Natural occurrence of microcystin synthetase deletion mutants capable of producing microcystins in strains of the genus *Anabaena* (Cyanobacteria)

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Microcystins form a large family of small cyclic heptapeptides harbouring extensive modifications in amino acid residue composition and functional group chemistry. These peptide hepatotoxins contain a range of non-proteinogenic amino acids and unusual peptide bonds, and are typically *N*-methylated. They are synthesized on large enzyme complexes consisting of non-ribosomal peptide synthetases and polyketide synthases in a variety of distantly related cyanobacterial genera. Here we report a 1236 bp in-frame deletion mutation in the *mcyA* gene of the microcystin biosynthetic pathway in nine strains of the genus *Anabaena*. The deletion removed almost the entire *N*-methyltransferase (NMT) domain. Strains of *Anabaena* carrying the in-frame deletion mutation incorporated mainly dehydroalanine (Dha) into the microcystins they produce while strains with full-length *mcyA* genes incorporated mainly *N*-methyldehydroalanine (Mdha). Interestingly, the strains of *Anabaena* lacking the NMT domain also incorporated elevated amounts of L-Ser, the precursor of Mdha and Dha, into the microcystin they produced relative to strains carrying functional NMT domains. We provide evidence for the in-frame deletion of the NMT domain without the co-conversion of the flanking adenylation domain. Our results demonstrate a further example of the strategies employed by cyanobacteria in the biosynthesis of microcystin variants.

INTRODUCTION

*Anabaena* is a common component of freshwater phytoplankton worldwide and strains of this genus produce a wide range of bioactive compounds, including neurotoxins and hepatotoxins (Sivonen & Jones, 1999). These cyanobacteria form filaments and contain gas vesicles allowing them to float to the surface during periods of calm weather to form thick surface scums (Stal *et al.*, 2003). Microcystin-producing strains of the genus *Anabaena* have been reported from Canada, Finland, France, Norway and Denmark (Sivonen & Jones, 1999). *Anabaena* is also common in brackish-water environments and contributes to bloom formation each summer in the Baltic Sea (Karlsson *et al.*, 2005; Stal *et al.*, 2003). These blooms are amongst the largest mass occurrences of cyanobacteria in the world (Kahru, 1997) and have recently been demonstrated to contain microcystin-producing *Anabaena* (Halinen *et al.*, 2007).

Microcystins are implicated in the toxicosis of wild and domestic animals worldwide (Ressom *et al.*, 1994). They are potent inhibitors of eukaryotic protein phosphatases 1 and 2A (MacKintosh *et al.*, 1990). These toxins penetrate the liver cell membranes via the bile acid route and lead to overphosphorylation and disruption of the cytoskeleton in hepatocytes (Carmichael, 1994). They cause hepatocyte necrosis, leading to pooling of blood in the liver. In acute poisoning, death is the result of intrahepatic haemorrhage and hypovolemic shock (Carmichael, 1994). Microcystins

Abbreviations: Dha, dehydroalanine; Mdha, *N*-methyldehydroalanine; NMT, *N*-methyltransferase; SAM, *S*-adenosylmethionine.

A supplementary table with details of the microcystin variants produced by the 26 strains of *Anabaena* studied is available with the online version of this paper.
are also suspected to be tumour promoters (Nishiwaki-Matsushima et al., 1992), and the use of water contaminated with the toxin in renal dialysis was held responsible for the deaths of 60 patients in Brazil (Jochimsen et al., 1998). The World Health Organization has set a provisional guideline of 1 μg microcystin-LR per litre of drinking water (WHO, 1998).

Microcystins are small cyclic heptapeptides with an unusual chemical structure and a number of non-proteinogenic amino acids (Sivonen & Jones, 1999). They share the common structure cyclo-d-Ala<sup>1</sup>-X<sup>2</sup>-d-MeAsp<sup>3</sup>-Z<sup>4</sup>-Adda<sup>5</sup>-d-Glu<sup>6</sup>-Mdha<sup>7</sup> where d-MeAsp is d-erythro-β-methylaspartic acid, Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-(4E,6E)-decadienoic acid, d-Glu is d-iso-glutamic acid, and X and Z are variable L-amino acids (Fig. 1). Microcystins typically contain three variable methyl groups, including N-methyl, O-methyl and C-methyl groups (Fig. 1). Methylation is a relatively common modification in biologically active natural peptides and is thought to improve stability against proteolytic degradation (Finking & Marahiel, 2004; Sieber & Marahiel, 2005).

