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ORIGINAL ARTICLE

Nodularin, a cyanobacterial toxin, is synthesized *in planta* by symbiotic *Nostoc* sp.

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The nitrogen-fixing bacterium, *Nostoc*, is a commonly occurring cyanobacterium often found in symbiotic associations. We investigated the potential of cycad cyanobacterial endosymbionts to synthesize microcystin/nodularin. Endosymbiont DNA was screened for the aminotransferase domain of the toxin biosynthesis gene clusters. Five endosymbionts carrying the gene were screened for bioactivity. Extracts of two isolates inhibited protein phosphatase 2A and were further analyzed using electrospray ionization mass spectrometry (ESI-MS)/MS. *Nostoc* sp. '*Macrozamia riedlei* 65.1' and *Nostoc* sp. '*Macrozamia serpentina* 73.1' both contained nodularin. High performance liquid chromatography (HPLC) HESI-MS/MS analysis confirmed the presence of nodularin at $9.55 \pm 2.4\,ng\,\mu g^{-1}$ chlorophyll *a* in *Nostoc* sp. '*Macrozamia riedlei* 65.1' and $12.5 \pm 8.4\,ng\,\mu g^{-1}$ Chl *a* in *Nostoc* sp. '*Macrozamia serpentina* 73.1' extracts. Further scans indicated the presence of the rare isoform [L-Har^2] nodularin, which contains L-homoarginine instead of L-arginine. Nodularin was also present at $1.34 \pm 0.74\,ng\,ml^{-1}$ (approximately 3 pmol per g plant ww) in the methanol root extracts of *M. riedlei* MZ65, while the presence of [L-Har^2] nodularin in the roots of *M. serpentina* MZ73 was suggested by HPLC HESI-MS/MS analysis. The *ndaA-B* and *ndaF* genomic regions were sequenced to confirm the presence of the hybrid polyketide/non-ribosomal gene cluster. A seven amino-acid insertion into the NdaA-C1 domain of *N. spumigena* NSOR10 protein was observed in all endosymbiont-derived sequences, suggesting the transfer of the *nda* cluster from *N. spumigena* to terrestrial *Nostoc* species. This study demonstrates the synthesis of nodularin and [L-Har^2] nodularin in a non-*Nodularia* species and the production of cyanobacterial hepatotoxin by a symbiont *in planta.*

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Introduction

Toxin-producing cyanobacteria are recognized as a threat to public health globally, particularly in regions without access to high quality potable water. Increased aquatic eutrophication has resulted in massive blooms of cyanobacteria both in freshwater and marine environments. The most commonly produced cyanobacterial toxins are the hepatotoxins, microcystin and nodularin (Fristachi and Sinclair, 2008), both capable of inhibiting protein phosphatase 1 and 2A to varying degrees (Mackintosh *et al.*, 1990) and inducing protein hyperphosphorylation, cytoskeletal collapse and massive hepatic bleeding (Kuiper-Goodman *et al.*, 1999). Both toxins are also recognized as potential tumor promoters and carcinogens, and hence it is of importance to identify the production of these toxins in the environment to eliminate even low-level exposure to humans (Humpage, 2008).

Nodularin is a cyclic pentapeptide (Figure 1) synthesized by the planktonic cyanobacterial species *Nodularia spumigena* and the benthic species *Nodularia sphaerocarpa* PCC7804 (Beattie *et al.*, 2000; Moffitt *et al.*, 2001). It occurs primarily in brackish water blooms worldwide, including the Baltic Sea and coastal waters off New Zealand, Australia, South Africa and the United Kingdom (Beattie *et al.*, 2000). Variants of the nodularin toxin have been reported, namely [L-Har^2] nodularin (Beattie *et al.*, 2000; Saito *et al.*,

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2001), [6(Z)-Adda[3]] nodularin, [D-Asp[1]] nodularin and [DMAdda[3]] nodularin (Namikoshi *et al.*, 1994). The toxin motuporin, isolated from the marine sponge *Theonella swinhoei,* is thought to be another variant. It has arginine replaced by valine at position 2 (De Silva *et al.*, 1992). The producer of this toxin is unknown but thought to be a cyanobacterial symbiont. Nodularin synthesis has not been reported in any other cyanobacterial genus. Conversely, numerous cyanobacterial genera, including *Microcystis*, *Anabaena, Phormidium, Nostoc* and *Planktothrix,* produce the heptapeptide microcystin-LR, similar in structure to nodularin with an additional two amino-acid residues, D-alanine and D-leucine, and with *N*-methyldehydroalanine replacing *N*-methyldehydrobutyrine (Rinehart *et al.*, 1988).

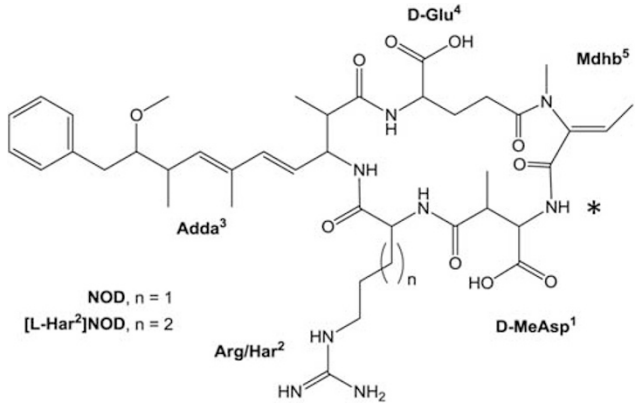


Figure 1 The chemical structure of the pentapeptide nodularin and its variant [L-Har[2]] nodularin indicating the numbering of the amino acids. The * indicates where the additional amino acids, D-alanine and D-leucine are inserted for the heptapeptide toxin, microcystin. Adda, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid; Arg, arginine; D-Glu, D-glutamate; D-MeAsp, D-*erythro*-β-methyl-aspartic acid; Har, homoarginine; Mdhb, *N*-methyldehydrobutyrine (Rinehart *et al.*, 1988).

Nodularins and microcystins are biosynthesized via modular megasynthases (Figure 2A), which consists of both non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) modules (Tillett *et al.*, 2000; Moffitt and Neilan, 2004). Each module contains enzymatic domains responsible for substrate selection and activation, modification and condensation. Within NRPS modules these functions are catalyzed by adenylation, thiolation, methyltransferase, epimerase and condensation domains. The 48-kb gene cluster encoding the synthesis of nodularin (*nda*) (Moffitt and Neilan, 2004) resembles the microcystin synthetase cluster (*mcy*) (Tillett *et al.*, 2000), with the exception of two NRPS modules that are absent in *nda* (Figure 2A). Christiansen *et al.*

