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The Genetic Basis for *O*-Acetylation of the Microcystin Toxin in Cyanobacteria

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SUMMARY

Microcystins are a family of cyclic peptide toxins produced by cyanobacteria. They are responsible for the toxicosis and death of wild and domestic animals throughout the world. They display extensive variation in amino acid composition and functional group chemistry. *O*-acetylated microcystins are frequently produced by free-living and symbiotic strains of the genus *Nostoc*. Here, we show that the production of acetylated microcystins is catalyzed by an acetyl-coenzyme A-dependent *O*-acetyltransferase (McyL) encoded in the 57 kb microcystin synthetase gene cluster of *Nostoc* sp. 152. Phylogenetic analysis demonstrates that McyL belongs to a family of enzymes that inactivate antibiotics through *O*-acetylation. The McyL enzyme has a relaxed substrate specificity, allowing the preparation of semisynthetic microcystins. This study sheds light on the evolutionary origins and genetic diversity of an important class of enzymes involved in antibiotic resistance.

INTRODUCTION

Microcystins are a family of small peptide toxins that are produced by a range of cyanobacteria in fresh and brackish water bodies worldwide. The microcystins exert their effects through inhibition of members of the PPP family of protein phosphatases (Gulledge et al., 2002). These enzymes are crucial for the regulation of a myriad of biological processes through the dephosphorylation of proteins (Moorhead et al., 2009). Microcystins are taken up mainly by the liver, and acute exposure to high concentrations of these toxins causes liver damage, while chronic exposure may promote liver tumor formation (Kuiper-Goodman et al., 1999). They are responsible for the toxicosis and death of wild and domestic animals throughout the world (Ressom et al., 1994; Sivonen, 2009).

The microcystin family is chemically diverse, and there are over 100 structural variants of microcystins, differing in the type of amino acids incorporated into the microcystin or modifications to the peptide backbone (Sivonen, 2009; Kaasalainen et al., 2012). The general structure of microcystins can be summarized as cyclo-D-Ala^1-X^2-D-MeAsp^3-Z^4-Adda^5-D-Glu^6-Mdha^7, where X and Z are variable L-amino acids and Adda ((2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-(4*E*,6*E*)-dienoic acid) is an unusual nonproteinogenic amino acid characteristic of the toxin (Figure 1). Microcystins block the catalytic site of protein phosphatases through interaction of the glutamyl carboxylate and hydrophobic Adda of microcystin with the active site of the enzyme (Gulledge et al., 2003; Xing et al., 2006). Isomerization of the Adda moiety to 6*Z*-Adda microcystin renders the molecule nontoxic (Harada et al., 1990).

Aquatic strains of the genus *Nostoc* produce a series of *O*-acetylated or *O*-methylated Adda microcystin variants (Sivonen et al., 1990; Beattie et al., 1998; Genuário et al., 2010; Bajpai et al., 2009). Microcystins carrying an acetyl group instead of methyl group in the hydroxyl on Adda are collectively termed ADMAdda variants (Figure 1). Symbiotic strains of the genus *Nostoc* isolated from lichen symbiosis around the world produce a series of *O*-acetylated Adda microcystin variants or, less frequently, *O*-methylated variants (Oksanen et al., 2004; Kaasalainen et al., 2009, 2012). *O*-acetylated variants of microcystin exhibit similar levels of toxicity compared to the most toxic variants of microcystin (Sivonen et al., 1990, 1992; Beattie et al., 1998; Laub et al., 2002).

Microcystins are assembled on megadalton enzyme complexes consisting of nonribosomal synthetases and polyketide synthases (Tillett et al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004). *O*-methylation of the microcystin is carried out by the McyJ *O*-methyltransferase (Christiansen et al., 2003). However, the biosynthetic origins of the *O*-acetylated microcystins remain unclear. Here, we show that the production of *O*-acetylated variants of microcystin in *Nostoc* is the result of specific enzymatic *O*-acetylation of the microcystin catalyzed by the McyL *O*-acetyltransferase.

RESULTS

Microcystin Biosynthetic Gene Cluster

The microcystin (*mcy*) gene cluster from *Nostoc* sp. 152 was located in two overlapping fosmids clones using a PCR-based method to screen a fosmid library. The DNA sequences of the two fosmids, pDF1346 (39307 bp) and pDF1720 (38617 bp), were determined by shotgun Sanger sequencing to greater than 9X coverage. The two fosmid clones were assembled into a 66.8 kb contiguous region that contained the 57 kb *mcy*

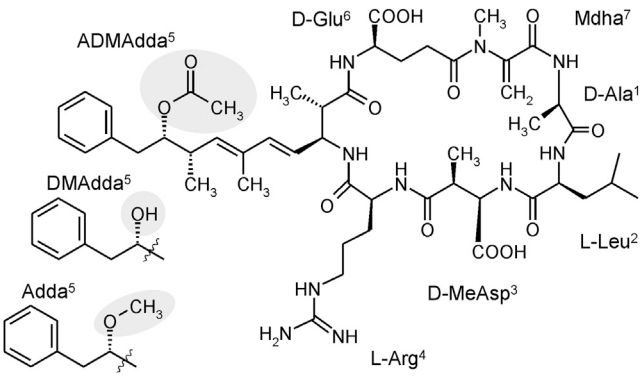

Figure 1. The Structure of [ADMAdda^5] MC-LR Microcystin Produced by *Nostoc* sp 152

