There are, however, a number of open questions. The prediction of individual enzymatic steps toward toxin production is still largely based on bioinformatic analysis. The increasing number of biochemically characterized NRPS and PKS domains together with the availability of crystal structures for most of the domain types has indeed allowed for the assignment of specificity conferring code amino acids for the substrate activating domains and enables a more and more reliable prediction of domain characteristics (Jenke-Kodama & Dittmann, 2009). The bioinformatic analysis, however, is limited to common substrates and NRPS and PKS type domains. Pathways that include a high number of tailoring enzymes and further non-NRPS or PKS type enzymes are difficult to reconstruct. From the biosynthetic routes introduced in this review, this especially concerns the saxitoxin pathway. The complete reconstruction of the alkaloid biosynthesis will require biochemical characterization of selected enzymes performing key steps during saxitoxin assembly and may necessitate further feeding assays. Understanding the complete saxitoxin pathway will help to interpret the differences observed in the saxitoxin biosynthetic gene compliment in individual producers.

Further research is also needed to understand the evolutionary history of cyanobacterial toxins. Different scenarios are proposed for the evolution and diversification of the toxin biosynthesis gene clusters. Whereas the data basis for microcystin and nodularin provides a growing body of evidence for a common ancestor of *mcy* and *nod* genes and a history of frequent gene losses, the situation

is still unclear for the other toxin families. To resolve the origin of the different pathways and to uncover the forces leading to their sporadic distribution and diversification the discovery of additional toxin producers among a larger number of genera will be necessary. The increase in the number of complete genomes available is likely to lead to the discovery of new toxin producers. One also has to keep in mind that a common ancestry and the diversification via recombination and horizontal gene transfer do not necessarily exclude each other; rather, it is likely that a combination of mechanisms leads to the final shape of the individual gene clusters. As NRPS and PKS gene cluster contain repetitive sequence elements they can serve as ideal platforms for recombination events leading to changes in the actual chemistry of the products. The ongoing diversification is reflected by the increasing numbers of isoforms detected for most of the toxin families.

The overall impact of research on toxin biosynthesis genes and their evolution will depend on the successful transfer of the knowledge to everyday field work, water management strategies and risk assessment. The most important step toward this goal is the advancement of techniques for detection and quantification of toxic cyanobacteria. For microcystin and nodularin producing cyanobacteria resulting bias can be minimized by combining the molecular detection methods with chemical detection methods and by using conserved parts of the gene clusters. The establishment of molecular techniques for the detection and quantification of cylindrospermopsin, anatoxin, saxitoxin, and lyngbyatoxin genes, however, are in their infancy. An emerging trend is the development of techniques for the simultaneous detection of genes involved in the production of different toxins. Quantitative multiplex PCR has been recently developed which enables the simultaneous assessment of the level of toxin (microcystin, nodularin, cylindrospermopsin, and saxitoxin) producing species in water samples (Al-Tebrineh *et al.*, 2011; Baron-Sola *et al.*, 2012). Attempts to establish protocols for the reliable detection of anatoxin producing strains started with PCR-RLFP screening of laboratory strains of *Anabaena* and *Oscillatoria* and the application of the protocol to Finnish fresh waters and Baltic Sea samples (Rantala-Ylinen *et al.*, 2011).

Finally, a close collaboration between academic groups and water authorities will be needed to verify the reliability and robustness of techniques for the detection and quantification of toxic cyanobacteria.

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