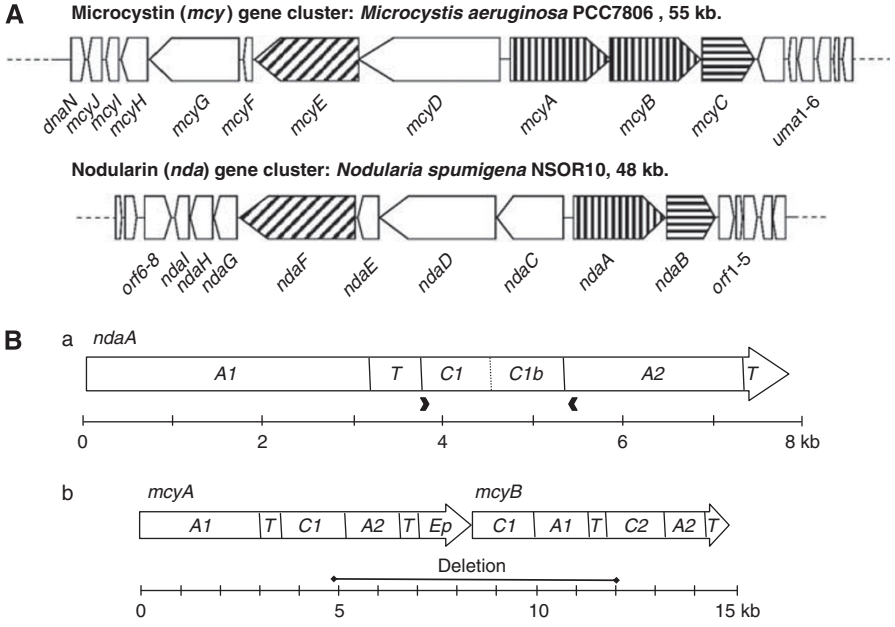


Figure 2 (**A**) The diagrammatic representation of the regions of the microcystin and nodularin gene clusters, of *M. aeruginosa* PCC7806 and *Nodularia spumigena* NSOR10, respectively, investigated in this study. Primers HEPF/HEPR (Jungblut and Neilan, 2006) were used to target the aminotransferase region of the *mcyE* and *ndaF* genes (diagonal hatching). Analysis of the evolutionary relationship between *nda* and *mcy* gene clusters was performed on the *ndaA* and *mcyA*/*B* gene regions indicated by perpendicular hatching. Analysis of the adenylation-binding domain encoded by *ndaB* and *mcyC* (horizontal hatching) was also performed. The diagram is not drawn to scale. (**B**) A diagram representing the nodularin biosynthetic gene, *ndaA*, and its proposed evolution from the microcystin biosynthetic genes *mcyA* and *mcyB*. Each gene encodes two non-ribosomal peptide synthetase (NRPS) modules. The approximate size and location of the encoded adenylation (A), condensation (C), thiolation (T), epimerase (Ep) domains within each module is shown and numbered according to their order of appearance. (**a**) The nodularin synthetase gene, *ndaA*, contains two partial condensation domains, C1a and C1b, that are proposed to have evolved from *mcyA-C1* and *mcyB-C2* following deletion of a fragment from the microcystin biosynthetic gene cluster. Forward and reverse primers, designed in this study for the amplification and sequencing of the *Nostoc ndaA* condensation domain, are indicated by the arrows. (**b**) The region from *mcyA-C1* and *mcyB-C2* (solid line) is proposed to have been deleted during the evolution of the *ndaA* gene.

(2003) proposed that the *mcy* gene cluster arose from an insertion of two additional activation domains into the *nda* cluster. In contrast, investigation of the co-evolution of housekeeping genes and the toxin gene clusters (Rantala *et al.*, 2004) and the complete gene cluster (Moffitt and Neilan, 2004) suggested that the *nda* cluster arose from an ancestral *mcy* cluster by a deletion event spanning the condensation domain of the first module of *mcyA* (*mcyA-C1*) to the condensation domain of the first module of *mcyB* (*mcyB-C1)* (Figure 2B). The plasticity of the *mcy* gene cluster is well known, with various deletions and recombination events allowing the synthesis of a diverse array of microcystin variants. Deletion mutants of the *N*-methyltransferase domain of *mcyA* in microcystin producing *Anabaena* strains were still able to synthesize microcystin (Fewer *et al.*, 2008). Independent recombination of adenylation domains without their associated condensation domains resulted in the generation of functional new peptide synthetases (Fewer *et al.*, 2007), thereby confirming that gene reorganization is a common means of diversification of non-ribosomal peptide synthesis in the cyanobacteria.

Nostoc strains are known to associate symbiotically with a wide range of hosts, such as diatoms, bryophytes (lichens and hornworts), pteridophytes (*Azolla*), gymnosperms (cycads) and angiosperms (*Gunnera*) (Rai *et al.*, 2000; Bergman *et al.*, 2007). A total of 24 endosymbiont species, mostly *Nosto*c spp., were recently isolated from the coralloid roots of 74 cycad plants (Gehringer *et al.*, 2010). It has been previously demonstrated that the symbiotic cyanobacteria in *Macrozamia riedlei*, while photosynthetically inactive, fixed nitrogen and transferred it to the host cycad plant in the form of citrulline and glutamine (Lindblad *et al.*, 1991). Microcystin-producing *Nostoc* symbionts of lichens have also been identified (Oksanen *et al.*, 2004; Kaasalainen *et al.*, 2009), with both strains producing several variants of microcystin. Microcystins are known to disrupt many plant physiological processes, including CO_2 fixation, sucrose synthesis, starch storage (McElhiney *et al.*, 2001) and photosynthesis (Abe *et al.*, 1996; Pflugmacher *et al.*, 2007) by inhibiting plant protein phosphatases (Peuthert *et al.*, 2008). Microcystin and nodularin exposure causes oxidative stress and stunted growth in plants exposed to the toxin in the rhizosphere (Gehringer *et al.*, 2003; Lehtimaki *et al.*, 2011), as well as inhibiting germination and seedling growth of several agricultural plants (Saqrane *et al.*, 2008). Accumulation of microcystin and nodularin has also been shown in plants irrigated with toxin-containing water (Mohamed and Al Shehri, 2009). The aims of the present study were to identify the potential for hepatotoxin production in commonly occurring terrestrial cyanobacterial species known to associate with gymnosperms (Cycadaceae), given the evidence that lichen symbionts are capable of synthesizing microcystin (Oksanen *et al.*, 2004; Kaasalainen *et al.*, 2009). In addition, we wished to determine whether this toxin was produced *in planta* and, if so, whether the levels of toxin production were high enough to affect plant physiology.

Materials and methods

Culture conditions

Cyanobacterial isolates (Gehringer *et al.*, 2010) were grown on BG11 medium and incubated at 22 °C on a 16:8 h light:dark cycle of 2000 lux. Isolates used for toxin detection were grown in 500 ml flasks with gentle agitation as above. Cells were harvested in late exponential phase (day 10).

Toxin biosynthesis gene detection

DNA was extracted from fresh culture material using the XS buffer extraction method (Tillett and Neilan, 2000) and screened using the primer pair HEPF/R targeting the *mcyE*/*ndaF* gene (Jungblut and Neilan, 2006; Figure 2A). Briefly, 50 ng of DNA was used in a 20 μl reaction containing 5 pmol of each primer, 1 × F1 *Taq* polymerase buffer, 0.2 U F1 *Taq* (Fisher Biotec, Wembley, WA, USA), 200 μM of each deoxynucleotide triphosphate and 2.5 mM $MgCl_2$. The reaction was heated to 95 °C for 2 min followed by 35 cycles of 30 s at 95 °C, 40 s at 55 °C and 50 s at 72 °C with a final extension of 5 min at 72 °C. PCR products were precipitated (Gehringer *et al.*, 2010) and sequenced using the PRISM Big Dye Terminator V3.1 system (Applied Biosystems, Mulgrave, VIC, Australia). Total genomic plant DNA was extracted from the leaves of the cycad plants containing toxin-producing symbiont species, using the Qiagen Plant DNAEasy Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. This DNA was used as a negative control for the HEPF/R PCR.

Characterization of the Nostoc nda cluster via gene walking

Elucidation of the unknown regions of DNA flanking the putative nodularin aminotransferase biosynthesis gene of *ndaF*, as well as the complete *ndaA* and *ndaB* genes, were determined by an adaptor-mediated (pan-handle) PCR method that was modified as previously described (Siebert *et al.*, 1995). A detailed description is provided in the supporting text. The *ndaA* and *ndaB* genes were identified using universal degenerate primers (MTF2: 5′-GCNGG(C/T)GG(C/T)GCNTA(C/T)GTNCC-3′ and MTR: 5′-GCNGG(C/T)GG(C/T)GCNTA(C/T)GTN CC-3′) targeting peptide synthetases (Neilan *et al.*, 1999). The primer pair NdaA C1F2 (5′-GACGAATC AAAATGGCGAAT-3′) and NdaA C1bR (5′-AATTGG CTGGAAGATGCAAG-3′) was designed from the *ndaA* sequence (Figure 2B) obtained from *Nostoc* sp. '*M. serpentina* 73.1' to amplify the partial

condensation domain sequences. The primers were used at 5 pmol each in a 20 μl reaction containing 100 ng of DNA, 1 × F1 *Taq* polymerase buffer, 0.2 U F1 *Taq* (Fisher Biotech), 200 μM of each dNTP and 2.5 mM $MgCl_2$. The amplification cycle was initially 2 min at 95 °C followed by 30 cycles of 20 s at 95 °C, 20 s at 58 °C and 90 s at 72 °C with a final extension of 5 min at 72 °C. PCR products were precipitated and sequenced with both primers as described above.