Microcystins are synthesized by a thiotemplate mechanism on large synthetases in a programmed biosynthetic event (Dittmann et al., 1997). In Anabaena this enzyme complex is encoded in a 55 kb gene cluster containing 10 genes (mcyA–J) encoding peptide synthetases, polyketide synthases and tailoring enzymes (Rouhiainen et al., 2004). Non-ribosomal peptide synthetases possess a highly conserved modular structure with each module consisting of catalytic domains responsible for the adenylation, thioester formation and condensation of specific amino acids (Marahiel et al., 1997). The arrangement of these domains within the multifunctional enzymes determines the number and order of the amino acid constituents of the peptide product (Sieber & Marahiel, 2005). Additional domains for the modification of amino acid residues such as epimerization, heterocyclization, oxidation, formylation, reduction or N-methylation may also be included in the module (Lautru & Challis, 2004; Marahiel et al., 1997; Sieber & Marahiel, 2005). Microcystins typically contain the unusual non-proteinogenic amino acid N-methyldehydroalanine (Mdha) (Fig. 1). N-Methylation of the microcystins is achieved by the N-methyltransferase (NMT) domain in the first module of McyA (Nishizawa et al., 1999; Till et al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004). The NMT domain catalyses the transfer of the S-methyl group from S-adenosylmethionine (SAM) to the α-amino group of the thioesterified amino acid (Sieber & Marahiel, 2005).

The strategies employed by microcystin-producing cyanobacteria to achieve chemical variation in amino acid residue composition and functional group chemistry are not fully clear at present. Strains of the genus Anabaena produce a broad range of N-methylated microcystins (Fewer et al., 2007; Halinen et al., 2007; Harada et al., 1991; Krishnamurthy et al., 1989; Sivonen et al., 1992a, b). It has been shown in several studies that strains of the genus Anabaena produce mixtures of microcystins, some of which lack the N-methyl group (Halinen et al., 2007; Namikoshi et al., 1992a, b; 1998; Sivonen et al., 1992a, b). However, a number of Anabaena strains produce microcystins all of which lack the N-methyl group (Halinen et al., 2007; Namikoshi et al., 1992a, 1998; Sivonen et al., 1992a). Here we demonstrate that the exclusive production of microcystins lacking the N-methyl group in strains of Anabaena is associated with a natural in-frame deletion removing almost the entire NMT domain from mcyA.

![Fig. 1. The microcystin chemical structure can be generalized as cyclo-d-Ala<sup>1</sup>-X<sup>2</sup>-d-MeAsp<sup>3</sup>-Z<sup>4</sup>-Adda<sup>5</sup>-d-Glu<sup>6</sup>-Mdha<sup>7</sup>, where X and Z denote the highly variable L-amino acids present at the second and fourth positions. The arrow indicates the N-methyl group of Mdha.](image-url)

### METHODS

**Culture preparation.** We selected 26 microcystin-producing strains of the genus Anabaena from Denmark, Finland, Norway and the Baltic Sea (Table 1). To obtain sufficient biomass for LC-MS analysis the strains were grown under continuous white light at a photon irradiance of 7 μmol m<sup>−2</sup> s<sup>−1</sup> in 40 ml ZB medium lacking a source of combined nitrogen (Kotai, 1972). Cells were collected from 21-day-old cultures by centrifugation at 7000 g for 7 min followed by the removal of excess medium. This step was repeated and the cells were stored at −20 °C.

**Assignment of microcystin structures.** Cells were freeze-dried to approximately 3–20 mg dry weight and homogenized with 0.5 mm diameter glass beads and 1 ml 75/25 DMSO/acetoniitrile. The mixture was shaken in a FastPrep FP120 cell disruptor (Savant Instruments) and then centrifuged at 10 000 g for 3 min. Whole-cell extracts were analysed by injecting 5 μl of this extract into a Agilent 1100 series modular HPLC system (Agilent Technologies) equipped with a diode array detector and a mass spectrometer (Agilent XCT Plus Ion Trap). Whole-cell extracts were separated on a Luna C18(2) column (2 × 150 mm, 5 μm, Phenomenex) using the following linear mobile phase gradient: 10% (0 min) to 50% (60 min) 2-propanol in water (+0.1% HCOOH) at a flow rate of 0.15 ml min<sup>−1</sup> at 40 °C. Electrospray ionization was performed in positive-ion mode. Nebulizer gas (N<sub>2</sub>) pressure was 30 p.s.i. (207 kPa); drying gas flow and temperature were 8 l min<sup>−1</sup> and 350 °C, respectively.