Microcystins typically contain the unusual β amino acid 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyl-deca-4,6-dienoic acid (Adda) as well as the non-proteinogenic amino acids Mdha and D-MeAsp. The microcystins produced by strains of the genus *Nostoc* are often *O*-acetylated (ADMAdda, O-acetyl-O-demethyl Adda) or lack both the *O*-methyl and *O*-acetyl groups (DMAdda, O-demethyl Adda).

See also Table S1.

gene cluster (Figure 2). The *mcy* gene cluster in *Nostoc* sp. 152 consists of ten proteins with a central bidirectional promoter (Figure 2). There were eight open reading frames flanking the *mcy* gene cluster (Figure 2). These encode housekeeping proteins and a variety of hypothetical open reading frames unlikely to be involved in the biosynthesis of microcystin (Table 1).

The *mcy* gene cluster encoded McyG, McyD, McyE, McyA, McyB, and McyC, which together are responsible for the stepwise assembly and cyclization of peptide intermediates to form microcystin (Tillett et al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004). The McyG, McyD, and McyE enzymes are responsible for the synthesis of DMAdda (Figure 2). The gene encoding the McyJ *O*-methyltransferase is absent from the *mcy* cluster of *Nostoc* sp. 152 (Figure 2). The McyA, McyB, and McyC enzymes encode six nonribosomal peptide synthetase (NRPS) elongation modules responsible for the activation of amino acids and extension of the microcystin intermediate (Table 2). The *mcy* gene cluster also encodes two known tailoring enzymes, McyI and McyF. McyH contains a membrane-spanning and an ATP-binding domain of ATP-binding cassette (ABC) transporters and is widely anticipated to be involved in the efflux of the toxin. The gene order is colinear with the hypothetical order of the enzymatic reactions for microcystin biosynthesis (Tillett et al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004). An open reading frame encoding a bimodular patatin-like protein is encoded between the *mcyH* and *mcyI* genes but with an unclear role in the biosynthesis of microcystin (Table 1). Downstream of the *mcy* gene cluster is a 630 bp gene encoding a 25 kDa protein, which we named McyL, as we suspected it to catalyze the *O*-acetylation of the microcystins produced by *Nostoc* sp. 152 (Figure 2).

The Genetic Basis for Acetylation of Microcystins

The McyL protein contains five imperfect tandem repeats with the [LIV]-[GAED]-XX-[STAV]-X hexapeptide motif, which, in the tertiary structure, forms a left-handed parallel β helix and assembles into catalytic trimers (Raetz and Roderick, 1995; Sugantino and Roderick, 2002). Hexapeptide enzymes often function as acyltransferases and participate in a variety of enzymatic processes, including cell wall biosynthesis, amino acid metabolism, and detoxification of xenobiotics, including chloramphenicol and streptogramins (Murray and Shaw, 1997; Beaman et al., 1998). The McyL enzyme was most similar to the xenobiotic acetyltransferase family (XAT), and this similarity implied that McyL

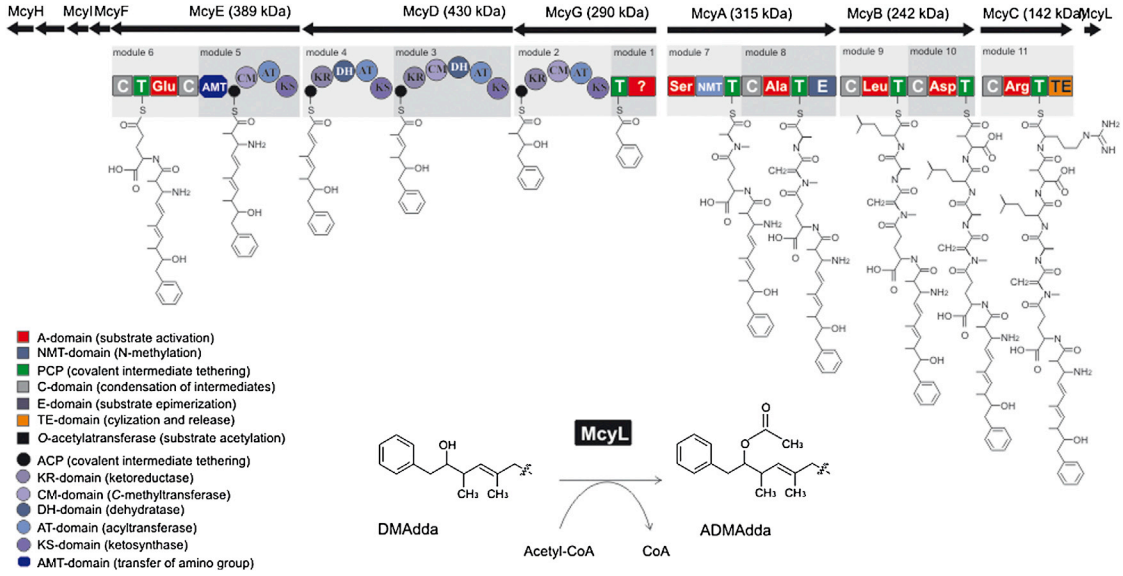

Figure 2. A Model for the Biosynthesis of the *O*-Acetylated [ADMAdda^5] MC-LR Microcystin Variant

The 57 kb microcystin synthetase gene cluster of *Nostoc* sp. 152.