DNA and amino-acid sequence analysis

The aminotransferase gene sequences and the 16S rRNA gene sequences of the isolates carrying the *mcyE*/*ndaF* genes, those of their nearest neighbors and representative reference strains were aligned using Clustal W (Thompson *et al.*, 1997), end gaps removed and the sequences re-aligned. Pairwise percentage identities were calculated for sequences within the alignments. Maximum likelihood trees were generated using the PhyML-3 package (Guindon *et al.*, 2009) using the HKY85 substitution model with 1000 bootstrap resampling events. No amplification products were obtained from plant DNA using the HEPF/R primer pair. The NRPS/PKS analysis tool available at http://nrps.igs.umaryland.edu/nrps/ (Bachman and Ravel, 2009) identified the location of the NRPS domain sequences. The duplicated region within the *ndaA* condensation domain and previously described conserved core motifs (Rausch *et al.*, 2007) were identified manually using the Ugene program (http://ugene.unipro.ru/). The *Nodularia spumigena* NSOR10 and *Nostoc* sp. '*M. serpentina* 73.1' nodularin synthetase protein NdaA contains a condensation domain sequence NdaA-C1, located at positions 1278–1711 and 1292–1731, respectively. A second partially duplicated condensation domain, identified downstream of NdaA-C1, NdaA-C1b was located between 1712 and 1973 and 1732 and 2017 in *Nodularia spumigena* NSOR10 and *Nostoc* sp. 73.1, respectively. The *Microcystis aeruginosa* PCC7806 condensation domain of McyA, McyA-C1, was located between positions 1280 and 1706, and the second condensation domain of McyB, McyB-C2, was located between positions 1062 and 1492. The binding pocket sequences for the adenylation domain binding pockets of NdaB and McyC for the strains, indicated in Table 1, were determined using the NRPS/PKS synthase analysis tool available at http://nrps.igs.umaryland.edu/nrps/ (Bachman and Ravel 2009, p189) and the NRPS predicter tool (Rausch *et al.*, 2005). Using a statistical model of multiple protein alignments, the software predicts PKS and NRPS units from an input protein sequence. It then identifies the potential eight amino-acid specificity domain derived from a database of NRPS adenylation domains previously characterized biochemically. Sequences generated in this project have been deposited in GenBank with accession numbers listed in Table 1.

Protein phosphatase 2A (PP2A) inhibition assay

A bioassay was performed on cyanobacterial extracts to determine whether the five isolates carrying the *mcyE*/*ndaF* gene regions were producing bioactive toxin or not. Approximately 500 mg of fresh culture material of these five isolates were resuspended in 500 μl of 70% methanol and lyzed by bead beating (FastPrep, Savant, Thermo Fisher Scientific, Waltham, MA, USA). After centrifugation to remove cell debris, the supernatant was extracted with an equal volume of chloroform and used in the colorimetric PP2A inhibition assay, as described in Ward *et al.* (1997) and detailed in the supplementary text.

In vitro toxin identification

As both microcystin and nodularin inhibit PP2A, it was necessary to identify which toxin was present in those samples showing bioactivity. Extracts shown to inhibit PP2A were desiccated and resuspended in 45% methanol for the identification using electrospray ionization mass spectrometry (ESI-MS). Samples were analyzed using an Ultima API hybrid QTof tandem mass spectrometer (Micromass, Manchester, UK) instrument via nanospray injection into the ESI source. The mass spectra between 100 and 1500 $m\,z^{-1}$ were acquired in the positive ion mode with a capillary voltage of 3 kV. Major species were subjected to ESI-MS/MS analysis by increasing the collision energy to 60 eV in order to identify the product ions and deduce the compound structure (Mazur-Marzec *et al.*, 2006).

New extracts of fresh culture material were made for HESI-MS/MS analysis. Approximately 500 mg of cells from the *Nostoc* spp. cultures were resuspended in 500 μl of 90% methanol. Cells were disrupted in a FastPrep bead beater (Savant) and stored in the dark overnight. The cell debris was pelleted by centrifugation and the OD_{665} reading of the cleared extract was taken to determine the chlorophyll *a* (*Chl a*) content (Meeks and Castenholz, 1971). One millitre of 70% methanol was added to the remaining cell debris and left overnight to complete the toxin extraction. Both the 70 and 90% methanol extracts were pooled, diluted 1:3 in water and concentrated on a C18 column (Spoof *et al.*, 2003).

The uncertainty of detection is reported as a 95% confidence interval, based on the standard deviation of the mean. A calibration equation was derived from the linear regression of five nodularin standard solutions. The standard errors of the estimated concentrations from the calibration equation were between 5 and 9%.

In planta toxin identification

In order to determine if toxin was produced *in planta*, approximately 500 mg of the green cyanobiont ring, containing both plant root material and cyanobionts, was extracted with 1 ml of 70%

Table 1 Toxin profiles and sequence data of the cycad endosymbionts investigated in this study, as well as the reference data used for phylogenetic analysis

Cyanobacterium	*Origin*	*Microcystin or nodularin isoform produced*	*Mol. weights*	*16S rDNA NCBI accession number*	mcyE/ndaF *NCBI accession number*	mcyABC/ndaAB *NCBI accession number*	*NdaB/McyC adenylation domain-binding pocket residues*
Nostoc sp. '*Macrozamia serpentina* 73.1'[a]	Cycad symbiont, Australia	NOD, [L-Har[b]] NOD	824, 838	GU254527[a]	**JF342710**	**JF342711**	DVWNFGFV
Nostoc sp. '*Macrozamia riedlei* 65.1'[a]	Cycad symbiont, Australia	NOD, [L-Har[b]] NOD	824, 838	GU254524[a]	**JF342715**	**JQ010858**	
Nostoc sp. '*Bowenia serrulata* 1.3'[a]	Cycad symbiont, Australia	None detected	—	GU254528[a]	**JF342713**	**JQ010856**	
Nostoc sp. '*M. mountperriensis* 62.1'[a]	Cycad symbiont, Australia	None detected	—	GU254526[a]	**JF342714**	**JQ010857**	
Nostoc sp. '*Macrozamia macleayi* 74.2'[a]	Cycad symbiont, Australia	None detected	—	GU254516[a]	**JF342717**		
Anabaena sp. 90[b]	Lake Vesijärvi, Finland	[D-Asp[c]] MCYST-LR, MCYST-LR, MCYST-RR[b]	980, 994, 1037	AJ133156[b]	AY212249[c]	AY212249[c]	DVW**C**FG**L**V
Anabaena sp. 318[d]	Helsinki, Finland	[D-Asp[c]] MCYST-LR, MCYST-LR, [D-Asp[c]] MCYST-HtyR, MCYST-HtyR[d]	980, 994, 1044, 1058	EF547196[d]	EU916758[d]	*mcyA* EU122319[d], *mcyB* EU009899[e], *mcyC* EU009917[e]	DVW**C**FG**L**V
Nodularia spumigena NSOR10[f]	Orielton Lagoon, Australia	NOD[f]	824	AF268014[f]	AY210783[g]	AY210783[g]	DVWNFGFV
Nodularia spumigena CCY9414[h]	Bornholm Sea, Finland	NOD[i]	Unknown	NZ_AAVW00000000	NZ_AAVW00000000	NZ_AAVW00000000	DVWNFGFV
Nodularia spumigena HEM[j]	Baltic Sea, Finland	NOD[k]	824	AF268005[f]	AY817170[l]		
Nodularia sphaerocarpa PCC7804[h]	Thermal spring, France	NOD, [L-Har[b]] NOD[m,n]	824, 838	AF268019[f]	AY817171[l]		DVW**S**FGFV
Nodularia spumigena BY1[j]	Baltic Sea, Finland	NOD[j]	824	AF268004[f]	AY817169[l]		
Nostoc sp. 152[b]	Lake Sääskjärvi, Finland	[DMAdda[e]] MCYST-LR, [D-Asp[c], ADMAdda[e]] MCYST-LR, [ADMAdda[e]] MCYST-LR, [D-Asp[c], ADMAdda[e]] MCYST-LHar, [ADMAdda[e]] MCYST-LHar, [D-Ser[a], ADMAdda[e]] MCYST-LR, [ADMAdda[e], MeSer[g]] MCYST-LR[b]	980, 1008, 1022, 1022, 1036, 1038, 1040	AJ133161[k]	AY817163[l]	*mcyA* EU431192[o], *mcyB* EU151875[o], *mcyC* EU151868[o]	DVWNFGF**I**
Nostoc sp. IO-102-I[p]	*Pannaria pezizoides* symbiont, Finland	[DMAdda[e]] MCYST-LR, [D-Asp[c], ADMAdda[e]] MCYST-LR, [ADMAdda[e]] MCYST-LR, [ADMAdda[e]] MCYST-XR, [ADMAdda[e]] MCYST-XR, [ADMAdda[e]] MCYST-XR[p]	980, 1008, 1022, 1022, 1036, 1076	AY566855[p]		*mcyA* AY566856[p], *mcyB* EU151876[o], *mcyC* EU151868[o]	
Microcystis aeruginosa K-139	Aquatic bloom, Japan	MCYST-LR[q]	994	—	AB032549[q]	AB019578[q]	
Microcystis aeruginosa PCC7806	Braaken reservoir, the Netherlands	MCYST-LR[r]	994	AF139299[s]	AF183408[s]	AF183408[s]	DVW**TI**G**A**V
Microcystis aeruginosa NIES-843	Lake Kasumigaura, Japan	MCYST[r]	Unknown	AP009552[t]	AP009552[t]	AP009552[t]	
Microcystis sp. CYN10	Lake Horowhennua, New Zealand	MCYST[u]	994	**JF342712**	FJ393328[u]	FJ393328[u]	
Phormidium sp. 1-6c[l]	Diamond Valley Lake, USA	MCYST[l]	Unknown	DQ235810[l]	AY817167[l]		