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Table 1. Details of Anabaena strains studied

The strain number, geographical origin, year of isolation, NCBI accession number for the mcyA gene, presence or absence of the NMT domain in mcyA and relative amounts (%) of amino acids present at the 7th position of the microcystins produced by each strain are listed.

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*MeSer-containing microcystins could not be assigned a complete structure, but MS² fragmentation of the protonated molecular ions m/z 999 (Rt 39.5 min) and m/z 1013 (Rt 40.3 and 41.9 min) supported the presence of MeSer in position 7 in these microcystins.

Capillary voltage was set to 5000 V, capillary exit offset to 300 V and skimmer 1 potential to 85 V with a trap drive value of 144. Spectra were recorded as averages of 4 using ultrasonic mode and a scan range from 100 to 2200 m/z. Tandem MS (MS²) spectra were recorded with auto MS mode with 1.0 V fragmentation amplitude. In auto MS mode a setting of 10 precursor ions was used with an isolation width of 4.0 m/z. The unusual β-amino acid Adda contains two conjugated π-bonds responsible for a UV absorbance maximum at 238 nm, so the UV spectra of microcystins are characteristic. Microcystins were distinguished from other peptides based on the characteristic UV absorbance maximum at 238 nm as well as their mass spectral characteristics as MH⁺ values corresponding to the range of published microcystins, loss of neutral fragment 134 in the ion source, occurrence of ions m/z 599 [(MeAsp)-Arg-Adda-(Glu)+H⁺] and m/z 375/361 [Adda–134-Glu-(M)dha] in the MS² spectrum. Structural identification of microcystin variants was based on interpretation of ions in MS³. MeSer-containing microcystins could not be assigned a complete structure, but MS² fragmentation of the protonated molecular ions m/z 999 (Rt 39.5 min) and m/z 1013 (Rt 40.3 and 41.9 min) supported the presence of MeSer at position 7 in these microcystins.

DNA preparation. The whole-cell extracts used in LC-MS were evaporated to dryness with a vacuum pump. The dried cell matter was used for DNA extraction with the Qiagen DNeasy Plant Mini kit according to the manufacturer’s instructions. The quality and quantity of DNA were measured on a UV spectrophotometer. Alternatively, cell lysates were prepared using an alkaline PEG lysis protocol (Chomczynski & Rymaszewski, 2006).

PCR amplification and sequencing. We performed PCRs in a 20 μl final volume containing approximately 20 ng DNA, 1× DynaZyme II PCR buffer, 250 μM of each deoxynucleotide, 0.5 μM of each oligonucleotide primer, and 0.5 units of DynaZyme II DNA polymerase (Finnzymes). PCRs were also carried out using the BD Advantage Tag polymerase (BD Biosciences), 1× Advantage PCR buffer and otherwise the same conditions as above. The primer pair NMT-F (5’-CTCACAATTTTCTCTTCTTCT-3’) / NMT-R (5’-GAGCGATTTGGTTCTGAGATGTTA-3’) was used in PCR to amplify the mcyA gene containing a portion of the C-terminal adenylation domain and the complete NMT domain with an expected size of 1682 bp. The following protocol was used: 95 °C for 3 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 2 min; followed by a final elongation of 72 °C for 10 min. The PCR products were purified using Montage PCR centrifugal filter devices (Millipore) or by the E.Z.N.A Gel Extraction kit (Omega Biotek). The purified PCR products were sequenced with the external primers NMT-F and NMT-R as well as the internal primers NMT-F2 (5’-CAGCGTCACGAGATAATT-3’) and NMT-R2 (5’-TGAATATCATGGGGGACATC-3’).
amplified the mcyA gene from Nostoc sp. 152 using the NMT-F/NMT-R primer pair using the same conditions as before but lowering the annealing temperature to 50 °C. Sequencing was conducted under BigDye terminator cycling conditions, and sequencing reactions were purified using ethanol precipitation and separated on an Applied Biosystems 3730xl DNA analyser. Chromatograms were checked and edited with the CHROMAS 2.2 program (Technelysium). Contig assembly and alignment of the sequences were performed with the BIOEDIT sequence alignment editor.