A linear model for the assembly of the [ADMAdda^5] MC-LR microcystin variant. McyL catalyzes the acetyl-CoA-dependent *O*-acetylation of DMAdda to produce ADMAdda.

Table 1. Proposed Functions of Proteins Encoded in the Microcystin Biosynthetic Gene Cluster and Three Genes Flanking the *mcy* Gene Cluster

Protein	Length (amino acids)	Proposed Function	Top BLAST Hit		
			Organism	Identity (%)	Accession Number
ORF4	978	GAF sensor-containing diguanylate cyclase	*Cyanothece* sp. PCC 7425	75	YP_002377105
ORF3	307	N-acetylmuramic acid 6-phosphate etherase	*A. variabilis* ATCC 29413	89	YP_320763
ORF2	137	hypothetical protein	*Nostoc* sp. PCC 7524	70	YP_007075536
McyH	601	ABC transporter	*N. spumigena* CCY9414	73	ZP_01629638
ORF1	698	patatin-like protein	*N. punctiforme* PCC 73102	59	YP_001865735
McyI	341	putative dehydrogenase	*N. spumigena* CCY9414	76	ZP_01629639
McyF	252	amino acid racemase	*Anabaena* sp. 90	77	AAO62581
McyE	3,475	NRPS-PKS (KS-AT-ACP-AMT-C-A-PCP-C)	*N. spumigena* CCY9414	85	ZP_01629641
McyD	3,873	PKS (KS-DM-CM-KR-ACP-KS-AT-DM-KR-ACP)	*N. spumigena* CCY9414	77	ZP_01629643
McyG	2,640	NRPS-PKS (A-PCP-KS-AT-CM-KR-ACP)	*N. spumigena* CCY9414	82	ZP_01629644
McyA	2,788	NRPS (A-NMT-PCP-C-A-PCP-E)	*Anabaena* sp. 90	77	AAO62586
McyB	2,133	NRPS (C-A-PCP-C-A-PCP)	*Anabaena* sp. 90	75	AAO62587
McyC	1,182	NRPS (C-A-PCP-Te)	*Anabaena* sp. 90	95	AAO62588
McyL	210	*O*-acetyltransferase	*X. oryzae*	38	AAV67422

is a coenzyme-A-dependent *O*-acetyltransferase catalyzing the acetylation of the microcystin toxin.

McyL Catalyzes CoA-Dependent *O*-Acetylation of Microcystin

In order to test this hypothesis, the gene encoding McyL was cloned and overexpressed in *Escherchia coli* BL21 to provide a soluble histidine-tagged protein variant (Figure 3). We suspected that the substrate for McyL would be DMAdda, and therefore, an in vitro *O*-acetylation assay was performed with an extract of *Nostoc* sp. UK222 (Figure 3). This strain, in comparison to other known microcystin producers, contained elevated amounts of DMAdda microcystins (Table S1 available online). This symbiotic strain, isolated from the *Peltigera degenii* lichen, produces six variants of microcystin: two of the variants contain DMAdda microcystins and account for approximately 6% of the microcystins, while the remaining four variants accounted for 94% of the microcystins, and each contained Adda (Table S1). The two DMAdda microcystins and acetyl-coenzyme A (CoA) acted as substrates and acyl donor in an *O*-acetylation in vitro assay (Figure 3). Ion chromatograms before and after the assay showed that DMAdda-containing microcystin concentrations decreased markedly (Figures S1B and S1D), and equivalent amount of ADMAdda-containing microcystins, which were originally absent, appeared (Figures 4A, 4B, S1C, and S1E). Product ion spectra of the ADMAdda variants proved the identity of these microcystins (Figure S2). As expected, no marked changes were seen in Adda-containing microcystins, as they are not substrates in the *O*-acetylation reaction (Figures S1D and S1F–S1H). This in vitro assay demonstrated that McyL transferred acetyl from an acetyl-CoA donor to the DMAdda microcystin variants.

A deacetylation assay was carried out with an extract of the *Nostoc* sp. 152 strain, which produces almost solely ADMAdda-containing microcystin variants (Figure 4). The deacetylation reaction was followed for 15 hr. This assay demonstrated that the *O*-acetylation reaction was reversible, and the McyL enzyme removed the acetyl from ADMAdda to create DMAdda in the presence of CoA (Figure 4). After 1 hr, the reaction reached equilibrium and the ADMAdda forms remained at 3% (SD 0.6) level for the remaining 15 hr reaction time.