Table 1 (Continued)

Cyanobacterium	*Origin*	*Microcystin or nodularin isoform produced*	*Mol. weights*	*16S rDNA NCBI accession number*	*mcyE/ndaF NCBI accession number*	*mcyABC/ndaAB NCBI accession number*	*NdaB/McyC adenylation domain-binding pocket residues*
Planktothrix rubescens NIVA-CYA 98	Lake Steinsfjorden, Norway	[Dha[g]] MCYST-LR, [Dha[g]] MCYST-RR [2259]	980.5, 1023.5	AB045951[v]	AM990462[v]	AM990462[v]	
Planktothrix argardii NIVA-CYA 126/8	Lake Langsjön, Finland	[D-Asp[c]] MCYST-LR , [D-Asp[c]] MCYST-RR[w]	980, 1023	EU266147[w]	Aj441056[w]	Aj441056[w]	**DPWV**FGLV
Gloeobacter violaceus PCC7421	Rock surface, Switzerland	None reported	—	AY768399			
Nostoc sp. PCC73102	Cycad, Australia	None reported	—	AF027655	Npun_R3446 (Glutamate-1-semialdehyde aminotransferase)		

The last column represents a comparison of the *N. Spumigena* NSOR10 NdaB/McyC adenylation domain-binding pocket residues with those from other [Arg[2]] and [L-Har[2]] nodularin and microcystin-producing endosymbionts. Amino-acid residues in bold differ from the *N. spumigena* NSOR10 sequence. [g]Gehringer *et al.* (2010); [b]Rouhiainen *et al.* (1995); [c]Rouhiainen *et al.* (2004); [d]Halinen *et al.* (2008); [e]Fewer *et al.* (2008); [f]Moffitt *et al.* (2001); [g]Tooming-Klunderud *et al.* (2008); [h]Fewer *et al.* (2009); [i]Barker *et al.* (1999); [j]Lehtimaki *et al.* (1994); [k]Lyra *et al.* (2001); [l]Jungblut and Neilan (2006); [m]Saito *et al.* (2001); [n]Beattie *et al.* (2000); [o]Fewer *et al.* (2007); [p]Oksanen *et al.* (2004); [q]Nishizawa *et al.* (1999); [r]Kuiper-Goodman *et al.* (1999); [s]Tillet *et al.* (2000); [t]Kaneko *et al.* (2007); [u]Rueckert and Cary (2009); [v]Rounge *et al.* (2009); [w]Christiansen *et al.* (2003).

methanol, diluted 1:3 in water, loaded onto preconditioned Sep-Pak C18 classic cartridges (Waters Corporation, Milford, MA, USA) and eluted with 90% (v/v) methanol. Following the solid phase extraction clean up, the eluents were filtered through Millex-HV 0.45 μm PVDF filters (Millipore, Billerica, MA, USA) (Spoof *et al.*, 2003). Toxic compounds were separated using reversed phase high performance liquid chromatography (HPLC) and identified using an Orbitrap LTQ XL (Thermo Fisher Scientific) ion trap mass spectrometer using a HESI source. The multiple reaction monitoring product ions used were determined from a commercial nodularin standard and from the ESI-MS/MS data for [L-Har[2]] nodularin.

Results

Detection of mcyE/ndaF and phylogenetic analysis

A total of 47 surviving cyanobacterial isolates including 44 *Nostoc* (19 strains) and 3 *Calothrix* isolates obtained during a previous cyanobiont diversity study (Table 2 in Gehringer *et al.*, 2010) were screened for the presence of the *mcyE*/*ndaF* toxin biosynthesis gene essential for microcystin or nodularin synthesis, respectively. Five of the isolates were found to contain regions indicative of the presence of the *mcy* or *nda* gene clusters responsible for the non-ribosomal synthesis of these toxins (Table 1). Neither an amplification product was obtained for the remaining 42 isolates nor for plant DNA controls. Phylogenetic analysis of the DNA sequences was performed to obtain an indication of the nature of potential toxin biosynthesis in these isolates. Three of the isolates clustered with the *mcyE* gene sequences of *Anabaena* sp. 90 and *Anabaena* sp. 318, both known to produce microcystin (Figure 3a, Table 1), with a high degree of statistical support (69%). This cluster was linked to the nodularin-producing cluster of *ndaF* sequences from *Nodularia* species and another symbiont *Nostoc* sp. (52% support at node). The sequence of the aminotransferase domain from *Nostoc* sp. '*Macrozamia macleayi* 74.2' was placed within the phylogenetic branch of *mcyE* genes from microcystin-producing *Microcystis* species (Figure 3a). None of the cyanobiont species grouped within the cluster of nodularin producers. Of interest is the positioning of the AMT region of *Nostoc* sp. 152, a known microcystin producer, with the nodularin producing *Nodularia* spp. Four of the sequences obtained from the endosymbiont *Nostoc* spp., namely JF342710, JF342713, JF342714 and JF342715 (Table 1), had deletions within the sequenced regions of the aminotransferase domains that could result in frameshifts and potentially inactivate biosynthesis.

Phylogenetic analysis of 16S rRNA genes from hepatotoxic genera (Figure 3b) clearly separated the filamentous genera of cyanobacteria, namely

Table 2 Summary of the quantification of nodularin production in methanol extracts of toxin producing endosymbionts and the coralloid roots from which they were isolated

Samples for methanol extraction and toxin analysis	*Total nodularin per ng ml^{-1} extract*	*Nodularin in ng µg^{-1} Chl a*	*Nodularin* per *g ww*	*[L-Har^2] nodularin*
Nostoc sp. '*M. riedlei* 65.1'	173 ± 45	9.55 ± 2.4	346 ng[a]	Yes
Nostoc sp. '*M. serpentine* 73.1'	116 ± 78	12.5 ± 8.4	232 ng[a]	Yes
M. riedlei MZ 65 coralloid root	1.34 ± 0.74	ND	3 pmol[b]	No
M. serpentine MZ 73 coralloid root	BDL	ND		Yes

Abbreviations: BDL, below the detection limit; ND, not determined. The final column indicates whether the presence of [L-Har^2] nodularin was indicated or not. One ml of extract was obtained from each sample of approximately 500 mg. [a]Approximate nodularin content as cells contained copious amounts of EPS; [b]Approximate amount of toxin per g plant ww as material contained both plant and bacterial matter.