Phylogenetic and structural analysis. The position of the NMT catalytic domain in peptide synthetases is highly conserved and located between the A8 and A9 core motifs of the adenylation domain (Marahiel et al., 1997). In order to characterize the deletion site in truncated mcyA gene sequences we aligned the McyA NMT and N-terminal adenylation domain amino acid sequences between the A8 and A9 conserved adenylation motifs against other adenylation domains, some of which contain this optional catalytic domain. We used conserved NMT domain motifs including the glycine-rich SAM-binding motif to guide the alignment (Marahiel et al., 1997; von Döhren et al., 1997).

In order to reconstruct the evolutionary history of the NMT domain we obtained and aligned a selection of NMT domain sequences from cyanobacteria, proteobacteria and chloroflexi. The set of NMT domains were identified in BLASTP searches using the NMT domain from Anabaena sp. 90 as a query (Rouhainen et al., 2004). We included NMT domains involved in the biosynthesis of anabamopeptilide, chondramide, tubulysin, myxochromides S and barbamide as well as NMT domains identified in non-ribosomal peptide synthetases in the genomes of Herpetosiphon auranticus ATCC 23779, Nostoc punctiforme PCC73102, Anabaena variabilis ATCC 29413 and Nodularia spumigena CCY9414. We also included the NMT domain from the McyA protein in seven microcystin producers and the NdaA protein from two nodulin producers. We excluded missing and ambiguous regions and used 296 aa in phylogenetic analyses. Maximum-likelihood phylogenies were inferred using PROML implemented in the PHYLIP package (Felsenstein, 1993) with a JTT substitution model in the CONSENSE programs and using the JTT substitution model in the PHYLIP package (Felsenstein, 1993). Phylogenetic trees were rooted with the NMT domain from a mixed polyketide synthetase–non-ribosomal peptide synthetase protein from the genome of Myxococcus xanthus DK 1622.

RESULTS

We identified strains of Anabaena carrying truncated mcyA genes via PCR with primers specific for regions of the mcyA adenylation domain flanking the NMT domain (Fig. 2). By sequence analysis we characterized a 1236 bp in-frame deletion in the mcyA gene in strains of Anabaena isolated from Denmark, Finland and the Baltic Sea (Table 1). Of the 26 strains included in this study, nine contained the 1236 bp deletion (Fig. 2, Table 1). In all cases the deletion occurred at the same position of the mcyA gene, removing 410 aa of the NMT catalytic domain from McyA. The deletion removed almost the entire NMT domain and two amino acids of the adenylation domain between amino acid positions 730 and 1130 of full-length McyA (CAD60101). Comparison with other cyanobacterial adenylation domain sequences suggests that only a short 13 aa remnant of the NMT domain was retained after the deletion event (Fig. 3). Anabaena strains isolated in different years from a number of different lakes in Denmark and Finland, as well as from the Baltic Sea, carried the in-frame deletion mutation in the mcyA gene (Table 1).

The 26 strains of Anabaena included in this study produced a broad range of microcystins (see supplementary Table S1, available with the online version of this paper). We identified between 2 and 14 microcystins in each strain and the dominant variant in most strains was MC-LR, [D-Asp]MC-LR or [Dha]MC-LR (Table S1). We observed variation in the type of amino acid incorporated at the X position (Table S1). Most strains produced microcystins containing L-leucine but some strains produced microcystins containing L-arginine at the X position and in trace amounts L-homoisoleucine, L-homotyrosine, L-phenylalanine or L-homophenylalanine at the X position (Table S1). A series of microcystins were identified in 17 strains of Anabaena, 85–100 % of which contained Mdha (Fig. 4).

[Fig. 2. A 1.5 % agarose gel illustrating the truncated (lanes 2–5, 8) and full-length (lanes 1, 6–7) mcyA PCR products from a selection of Anabaena strains: 1, 1TU44S16; 2, 1TU30S4; 3, 1TU31S9; 4, 202A1/35; 5, PH256; 6, 1TU44S9; 7, 1TU46S11; 8, 202A2/41. M, 1 kb DNA ladder (New England Biolabs).]