McyL Catalyzes Synthesis of an Unnatural Microcystin

O-acylation of the DMAdda microcystins was also performed using propionyl-CoA and butyryl-CoA in the absence of acetyl-CoA (Figure 5). DMAdda microcystin variants from *Nostoc* sp. UK222 extract were converted to *O*-propionylated (PDMAdda) and *O*-butyrylated (BDMAdda) microcystin variants in the presence of propionyl-CoA and butyryl-CoA, respectively (Figure 5). The identity of these semisynthetic microcystins was confirmed based on the ion mass of the protonated molecules and the product ion spectra, which showed the presence of the characteristic ions for the Adda structures. The formation of BDMAdda microcystin variants was comparable to the formation of ADMAdda variants, but PDMAdda variant levels were clearly lower (Figure 5). Neither *O*-propionylated nor *O*-butyrylated microcystins were detected from cell extracts of *Nostoc* sp. 152.

McyL Is a Member of XAT *O*-Acetyltransferase Family

The McyL enzyme shares 40% of its amino acid sequence with other members of the XAT family, including residues in the conserved CoA binding site and timer interaction sites. BLASTp analysis demonstrated that XAT *O*-acetyltransferase enzymes are very common in the genomes of bacteria. Almost 50% of the 1,890 complete bacterial genomes encoded one or more *O*-acetyltransferase homologs. However, McyL is distantly related to other members of hexapeptide family. The McyL proteins from *Nostoc* formed a sister clade to streptogramin and chloramphenicol genes (Figure 6).

DISCUSSION

Specific Synthesis of Microcystin Chemical Variants

O-acetylated microcystins are produced almost exclusively by strains of the genus *Nostoc* isolated from a range of aquatic

Table 2. Substrate Binding-Pocket Amino Acid Residues of the McyG, McyE, McyA, McyB, and McyC Adenylation Domains from Complete *mcy* Gene Clusters

Strain	McyG	McyE	$McyA_1$	$McyA_2$	$McyB_1$	$McyB_2$	McyC
Nostoc sp. 152	VGVWVAASGK	DPRHSGVVGK	DVWHISLIDK	DLFNNALTYK	DALFFGLIYK	DARHVGIFVK	DVWNFGFIDK
Anabaena sp. 90	----------	----------	----------	----------	-VW---VD-	----------	---C--L---
Planktothrix sp. CYA126/8	----------	----------	----------	------S--	------VD-	-P-----I-	-P-G--L---
Planktothrix rubescens CYA 98	----------	----------	-----GMV--	----------	------VD-	-P-----I-	-P-G--LV--
Microcystis aeruginosa PCC 7806	----------	----------	----------	----------	--W-L-NVV-	----------	---TI-A---
Microcystis aeruginosa K-139	----------	----------	----------	----------	--W-L-NVV-	----------	---TI-A-E-
Microcystis aeruginosa NIES 843	----------	----------	----------	----------	-GWTI-AVE-	----------	---TI-AVD-
	Adda	Glu	Mdha	Ala	X	B-MeAsp	Z

The variable X and Z positions in the chemical structure of microcystin are encoded by the $McyB_1$ and McyC adenylation domains.

and terrestrial habitats in free-living and lichen-associated growth states (Sivonen et al., 1990, 1992; Oksanen et al., 2004; Kaasalainen et al., 2009, 2012). Here, we show that the distribution of *O*-acetylated Adda variants in the genus *Nostoc* is the direct consequence of the gain of a gene encoding a specific tailoring enzyme. There are over 100 structural variants of microcystin differing in modifications to the peptide backbone or the type of amino acids incorporated into the microcystin (Sivonen, 2009; Dittmann et al., 2013; Kaasalainen et al., 2012). It is becoming clear that the bulk of this variation is the result of genetic changes, which affect the functioning of enzymes encoded in the *mcy* gene cluster. The *mcy* gene cluster is often spontaneously inactivated through deletions and insertions (Kurmayer et al., 2004; Fewer et al., 2011a). However, in other cases, the *mcy* gene is altered through a series of genetic recombinations and point mutations, which affect the functioning of the microcystin peptide synthetases and result in the chemical diversity observed in nature (Fewer et al., 2007, 2008; Tooming-Klunderud et al., 2008). This study provides a mechanism for the specific synthesis of chemical variation in microcystin structure by toxic cyanobacteria through the gain or loss of genes encoding tailoring enzymes. *O*-acetylated microcystins have also been reported in a single strain of *Planktothrix agardhii* PH123 (Laub et al., 2002), and given that the production of ADMAdda requires *O*-acetylation in *Nostoc* strains, it seems likely to be the case in this strain of *Planktothrix agardhii* as well.