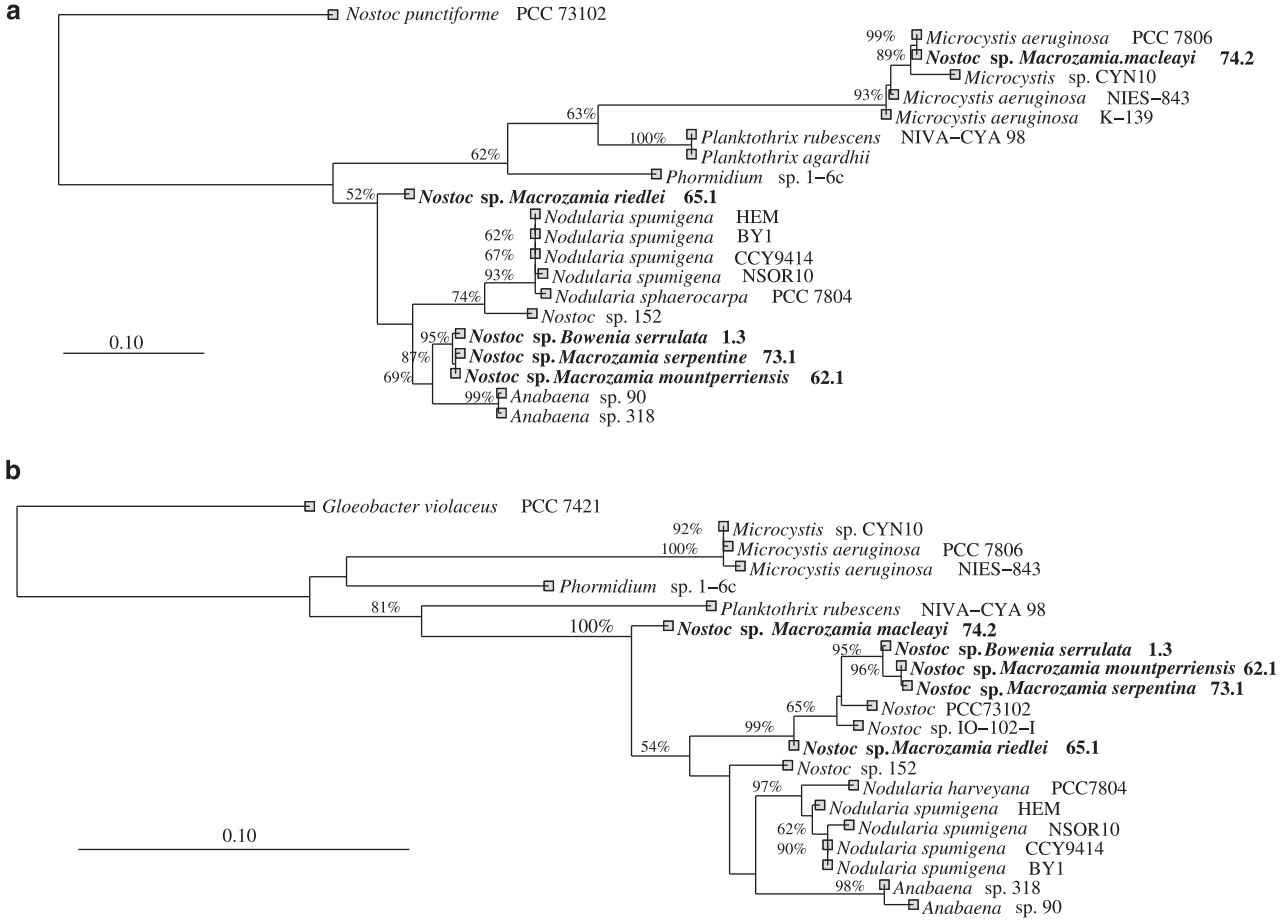


Figure 3 Maximum likelihood phylogenetic tree representing genetic similarities between potential toxin-producing *Nostoc* spp. and reference strains. **(a)** *mcyE*/*ndaF* sequence analysis of a 440-bp fragment of the aminotransferase region; **(b)** Partial 16S rRNA gene sequence comparison of a 770-bp fragment. Support values greater than 50% (1000 bootstrap events) are presented. Sequences in bold were generated during this study.

Phormidium, Planktothrix, Nostoc, Nodularia and *Anabaena,* from the unicellular microcystin-producing genus *Microcystis*. Within the filamentous cluster each species grouped coherently. The two microcystin-producing *Anabaena* species grouped with the nodularin-producing *Nodularia* species, followed by the *Nostoc* clade comprised of non-toxic (*Nostoc* PCC73102), microcystin-producing (*Nostoc* sp. IO-102-I and *Nostoc* sp. 152) and nodularin-producing *Nostoc* species (*Nostoc* sp. '*Macrozamia serpentina* 73.1' and *Nostoc* sp. '*Macrozamia riedlei* 65.1').

Molecular analysis of the Nostoc nodularin cluster

Sequencing of key genes in the non-ribosomal gene cluster would provide information regarding the biosynthetic product of the gene cluster. Pan-handle PCR and sequencing of the genetic region surrounding the AMT domain analyzed above and the

NRPS-encoding genes of *Nostoc* sp. '*M. serpentina* 73.1' (Table 1), indicated the presence of three open reading frames, encoding three NRPS modules and one hybrid NRPS/PKS module. The domain structure of the modules was identical with the corresponding modules in *N. spumigena* NSOR10 within proteins NdaA and NdaB. This analysis implied that *Nostoc* sp. '*M. serpentina* 73.1' produced nodularin and that additional chemical analysis was necessary to confirm this.

Further analysis of amino-acid residues that line the binding pocket of the adenylation domains and contribute to substrate specificity was performed (Challis *et al*., 2000). The NdaB adenylation domain carries binding pocket residues that are important in incorporating L-Arg or L-Har residues into nodularin. The binding pocket residues in *Nostoc* sp. '*M. serpentina* 73.1' were identical to those found in *N. spumigena* NSOR10 and *N. spumigena* CCY9414 (Table 1), further supporting the hypothesis that this strain produces nodularin, rather than microcystin.

Previously, we proposed that the *nda* gene cluster evolved from the *mcy* cluster following deletion of a gene fragment encoding two NRPS modules from within the *mcyA* and *mcyB* genes (Figure 2A) (Moffitt and Neilan, 2004). This hypothesis is based on the identification that within the condensation domain of the NdaA NRPS module (NdaA-C), highly conserved motifs are repeated. The protein sequence before and after the domain repeat of *Nostoc* sp. '*Macrozamia serpentina* 73.1' (referred to as NdaA-C1 and NdaA-C1b) was homologous to McyA-C and McyB-C2, respectively. As previous analyses have been restricted to the *nda* cluster from *N. spumigena*, we used this as an opportunity to further investigate this theory using the *nda* genes of the *Nostoc* strains. Newly designed primers targeting the condensation domains within the *ndaA* gene (Supplementary Figure S1B) were used to determine the partial condensation domain sequence in the four *ndaF* carrying endosymbiont species of *Nostoc*. Alignment of the DNA sequences for the region spanning the condensation motif repeat for *Nostoc* spp. '*Macrozamia serpentina* 73.1', '*M. mountperriensis* 62.1' and '*Bowenia serrulata* 1.3' show high identities (98–99%) with one another and an 84% identity with the similar sequence found in *N. spumigena* NSOR10 (Supplementary Fig. S1A). All three endosymbionts have a 21 bp insertion (highlighted red in Supplementary Fig. S1) when compared to the *N. spumigena* NSOR10 sequence. There is a three base AAC deletion, a two base TT and a single T deletion across all the three endosymbiont sequences when compared to the sequences from *N. spumigena* NSOR10 (highlighted in blue in Supplementary Figure S1A).

Careful analysis of the *Nostoc* NdaA-C domain sequence located a region that appeared to be duplicated in a similar manner to that described in the *N. spumigena* NdaA-C domain sequence (Figure 4b). Each of the *Nostoc* NdaA-C domain sequences were divided into two sections, NdaA-C1 and its duplicate NdaA-C1b (Figure 2B), and phylogenetic analysis (Figure 4a) revealed a similar clustering of *Nostoc* and *N. spumigena* NdaA-C domain sequences. The NdaA-C1 domain of three endosymbionts grouped with the McyA-C1 domains of the microcystin producers and NdaA-C1 domains of the nodularin-producing strains (Figure 4a). The NdaA-C1 region of *Nostoc* sp. '*Macrozamia riedlei* 65.1' could not be amplified and was excluded from analysis. The NdaA-C1 clade was distinct from the tightly grouped clusters observed for the McyB-C1 and McyC/NdaB condensation domains, with high support of the nodes (71% and 81%, respectively). The NdaA-C1b domains of all four *ndaF* carrying endosymbiont species were most similar to the NdaA-C1b homologs of *N. spumigena* NSOR10 and *N. spumigena* CCY9414, followed by the McyB-C2 domains of the microcystin-producing cyanobacterial species. To further demonstrate the similarity of the duplicated NdaA-C domain sequences to the respective Mcy domains, the conserved motifs within the condensation domains of NdaA of *Nodularia spumigena* NSOR10 and *Nostoc* sp. '*Macrozamia serpentine* 73.1' and McyA and McyB of *Microcystins aeruginosa* PCC7806 were aligned (Figure 4b) (Rausch *et al*., 2007). The motifs C1–3 and C5, 6 and 7 are highlighted in grey with residues corresponding to the conserved motifs bolded. Due to low sequence homology, conserved motif C4 could not be easily identified. The alignment demonstrated that NdaA-C1 is homologous to McyA-C, whereas NdaA-C1b is homologous to McyB-C2. A seven amino-acid insertion was observed within the NdaA-C1 region of *Nostoc* sp. '*Macrozamia serpentine* 73.1' just downstream of conserved domain C3 (Figure 4b). The nucleotide deletions resulted in two single amino-acid deletions in the NdaA-C1 and NdaA-C1b regions outside of the conserved domains when compared to *N. spumigena* NSOR10-derived sequence. These analyses indicated the synthesis of nodularin by some endosymbiont *Nostoc* spp.