[Fig. 3. Schematic illustrating the in-frame deletion mutation in strains of the genus Anabaena. The alignment includes the adenylation domains from the conserved core motifs A8–A9 and the NMT domain core motifs M1–M3 (Marahiel et al., 1997) and conserved NMT domain core motifs 1–6 (von Döhren et al., 1997). Anabaena strains carrying full-length mcyA genes (I): 90, 1TU44S16, 1TU44S9, 1TU46S11, 0TU33S16, 0TU43S7, 258, BIR246, 1TU35S12, 288, 0TU39S15b, 318, NIVA 83/1, NIVA 267/4, NIVA 269/2, NIVA 269/6, NIVA 270/1 and 1TU4436. Anabaena strains carrying truncated mcyA genes (II): PH256, 1TU30S4, 1TU31S9, 202A1/35, 202A2/41, 1TU32S11, 66A, 315 and 18B6.]

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However, a subset of these strains also produced trace amounts (0.1–1.4%) of microcystins containing Dha (Fig. 4, Table 1). The remaining 9 strains produced microcystins 88–100% of which contained Dha at the seventh position (Fig. 4). Interestingly, 3–7% of the microcystins produced by 7 of these 9 strains contained L-Ser instead of Mdha (Fig. 4). Anabaena sp. 18B6 and 315 produced microcystins which only contained Dha (Fig. 4). Microcystins containing L-Ser were present only in trace amounts in strains producing N-methylated microcystins (Table 1). Low amounts of microcystins containing L-MeSer could be identified in some strains (Table 1).

Phylogenetic analyses demonstrated that NMT domains from McyA form a monophyletic group (Fig. 5). We observed a primary split with Planktothrix and Microcystis and the heterocyst-forming strains Anabaena and Nostoc grouping separately (Fig. 5). We did not find support for the monophyly of nodularin and microcystin synthetase NMT domains (Fig. 5). The topology may suggest that these catalytic domains cluster in separate parts of the maximum-likelihood tree (Fig. 5). However, all but the terminal branches in the tree are unresolved, and a sister group relationship between nodularin and microcystin NMTs cannot be ruled out.
DISCUSSION

Microcystins are synthesized on large multienzyme complexes consisting of polyketide synthases and non-ribosomal peptide synthetases in *Anabaena* (Rouhiainen et al., 2004). Nearly all of the 48 catalytic reactions necessary for the biosynthesis of microcystin can be assigned to the 10 proteins encoded in this 55 kb gene cluster (Rouhiainen et al., 2004). The range of variation of microcystins produced by the strains of *Anabaena* included in this study is consistent with previous observations (Fewer et al., 2007; Halinen et al., 2007; Namikoshi et al., 1992a, b, 1998; Sivonen et al., 1992a, b). The 425 aa NMT domain of McyA is responsible for the N-methylation of the microcystin and is inserted between the core motifs A8 and A9 of the first adenylation domain in *mcyA* in *Anabaena* 90 (Rouhiainen et al., 2004). Here we identified an association between the incorporation of Dha into microcystins and the deletion of the NMT domain in 35% of the strains examined (Fig. 4). The 412 aa in-frame deletion removes almost the entire NMT domain and 2 aa of the adenylation domain, leaving only a small 11 aa NMT remnant (Fig. 3). All conserved NMT domain motifs (Marahiel et al., 1997; von Döhren et al., 1997) were absent in the deletion mutants, including the glycine-rich SAM-binding motif (Marahiel et al., 1997).

The sequence similarity between the regions flanking the deletion site in the 26 strains of *Anabaena* is between 98 and 100% (data not shown). The regions of *mcyA* genes flanking the deletion site differ from other *mcyA* genes by just three basepairs (data not shown). It is not clear how the in-frame deletion took place. Possible mechanistic explanations for the deletion event could be rare homologous, homeologous, or illegitimate repair of double-strand breaks in the NMT domain following DNA damage resulting in an in-frame fusion. Such a model would not invoke co-conversion of the adenylation domain through a homologous recombination event. Strains of cyanobacteria producing microcystin variants containing either Dha or Dhb exclusively are known from all major producers of microcystins (Sivonen & Jones, 1999). This suggests that in addition to *Anabaena* and *Planktothrix* some strains of *Microcystis* and *Nostoc* either contain inactive NMT domains or lack the domain completely. Microcystins containing Dhb in *Planktothrix* are thought to be the result of a homologous recombination event in the first module of *mcyA* which replaces the adenylation domain, leading to the change in substrate specificity and presumably the concomitant deletion of the NMT domain (Kurmayeur et al., 2005). Our results suggest a parallel but independent strategy for producing microcystins lacking the N-methyl group in *Anabaena* through deletion of the NMT domain alone without co-conversion of the adenylation domain.