Enzymatic Synthesis of Microcystins

The McyL enzyme catalyzes the CoA-dependent *O*-acetylation of microcystins. We exploited this dependency to carry out enzymatic synthesis of unnatural *O*-propionylated and *O*-butyrylated microcystins. However, we did not detect *O*-propionylated or *O*-butyrylated microcystins from cell extracts of *Nostoc* sp. 152, and this type of microcystin variant has not been reported from microcystin producing strains of the genus *Nostoc* (Sivonen et al., 1990, 1992; Oksanen et al., 2004; Kaasalainen et al., 2009, 2012). A large proportion of the intracellular CoA pool consists of acetyl-CoA and could explain the lack of specificity toward the other acyl-CoA substrates (Jackowski and Rock, 1986). The relaxed specificity of McyL toward acyl-CoA substrates may have applications in the development of novel protein phosphatase inhibitors.

Evolution of Antibiotic Resistance and the XAT Family of *O*-Acetyltransferases

The transfer of acyl groups is a common mechanism for the inactivation of antibiotics in bacteria. The covalent modification of vulnerable hydroxyl groups often results in inactive antibiotics, which cannot bind to their target (Shaw and Leslie, 1991; Murray and Shaw, 1997; Beaman et al., 1998). A large number of enzymes belong to the hexapeptide class of acyltransferases and catalyze the CoA-dependent acetylation of hydroxyl-bearing acceptors that include polysaccharides, sugars, and natural products (Murray and Shaw, 1997). The McyL enzyme is a member of the XAT family of hexapeptide acyltransferase. These enzymes are common in the genomes of bacteria and catalyze the inactivation of antibiotics such as chloramphenicol and streptogramin (Shaw

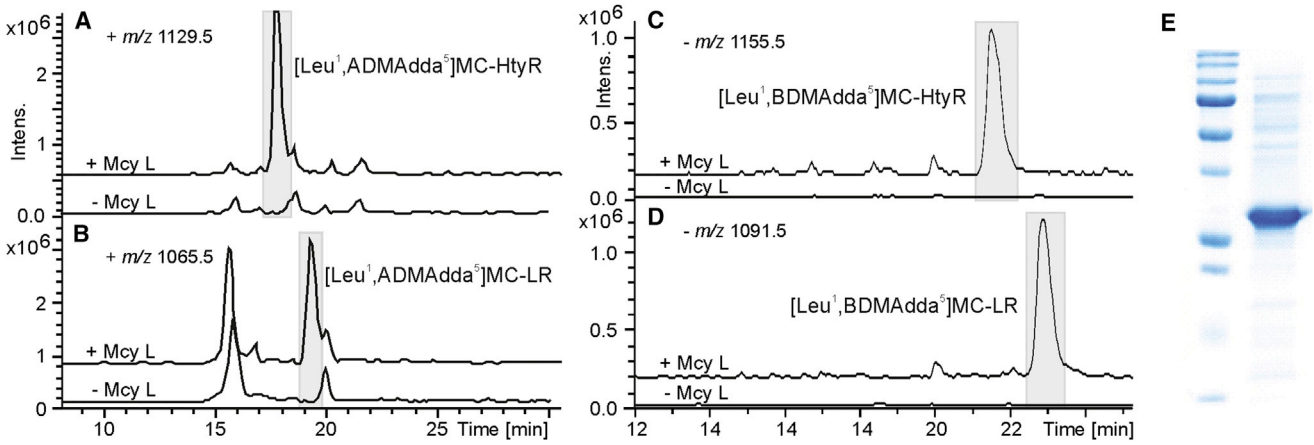

Figure 3. McyL Catalyzes CoA-Dependent *O*-Acylation of [DMAdda^5] Microcystins

(A and B) Extracted positive ion chromatograms (*m/z* 1,129.5 and *m/z* 1,065.5) of the *O*-acetylated [Leu^1, ADMAdda^5] MC-HtyR and [Leu^1, ADMAdda^5] MC-LR microcystin variants formed from the acetyl-CoA-dependent conversion of corresponding DMAdda-containing microcystin variants catalyzed by McyL.

(C and D) Extracted negative ion chromatograms (*m/z* 1,155.5 and *m/z* 1,091.5) of the *O*-butyrylated [Leu^1, BDMAdda^5] MC-HtyR and [Leu^1, BDMAdda^5] MC-LR microcystin variants formed from the butyryl-CoA-dependent conversion of corresponding DMAdda-containing microcystin variants catalyzed by McyL.

(E) Hexahistidine-tagged McyL (28 kDa) was eluted from a Ni^{2+}-affinity column and concentrated.

See also Figure S1.

and Leslie, 1991; Murray and Shaw, 1997). However, only the XAT enzymes encoded by cat3, satA, and vatB, which all inactivate antibiotics, have been studied biochemically. The substrate and function of the vast majority of XAT enzymes is unclear. To date, no XAT enzyme has been shown to be involved in the biosynthesis of natural products. Our results show that the McyL protein is a member of this XAT family of trimeric *O*-acetyltransferases. This is one of the few instances where the XAT *O*-acetyltransferase is directly associated with the biosynthesis of a natural product instead of inactivation of antibiotics and sheds light on the origin of such enzymes. The enzymes involved in biosynthesis of natural products might provide a pool of enzymes that could potentially inactivate antibiotics and should be taken into account in search for new antibiotics.