Bioactivity, identification and quantification of toxin from symbionts

In order to identify cyanobiont isolates actively producing toxin, crude methanol extracts of culture material were analyzed qualitatively in the PP2A inhibition assay. Strong PP2A inhibition corresponding to approximately 10 μM equivalent microcystin-LR (approximately 8 μg nodularin per ml or 8 μg nodularin per g ww) was identified in the methanol extracts of *Nostoc* spp. '*Macrozamia riedlei* 65.1' and '*Macrozamia serpentina* 73.1', confirming the presence of bioactive toxins. No bioactivity was detected in extracts from *Nostoc* sp. '*Bowenia serrulata* 1.3', *Nostoc* sp. '*M. mountperriensis* 62.1' or *Nostoc* sp. '*Macrozamia macleayi* 74.2'.

These samples were subsequently analyzed using HPLC, with an ESI-MS/MS, and were found to

contain nodularin, as indicated by the mz^{-1} of 825 Da. MS/MS analysis (Supplementary Figure S2A–D) confirmed the identity of nodularin by the dissociation of indicative fragmentation patterns at high collision energy (Mazur-Marzec *et al.*, 2006). The toxin extract of *Nostoc* sp. '*M. serpentina* 73.1' was also found to contain the nodularin variant, [L-Har^2] nodularin (Supplementary Figure S2E–H). The structure of this variant was confirmed by the presence of fragmentation peaks with an increased mz^{-1} of 14 Da when compared to the data for nodularin, that were only detected when arginine residues were present. Adda was ruled out as the variable residue as neither the $PheCH_2CH(OCH_3)$ nor $C_{11}H_{15}O$ fragments varied from the nodularin variant fragmentation patterns (Supplementary Figure S2). Other supporting evidence included the presence of L-Har in $[\text{L-Har} + H + NH_3]^+$ (Supplementary Figure S2, peak 5), which has a mz^{-1} of 188 instead of 174 $[\text{Arg} + H + NH_3]^+$, the difference of a 14 Da moiety corresponding to the mass of the additional CH_2 unit in [L-Har^2] nodularin.

HPLC, with a heated electrospray ionization source (HESI) coupled to tandem mass spectrometry (HPLC HESI-MS/MS), was used to quantify the amount of nodularin in newly obtained extracts obtained from the *Nostoc* sp. '*M. riedlei* 65.1' and *Nostoc* sp. '*M. serpentina* 73.1' against *Chl a* content. A high-resolution molecular ion scan and multiple reaction monitoring scans from the precursor molecular ion of mz^{-1} 825.45 Da were used to quantify the mass concentration of nodularin (Supplementary Figures S3–S5). From triplicate measurements of *Nostoc* sp. '*M. riedlei*' 65.1 and duplicate measurements of *Nostoc* sp. '*M. serpentina* 73.1', the mass concentrations of nodularin were $9.55 \pm 2.4\ \text{ng}\,\mu\text{g}^{-1}$ *Chl a* and $12.5 \pm 8.4\ \text{ng}\,\mu\text{g}^{-1}$ *Chl a*, respectively (Table 2). Further two scans, one for the precursor molecular ion mz^{-1} of 839.5 Da and one for the MS/MS product ion, mz^{-1} 767.0 Da (Supplementary Figures S5A and S5B, spectra e–f), indicated the likely presence of [L-Har^2] nodularin in both cultures. The absolute mass concentrations for [L-Har^2] nodularin are not presented as a standard for this isoform was not available.

Analysis of nodularin in cycad roots

The availability of small amounts of frozen coralloid root samples from which the toxin-producing endosymbionts had been isolated allowed us to address the question: Are these toxins produced *in planta*? Methanol extracts of the green symbiotic ring within the remaining coralloid roots were obtained. HPLC HESI/MS/MS analysis identified the presence of nodularin in the coralloid root extract obtained from *Macrozamia riedlei* MZ65 (Gehringer *et al.*, 2010) (Supplementary Figure S6), the same plant from which *Nostoc* sp. '*M. riedlei* 65.1' was isolated. HPLC HESI-MS/MS also indicated the presence of [L-Har^2] nodularin in the root extract of *Macrozamia serpentina* MZ73 (Gehringer *et al.*, 2010) (Supplementary Figure S7, spectra e–f) from which the endosymbiont *Nostoc* sp. '*M. serpentina* 73.1' was obtained.

Data from the *M. riedlei* 65.1 roots, using both the nodularin molecular ion and multiple reaction monitoring peak intensities, were mean centered and combined in a principal components analysis. Three replicate measurements of the root extract revealed a mean concentration of $1.34 \pm 0.74\ \text{ng}\,\text{ml}^{-1}$ (Table 2). Accurate quantitation of toxin content against *Chl a* was not feasible as the samples had been stored at −20 °C for 2 years. Sample size prevented quantitation of toxin against protein content.

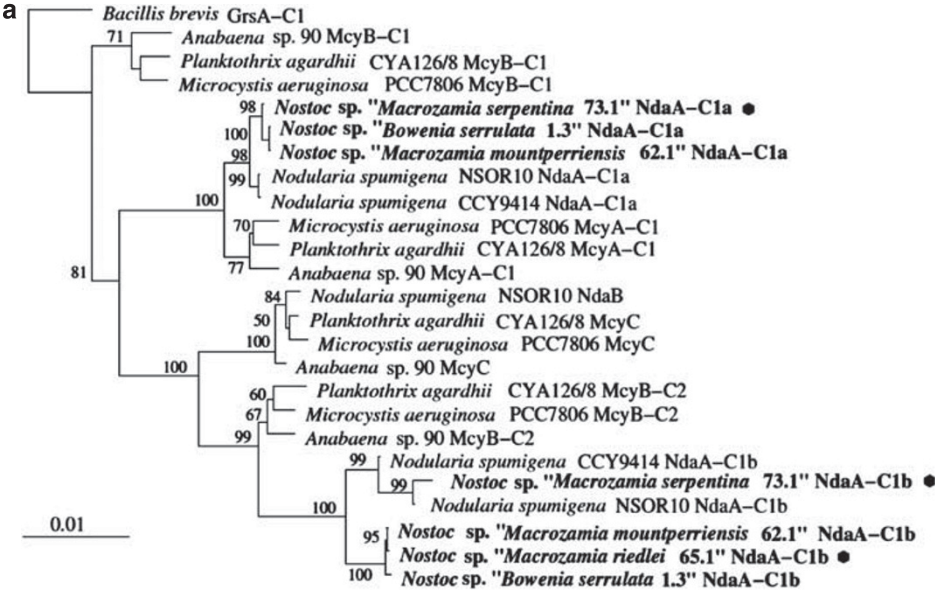
Discussion

Nodularin synthesis has previously been reported only in the aquatic *Nodularia spumigena* species and the benthic, mat-forming *Nodularia sphaerocarpa* strain PCC7804 (Beatti *et al.*, 2000; Saito *et al.*, 2001; Moffitt and Neilan, 2004; Fristachi and Sinclair, 2008). This study reports the synthesis of nodularin and [L-Har^2] nodularin synthesis by terrestrial *Nostoc* spp. Two of the terrestrial symbiont isolates investigated in this study, *Nostoc* sp. '*M. serpentina* 73.1' and *Nostoc* sp. '*M. riedlei* 65.1', were confirmed genetically and chemically as nodularin producers (Tables 1 and 2, Supplementary Figures S2 and S5). The only other terrestrial symbiotic cyanobacteria reported to produce hepatotoxins are *Nostoc* IO-102, isolated from a lichen, and *Nostoc* sp. strain UK18, isolated from *P. leucophlebia* cephalodia, both of which produce microcystin (Oksanen *et al.*, 2004; Kaasalainen *et al.*, 2009).