The N-terminal adenylation domain of McyA recognizes and activates l-Ser as an AMP-adenylate (Christiansen et al., 2003; Nishizawa et al., 1999; Rouhiainen et al., 2004; Tillett et al., 2000). The Ser residue undergoes N-methylation and dehydration to form Mdha (Christiansen et al., 2003; Nishizawa et al., 1999; Rouhiainen et al., 2004; Tillett et al., 2000). The order and timing of the N-methylation, dehydration and condensation reactions are not yet clear at present. Interestingly there is a 10–500-fold increase in the amount of l-Ser incorporated into the microcystins in the strains of *Anabaena* lacking a functional NMT domain (Table 1). This suggests that there may be a functional coupling of the dehydration and N-methylation reactions. Additional expression studies, enzyme characterization and kinetic experiments are required to unravel the order and timing of these catalytic reactions in the biosynthesis of Mdha.

The majority of strains in this study produce microcystins containing Mdha (Fig. 4). However, all strains produced mixtures of microcystins and we identified trace amounts of microcystins containing Dha in some strains even in the presence of a functional NMT domain (Fig. 4). A fundamental feature of modular non-ribosomal peptide synthetases is the highly predictable relationship between the domain order and the chemical functional groups of resultant non-ribosomal peptide products (Marahiel et al., 1997). The simultaneous production of Mdha- and Dha-containing microcystins in *Anabaena* could be the result of the inaction of the NMT domain during the assembly of Dha-containing microcystins. However, the concomitant biosynthesis of Dha- and Mdha-containing microcystin variants could also be the result of a limitation of the SAM precursor of the methyl group (Marahiel et al., 1997). Such mixtures of microcystins containing both Mdha and Dha are common in *Planktothrix* (Luukkainen et al., 1993) and *Microcystis* (Robillot et al., 2000), and inaction of the NMT domain may be a widespread feature of microcystin biosynthesis.

The cyclic pentapeptide nodularin and the cyclic heptapeptide microcystin share a similar chemical structure (Sivonen & Jones, 1999) and nodularin synthetase is believed to have evolved from a microcystin synthetase progenitor (Moffitt & Neilan, 2004; Rantala et al., 2004). The nodularin synthetase gene cluster is proposed to have arisen following deletion of two non-ribosomal peptide synthetase modules and a change in substrate specificity from l-Ser to l-Thr in the C-terminal adenylation domain of *NdaA* (Moffitt & Neilan, 2004). Interestingly, we find that the NMT domains from microcystin and nodularin synthetase proteins McyA and NdaA do not have the same topology as other regions of the *mcy* and *nda* gene clusters (Fig. 5) as might be expected in an evolutionary scenario where all nodularin and microcystin synthetase genes share the same evolutionary history (Rantala et al., 2004). There is no decisive evidence for a sister taxa relationship between the two domains (Fig. 5). Instead, the phylogenetic position of the NdaA NMT domains suggests that the proposed change in substrate specificity in the nodularin synthetase gene cluster from Mdha to Mdhb (Moffitt & Neilan, 2004; Rantala et al., 2004) may have been the result of a recombination event resulting in the replacement of both the N-terminal adenylation domain and the NMT domain in *ndaA*. 
Many important antibiotics, antimicrobial compounds, siderophores and toxins are synthesized on non-ribosomal peptide synthetase enzyme complexes (Marahiel et al., 1997; von Döhren et al., 1997). The modular structure of non-ribosomal peptide synthetases allows the rational design of novel peptides by targeted replacement of these catalytic domains (Stachelhaus et al., 1995). There is much current interest in engineering non-ribosomal peptide synthetases in order to create new peptides with potential biological activities (Sieber & Marahiel, 2005). Understanding the natural evolution of non-ribosomal biosynthetic pathways can provide insights useful in developing combinatorial engineering strategies and rational design of novel metabolites and pharmaceuticals. Our results demonstrate that it is possible to create functional non-ribosomal peptide synthetases through partial in-frame deletion of the NMT domain, and the deletion site identified here may prove useful in defining potential deletion strategies. This study further demonstrates in one of the few natural examples that the deletion of biosynthetic domains can lead to new biosynthetic outcomes. This naturally occurring mechanism is an important addition to the known evolutionary strategies for generating structural diversity in nonribosomal peptides such as module (Kurmay er et al., 2005) or domain replacement (Fewer et al., 2007; Mikalsen et al., 2003).

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