SIGNIFICANCE

Many important antibiotics, antimicrobial compounds, siderophores, and toxins are synthesized on nonribosomal peptide synthetase enzyme complexes (Marahiel et al., 1997). There is much current interest in engineering nonribosomal peptide synthetases in order to create new peptides with potential biological activities (Sieber and Marahiel, 2005). Here, we have evidence for the acquisition of an *O*-acetyltransferase, which allows the specific *O*-acetylation of microcystins in free-living and symbiotic strains of the genus *Nostoc*. This McyL enzyme catalyzes the CoA-dependent *O*-acetylation of microcystins. We exploited this dependency to carry out enzymatic synthesis of unnatural microcystins, which may have applications in the development of novel protein phosphatase inhibitors. This is an example of a XAT enzyme that is not involved in the inactivation of antibiotics and sheds light on the origin of such enzymes.

EXPERIMENTAL PROCEDURES

DNA Isolation and Fosmid Library Construction

The cyanobacterial strain *Nostoc* sp. 152 was isolated from Lake Sääksjärvi in 1986 and purified (Sivonen et al., 1990, 1992). This strain produces a range of microcystins containing *O*-acetylated and demethylated variants of Adda (Sivonen et al., 1990, 1992; Oksanen et al., 2004; Fewer et al., 2007). *Nostoc* sp. 152 was grown in Z8 medium (Kotai 1972) without nitrate at 22°C–25°C at a photon irradiance of 20–27 μmol m^{-2} s^{-1} for 21 days.

High molecular weight DNA for the construction of a fosmid library was obtained from *Nostoc* sp. 152 using enzymatic lysis and a phenol-chloroform extraction, as previously described (Rouhiainen et al., 2010; Fewer et al., 2011b). The high molecular weight DNA was sheared using a 22 gauge hypodermic needle and syringe, size-selected, end-repaired, and ligated into the copy control fosmid vector pCC1FOS according to instructions provided with the CopyControl Fosmid Library Production Kit (Epicenter). *Escherichia coli* strain EPI300, which was used as a host for DNA cloning and sequencing, was grown at 37°C overnight in Luria Bertani medium supplemented with chloramphenicol at a final concentration of 12.5 μg ml^{-1}. The titer from this method was very low, and the entire packaging extract was used to generate a 2,200 clone library (Fewer et al., 2011b). DNA manipulations, such as restriction digests, ligations, and transformations, were carried out using standard methods.

Screening the Fosmid Library

We introduced a three-step PCR screening system to identify fosmids carrying the microcystin synthetase gene cluster, as previously described (Fewer et al., 2011b). DNA was extracted from four pools of 500 fosmid clones and used as template in PCR, using primers specific for the *mcyD* microcystin synthetase gene (Rantala et al., 2004). The PCR was performed for 30 cycles consisting of 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s, with a final extension at 72°C for 10 min. Pools positive for fosmids bearing the *mcyD* gene were spread on selective plates, and the resulting fosmid clones were collected to construct five pools of 100 clones. Fosmid DNA was extracted and used as a template in PCR using the *mcyD*-specific primers. Pools positive for fosmids bearing the *mcyD* gene were plated and used to construct a third pool of ten fosmid clones. These pools were screened as before, and those positive for the *mcyD* gene were plated and screened directly by colony PCR in order to isolate the fosmid clones bearing the *mcyD* gene. These fosmid clones were

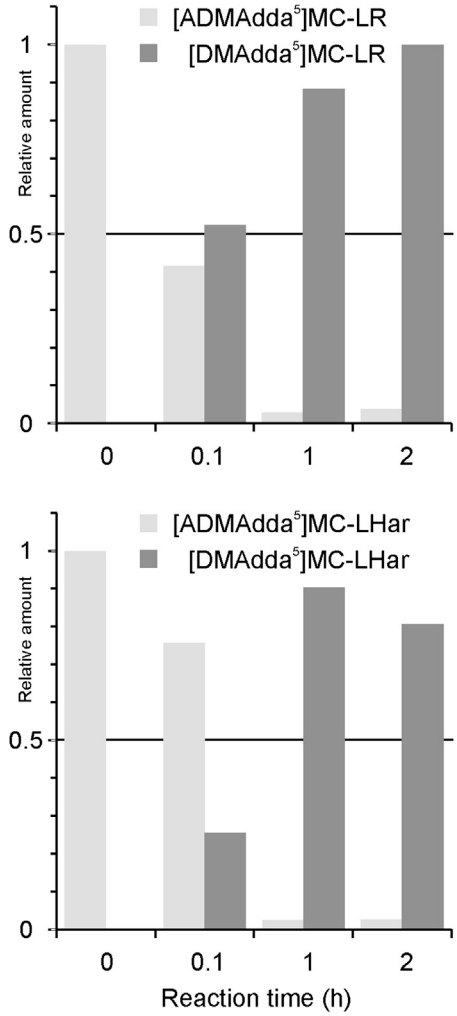

Figure 4. McyL Catalyzes the Deacetylation of Microcystins Containing ADMAdda

The McyL enzyme removes the *O*-acetyl group from the [ADMAdda^5] MC-LR and [ADMAdda^5] MC-LHar microcystin variants of *Nostoc* sp. 152 in the presence of CoA.