Phylogenetic analysis of the AMT domain (Figure 2A) indicated that five endosymbiont species may produce a hepatotoxin. It was unable to identify whether they were capable of producing either nodularin or microcystin, as illustrated with

Figure 4 (**a**): Maximum likelihood phylogeny comparing the sequence similarities in the condensation domains (C) of the newly described *Nostoc* sp. '*Macrozamia serpentina* 73.1' and representative condensation domains of NdaA, NdaB, McyA, McyB and McyC reference protein sequences. Bootstrap values greater than 50% after 1000 resampling events are indicated at the relevant nodes. Sequences indicated by a dot were from nodularin producers. (**b**) Protein sequence alignment of the conserved motifs C1-3 and C5, 6 and 7 (shaded in grey) of the condensation domains NdaA-C1 and NdaA-C1b from *Nodularia spumigena* NSOR10 (NSOR10) and *Nostoc* sp. '*M. serpentine* 73.1' (73.1), and the corresponding McyA-C1 and McyB-C2 domains of *M. aeruginosa* PCC7806 (PCC7806). Residues corresponding to the conserved motifs are in bold. A seven amino-acid insertion in the protein sequence of *Nostoc* sp. '*Macrozamia serpentina* 73.1' when compared to *N. spumigena* NSOR10 is boxed.

a



b

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NSOR10 NdaA-C1b     ------------------------------------------------------------
73.1 NdaA-C1b       ------------------------------------------------------------
PCC7806 McyB-C2     QETYETSHAQRRFYVLQQMDLNNVAYHIVSTLKIAGDFSPDVFEKAIQLLISRHESLRTSFI
NSOR10 NdaA-C1      EDAYPLARLQMGMLFHSEYSPGSAVYHNVNSFHLRVAFNESKWRIAIQELVATHAVLRTFFD
73.1 NdaA-C1        EDAYPLARLQMGMLFHSEYSPGSAVYHNVNSFHLRVVFDESKWRIAVQELVANHSVLRTFFD
PCC7806 McyA-C1     EDAYPLTALQLGMIFHSEYQGNLSVYHDVFTYHIRADFSFPALHSAIQEIVQRHPVLRTSFA
                                  C1                          C2

NSOR10 NdaA-C1b     ------------------------------------------------------------
73.1 NdaA-C1b       ------------------------------------------------------------
PCC7806 McyB-C2     LINGEPQQKIL------QNRPFDWEFKDWTNKPDEEILETIAKERKAFDLEKSPLVRSKIYK
NSOR10 NdaA-C1      VSNFSEPLQLVGRTVPVPLEVVDLRHLSATDQESVLDAWFTEQRQQHFRLDSPPLLRFHLHR
73.1 NdaA-C1        VSNFSEPLQLVRRIVRVPLVVEDLRHLSVADQESILDAWFVAERKQNFRLDSPPLLRFHIHR
PCC7806 McyA-C1     LFEYQEPLQLVHRQIDVPLGLDDLTHLSTSEQDTAIDDWIEREKIRTFDWNIPPLFRFHLHR

NSOR10 NdaA-C1b     --------------------FNLHLLEPD----QTAISNAQTSQFHLQQIALN-----TDAVW
73.1 NdaA-C1b       --------------------FTLHLLEPD----KTAISHAQQSQ-SIQQIELN-----TDAVW
PCC7806 McyB-C2     LSPNEYILELEIHHIICDGWSMSLLAKECLQYYSDLAKGLQPNIEPLPIQYK-----DYAGW
NSOR10 NdaA-C1      RTDETFQLTLIEHHAILDGWSVGLLFTELLQRYLSLLGEEIVLLNPPP-------NIAFRDF
73.1 NdaA-C1        RSDETFQLTLIEHHAILDGWSVGLLFTELLQRYLSFLSEEILPLNPSFALAQPAVGIGFRDF
PCC7806 McyA-C1     RSQDNFNLTFSFHHSILDGWSVASLLTELLQQYLYLLDKKVLPLSPTP-------ALSFRDF
                                        C3

NSOR10 NdaA-C1b     QSNIFPSEDSNKHLDYWRKQLEDGQIPRVHLPTDFHRP--PVKTFNGSRLNWTFTPEILSGL
73.1 NdaA-C1b       SSNILPNEKHKKHLDYWRQQLNDGQLPRVHLPTDFHRP--PTKTFNVSRLNWTFQPEIISGL
PCC7806 McyB-C2     QNNLLRSENNSKTLDYWREKLDNGQLTRVHLPTDFKRP--QIKTFKGSHLSWKFTQETISKL
NSOR10 NdaA-C1      VALEQTALNSPEYQQHWLEKLKDITITKLPRWSQSDRSSEPENLKTLGTHEVTLSLTVSHGL
73.1 NdaA-C1        IALEQKALNSQECRQHWLEKLNGITITKLPRWPQSYHSHEPE-LGTLGTQEITLSLTVSQGL
PCC7806 McyA-C1     VALEKKTIQSPECQNYWQEKLRDVTLTKLPQWSKSNQ------VNQDWDWLVPISSQVSQGL

NSOR10 NdaA-C1b     QMICQQTETTLFMGLIAAVKVLLYRYSGQQDITIGTEIASSN--------------TLVIRD
73.1 NdaA-C1b       RTICNQTETTLFMGLVAAVKVLLYRYSGQHDIAIGTEITTRNNYQLASSS--QFLNTVVIRD
PCC7806 McyB-C2     RKSCQENEITLFMALVAAVKILLYRYSGQHDITIGTEIATRNHPQLQ-SLIGLFLNTLVIRD
NSOR10 NdaA-C1      KQVANQAGVTLKSLLLAAHLKVLSVLFNQSDVLTGLVSNGRSEEADGERVLGLFLNTLPLRL
73.1 NdaA-C1        KQVANKAGVTLKSVLLAAHMKVLSVLFNQSDVLTGMVSNGRPEEADGERVLGLFLNTLPLRL
PCC7806 McyA-C1     KQLGKQVGVPLKSVLLAAHFRVLSLLNNQRDIVTGLVSNGRLEAADGEKILGLFLNTLPLRL
                                                                  C5

NSOR10 NdaA-C1b     KLEPEQGYKNLLIQVSQTITEAFEHSNYPFELLVKELATSSEINRTPFFDVLVLLQNLSPSV
73.1 NdaA-C1b       EIEPDRGYQNLLIKVRQTVTEALEHSAYPFDILVKELANSSEINRTPLFDVLVLLQNISQPV
PCC7806 McyB-C2     QIEPEKGYKNLLAKVRQTVTEALEHSDYPFDILVEKLAVSREINRTPLFDTLVLLQNFEQSV
NSOR10 NdaA-C1      QLLGGT-WIDLMQETFKAERESLPFRRYPLADLQWNLGGQPLFETSFNFTHFHVYHSILGLP
73.1 NdaA-C1        QLLGGT-WIDLMQETFKAERESLPFRRYPLADLQWNLGGQPLFETSFNFTHFHVYQSILGLP
PCC7806 McyA-C1     ELSGGP-WSDLVKQAFDVERECLSWRRYPLAELQK--SGQPLFDTAFNFIHFHVYQGIIGVK
                                              C6&7

NSOR10 NdaA-C1b     D---------------YQFDLSFVFVEQNNELKLDLIYNTDIFQADRIKKALIHLDKLLTEM
73.1 NdaA-C1b       EEI--QIKSLGSLDDLNKFDLSFVFIEQNNELSLALIYNTDIFQAERIQQALRHLDKLLIEM
PCC7806 McyB-C2     GLENIQIKSLDSLTPTSKFDLSFVFSEDEEKLRLELIYNTDLFQEERMKKCLIHFDKLLNEM
NSOR10 NdaA-C1      GLQVLESKFLGKTN--FTLGVHFSLDLFSSQINLTLKYNASELCQQQAIAIGNYYTNTLTAI
73.1 NdaA-C1        GLQVLDSKFLGKTN--FTLGVHFSLDLVSSQIHLTLKYNASELCQEQAIAIGNYYTSTLAAI
PCC7806 McyA-C1     DLEVLGGKFFNQTN--FTLLANFSLHPLSSQIELTLKYDGNYLGEKQMELIGGYYEKTLIAM

NSOR10 NdaA-C1b     LANPSQAVCAI
73.1 NdaA-C1b       IVNPSQPVCKI
PCC7806 McyB-C2     LSNPAQPVKDI
NSOR10 NdaA-C1      ASDPLQRYDS-
73.1 NdaA-C1        ASDPSQRYES-
PCC7806 McyA-C1     ATEGLERYETC
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the positioning of the AMT sequence from the microcystin producer, *Nostoc* 152 with the nodularin-producing *Nodularia* species. The positioning of the AMT domain sequence of *Nostoc* sp. *Macrozamia macleayi* 74.2 within the microcystin producing *Microcystis* cluster would suggest that this endosymbiont carries at least part of the *mcy* cluster. This positioning on the AMT tree would be consistent with the studies of Oksanen *et al*. (2004) and Kaasalainen *et al*. (2009) who demonstrated microcystin production in *Nostoc* symbionts found in lichens. A bioassay utilizing PP2A indicated that only extracts from two cyanobiont isolates inhibited the eukaryotic protein phosphatase, namely *Nostoc* sp. '*M. serpentina* 73.1' and *Nostoc* sp. '*M. riedlei* 65.1'. The lack of bioactivity in the remaining three AMT-carrying symbiont isolates would suggest that they may carry non-functional *mcy*/*nda* gene clusters, express toxin at levels below assay detection limits or produce a novel microcystin or nodularin congener that does not inhibit PP2A. In addition, two of the *Nostoc* isolates demonstrating no bioactivity in the PP2A assay, *Nostoc* spp. '*Bowenia serrulata* 1.3' and '*Macrozamia mountperriensis* 62.1', also had frame-shift mutations in their aminotransferase sequences that may have contributed to their non-toxic phenotype.