See also Figure S2.

grown overnight at 37°C overnight in Luria Bertani medium supplemented with chloramphenicol at a final concentration of 12.5 μg ml^{-1}. Fosmid DNA isolation was carried out using a commercially available kit (QIAGEN). The fosmids were end-sequenced using 400 ng of fosmid DNA as a template for cycle sequencing reaction using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and analyzed on the ABI 310 Genetic Analyzer. Two fosmid clones, pDF1346 and pDF1720, were verified to contain the entire *mcy* gene cluster and sent for shotgun sequencing at Beijing Genomics Institute.

Sequence Analysis

The pDF1346 and pDF1720 fosmid clones were shotgun-sequenced to greater than 9X coverage. Analyses of DNA and protein sequences were carried out using the National Center for Biotechnology Information BLAST server. Protein-encoding open reading frames (ORFs) were predicted using Glimmer 2.0. Catalytic domain prediction was carried out using conserved motifs characteristic of NRPS and polyketide synthase (PKS) proteins (Marahiel et al., 1997) in combination with BLAST searches and the Conserved Domains Database. Prediction of adenylation domain substrate specificity was carried out using NRPS predictor (Rausch et al., 2005).

Amplification and Cloning of mcyL for Overexpression

We amplified the *mcyL* gene of the *mcy* gene cluster from *Nostoc* sp. 152 by PCR using oligonucleotide primers mcyL_F_pET (5′-CACCATGGCT TACCATACCTACAAATC-3′) and mcyL_R_pET (5′-GTTGTTAACGTCTTC CTGTAGAAG-3′). The PCR reactions were done in a volume of 50 μl consisting of *Pfu* buffer (20 mM Tris-HCl [pH 8.8], 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1 mg/ml BSA, 0.1% [v/v] Triton X-100, 2 mM $MgSO_4$, Thermofisher), 2.5 μM primers, 0.2 mM dinucleotide triphosphate, 1.25 U of *Pfu* DNA polymerase (Thermofisher) and 20–50 ng of genomic DNA. The following protocol was used: 3 × (95°C, 1 min; 50°C, 30 s; 72°C, 2 min); 25 × (95°C, 30 s; 54°C, 30 s; 72°C, 2 min); and 72°C, 5 min. PCR products were purified with the QuickStep 2 PCR Purification system (Edge Biosystems), ligated to pET101D expression vector (Invitrogen) according to the manufacturer's instructions, and used to transform the TOP10 strain. The resulting plasmids were purified, end-sequenced using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems), and analyzed on the ABI 310 Genetic Analyzer in order to verify that the inserts had the correct orientation relative to the T7 promoter on pET101D. Plasmids were used to transform the *Escherichia coli* BL21 (DE3) cloning strain for expression.

Overexpression and Purification of the McyL Protein

The plasmid clones were used to transform the *E. coli* BL21 Star (DE3) expression host (Invitrogen). Transformants were grown in Luria-Bertani medium supplemented with 100 μg ml^{-1} ampicillin. Transformants were grown at 37°C overnight with shaking at 160 rpm, and 600 μl was used to innoculate 30 ml of 2 × yeast extract-Tryptone medium containing 50 μg ml^{-1} carbenicillin. Cultures were then grown at 37°C by shaking at 160 rpm until they reached an optical density measured at a wavelength of 600 nm of 0.7–1.0 and then induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside. Shaking was continued at 23°C–24°C overnight for 18–20 hr at 100 rpm. Cells were collected by centrifugation at 4000 g, 4°C for 5 min; resuspended in 1.5 ml of lysis buffer containing 50 mM sodium phosphate, 300 mM NaCl, 100 mM KCl, 10 mM imidazole, 0.5% TRITON X-100, 1 mM β-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, and Complete Mini protease inhibitor cocktail (Roche); and placed on ice. The cells were sonicated in ice (Braun Labsonic U sonicator), power level 50%, and at 0.7 repeating duty cycles 7 × 10 cycles, respectively, and the soluble proteins were recovered by centrifugation at 12,000 g for 20 min. Expression level and protein purity were tested with the SDS-PAGE runs. Soluble protein was purified with the His affinity tag method using His Pur Ni-nitrilotriacetic acid Spin Columns (Thermo Scientific) and PBS buffer containing 250 mM imidazole for elution. The buffer was changed to 50 mM TRIS pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol using Zeba Desalt Spin Columns (Thermo Scientific). Amicon Ultra-4 30K Centrifugal Filter Units (Merck Millipore) were used to concentrate the preparations to final volume of 1 ml. Protein concentration of the preparations was measured with the two-dimensional Quant kit (GE Healthcare).