The synthesis of both nodularin and [L-Har^2] nodularin in these cyanobacterial isolates presented the opportunity to study the mechanistics of toxin production. Sequencing of the partial *nda* cluster in *Nostoc* sp. '*Macrozamia serpentina* 73.1' revealed identical domain structures for NdaA and NdaB as seen in *N. spumigena* NSOR10. However, additional sequencing is required to confirm if the remaining PKS and NRPS domains are conserved in *Nostoc* sp. '*Macrozamia serpentina* 73.1'. It has been proposed that changes in the substrate-binding pocket of the terminal adenylation domain of NdaB may allow for more flexibility of binding for the activation and incorporation of L-Har into nodularin during biosynthesis (Moffitt and Neilan, 2004). *Nostoc* sp. '*M. serpentina* 73.1', that produces both nodularin and [L-Har^2] nodularin, has an NdaB-binding pocket sequence identical to that found in nodularin producers but differs from the other [L-Har^2] nodularin producer, *Nodularia sphaerocarpa* PCC7804 (Table 1). Our analysis indicated that the asparagine to serine substitution is not essential for [L-Har^2] nodularin synthesis, and that the binding pocket sequence DVWNFGFV provides sufficient molecular space for the incorporation of the larger L-Har residue.

The identification of a novel nodularin-synthesizing cyanobacterial species other than *Nodularia* spp. raises questions as to the evolutionary origin of the *nda* cluster. This cluster differs from the *mcy* gene cluster as it does not carry the NRPS modules responsible for the incorporation of the amino acids D-Ala and L-Leu (Moffitt and Neilan, 2004). Analysis of the partial condensation domain DNA sequences of the *ndaA-C1* and *ndaA-C1b* genes of the toxin-producing *Nostoc* sp. '*M. serpentina* 73.1', as well as the AMT carrying endosymbiont strains *Nostoc* sp. '*Bowenia serrulata* 1.3' and *Nostoc* sp. '*Macrozamia riedlei* 62.1', revealed the identical condensation motif repeat present in *N. spumigena* NSOR10 (Figure 4b and Supplementary Figure S1A). This suggested that the genetic event generating the *nda* cluster was the same for all nodularin-producing strains investigated to date. An identical insertion of seven amino acids was observed in the NdaA-C1 domains of all *nda*-carrying endosymbiont strains sequenced and was not present in the NdaA-C1 domain of *N. spumigena* NSOR10. This seven amino-acid insertion was also not observed in the McyA-C1 protein sequence derived from *M. aeruginosa* PCC7806 (Figure 4b) from which the NdaA-C1 domain is thought to have originated. This would suggest that the seven amino-acid insertion probably occurred after the proposed *mcy* truncation event and after transfer of the *nda* cluster into *Nostoc* and *Nodularia* species. The protein alignment in Figure 4b and DNA alignment of Supplementary Fig S1A would suggest that the *nda* cluster was first transferred into *Nodularia spumigena* species, as all the endosymbionts carry the identical 21 bp insertion and 6 nucleotide deletions. Analysis of the condensation domains of NdaA, McyA and McyB (Figure 4) demonstrate high similarity between the endosymbiont-derived protein sequence and that of *N. spumigena* NSOR10. This suggests that the duplicated sequence within the NdaA-C domain of *Nostoc* sp. '*Macrozamia serpentina* 73.1' may have evolved from the fusion of a McyA-C domain and the McyB-C2 domain following deletion of two NRPS modules from *mcy*, in the same manner as proposed for the nodularin-producing *N. spumigena* (Moffitt and Neilan, 2004). This study thereby provides support for the common origin of the *nda* cluster, however, it cannot offer support as to the order of recombination and transfer of the *nda* cluster to *Nodularia* spp.

The observation that the toxin-producing endosymbiont AMT regions do not show good sequence similarity to the AMT sequences found in nodularin-producing *Nodularia* spp. (Figure 2A), would suggest that the evolution of the *nda* cluster in *Nostoc* spp. is not straightforward. Additional sequencing of the *nda* clusters is required before further sequence and phylogenetic analysis can be undertaken. Whether the genetic event generating the *nda* cluster occurred in a *Nodularia, Microcystis* or another species of cyanobacteria, remains an enigma. What is evident from the alignment of the DNA sequences spanning the potential deletion site is that all *nda* carrying endosymbiont *Nostoc* species and the *nda* carrying *N. spumigena* NSOR10 show high similarity, thereby suggesting a similar evolutionary origin.

Tandem MS analysis indicated the production of nodularin and not microcystin in the two strains

showing bioactivity in the PP2A assay. Data on product ion spectra (S2) confirmed that the synthesis of [L-Har^2] nodularin as well. Nodularin was further confirmed by HPLC HESI-MS/MS at low concentrations (Table 2) in extracts of the cyanobionts cultures. The levels of toxin produced by these endosymbionts are low, with a μg:μg ratio of nodularin:*Chl a* of 0.01:1, when compared to the nodularin producing abilities of *Nodularia* KAC66, an aquatic isolate, with ratios ranging between 0.4:1 and 0.7:1 (Stolte *et al.*, 2002). The discrepancy between nodularin levels detected by the PP2A assay (about 8 μg ml^{-1}) versus the HPLC HESI-MS/MS (over 100 ng ml^{-1}) could be ascribed to differences in the assay procedures. The PP2A would reflect the influence of all the hepatotoxins in an extract on the enzyme, that is, nodularin and the potential inhibition by [L-Har^2] nodularin, whereas the HESI-MS/MS reflects the nodularin concentration only. The PP2A assay was standardized against microcystin-LR, whereas nodularin was used to establish a standard curve for HESI-MS/MS. In addition, the HESI-MS/MS nodularin quantitation was completed 2 years after the PP2A quantitation and the cultures may have altered their toxin profile and levels of production.

Nodularin was detected *in planta* in extracts of the cycad roots that yielded the endosymbiont *Nostoc* sp. '*M. riedlei* 65.1', while [L-Har^2] nodularin was only present in root extracts that contained *Nostoc* sp. '*M. serpentina* 73.1' (Supplementary Figures S6 and S7). Nodularin production was not observed in the root extracts of *Macrozamia serpentina* (MZ73) (Supplementary Figure S7, spectra a–d). Although the symbiont *Nostoc* sp. '*M. serpentina* 73.1' synthesized nodularin, the quantities were significantly lower than that seen for [L-Har^2] nodularin (Supplementary Figure S5, Table 2). The lack of amplification of *mcyE*/*ndaF* genes from plant material not containing cyanobacterial symbionts indicates that this toxin cannot be of plant origin. The concentration of nodularin in the coralloid root tissue of *Macrozamia riedlei* (MZ65) can be approximated as 3 pmol g^{-1} (for a single sample of approximately 500 mg). Lehtimäki *et al.* (2011) demonstrated signs of oxidative stress in young spinach plants irrigated with nodularin-containing water and detected toxin in young roots in the range of 0–24 pmol g^{-1}. This would suggest that, at least locally, nodularin would induce oxidative stress in the coralloid root tissue. Whether the toxin is transported to the rest of the plant or restricted to the coralloid root, remains to be elucidated.

Research into the toxic effects of nodularin has been largely over-shadowed by studies on the biological effects of microcystin (Humpage, 2008). Given that both toxins inhibit protein phosphatases, it is thought that they exhibit similar biological activities (Mackintosh *et al.*, 1990; Honkanen *et al.*, 1991). The different structures of nodularin has leant itself to the theory that it can target different tissues than those effected by microcystin (Ohta *et al.*, 1994) and therefore induce different toxicological effects in exposed animals and potentially plants. This study has demonstrated the production of nodularin *in planta* at levels capable of inducing oxidative stress in spinach plant root tissue (Lehtimaki *et al.*, 2011). Whether these effects can be extended to include cycad roots remains to be determined. The nodularin-producing *Nostoc* spp. produce toxin at levels over 100-fold lower than that observed for the aquatic, nodularin-producing *Nodularia* sp. Toxin released into an aquatic environment would be immediately diluted, thereby reducing the immediate toxic effects on the aquatic biota. In contrast, toxin released into a drier environment, and possibly *in planta*, would not be diluted and may accumulate. Whether these levels of hepatotoxin production are significant in a terrestrial environment remains to be investigated. Further research into the ancient relationship between cyanobacteria and the Cycadacea should reveal the molecular interactions between toxin and host plant, and also highlight the evolutionary importance of secondary metabolism in symbioses and the terrestrial environment.

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