In Vitro *O*-Acylation

The substrate for the McyL enzyme was suspected to be DMAdda. However, DMAdda, when detected, is produced in trace amounts by cyanobacteria (e.g., Fewer et al., 2007). Approximately 6% of the microcystins produced by *Nostoc* sp. UK222 contained DMAdda, and ADMAdda microcystins were totally absent (Table S1). These microcystins were used as an acceptor together with acetyl-CoA as a donor in an in vitro acetylation assay. We used 100 mM Tris (pH 8.3), 0.02 mM acetyl-CoA (Sigma-Aldrich), 2 μl BSA, 10 μl purified McyL, 10 μl microcystin extract in a final reaction volume of 100 μl. Reactions were performed in 300 μl clear glass vials. The enzymatic reaction was initiated by adding purified McyL enzyme and allowed to proceed for 30 min at 25°C. We also used n-propionyl-CoA (Sigma-Aldrich) and n-butyryl-CoA (Sigma-Aldrich) in order to investigate the acceptor of the McyL enzyme. Deacetylation experiments were performed as the *O*-acylation experiments using CoA as the acyl group acceptor. All liquid chromatography (LC)-mass spectrometry work was performed as described previously using positive and negative ionization and differing LC gradient profiles (Kaasalainen et al., 2009).

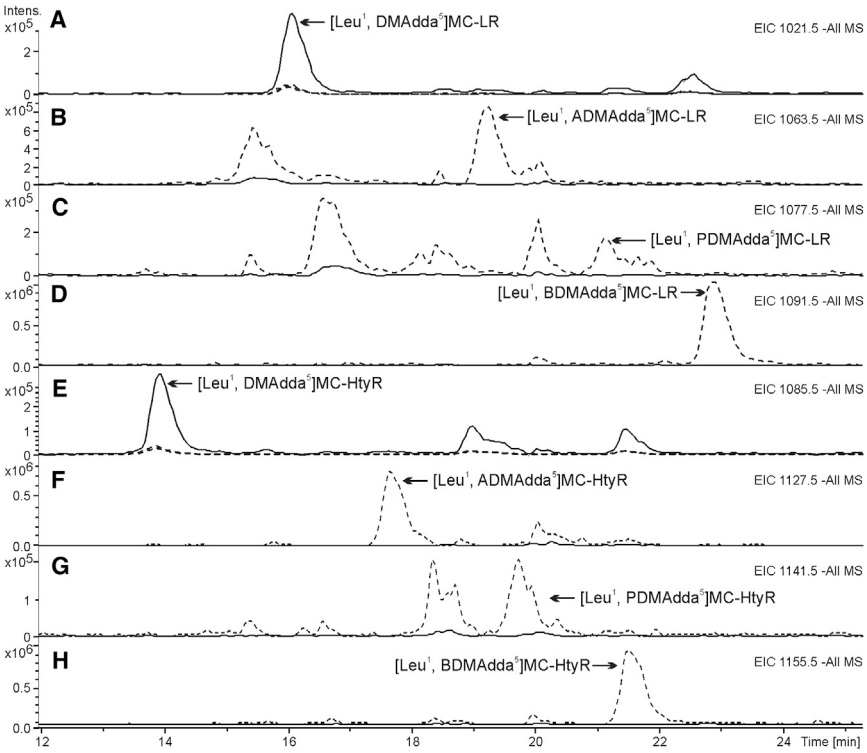

Figure 5. The *O*-Acetylation, *O*-Propionylation, and *O*-Butyrylation of Two DMAdda-Containing Microcystin Variants

Negative ion chromatograms with solid lines before and with dashed lines after the reaction.

(A) Extracted ion chromatograms of [Leu^1, DMAdda^5] MC-LR microcystin, which was used as substrate for McyL.

(B–D) Extracted ion chromatograms of the corresponding *O*-acetylated, *O*-propionylated, and *O*-butyrylated microcystin derivatives formed from the McyL-catalyzed conversion of [Leu^1, DMAdda^5] MC-LR.

(E) Extracted ion chromatograms of [Leu^1, DMAdda^5] MC-HtyR microcystin, which was used as substrate for McyL.

(F–H) Extracted ion chromatograms of the corresponding *O*-acetylated, *O*-propionylated, and *O*-butyrylated microcystin derivatives formed from the McyL-catalyzed conversion of [Leu^1, DMAdda^5] MC-HtyR.

Phylogeny

Sequence analysis suggested that the McyL protein belongs to the xenobiotic acetyltransferase family of enzymes that inactivate a variety of antibiotics by coenzyme-A-dependent *O*-acetylation. The evolutionary origins of the McyL enzyme were examined by constructing an amino acid alignment of XAT acetyltransferases from the genomes of a range of bacteria using conserved motifs to guide the alignment. Ambiguous regions and gaps were excluded, and 155 positions were subjected to phylogenetic analysis. A maximum-likelihood tree was constructed using ProtML using the Jones-Taylor-Thornton (JTT-F) model of amino acid substitution and ten random sequence addition searches with global rearrangements (Felsenstein, 1993). The phylogenetic tree was midpoint-rooted using the RETREE program. One thousand likelihood bootstrap replicates were performed under a JTT-F and uniform rate model with five random sequence additions per replicate and global rearrangements using SEQBOOT and PROTML.

ACCESSION NUMBERS

The GenBank accession number for the entire sequence of the gene cluster for microcystin production in *Nostoc* sp. 152 reported in this paper is KC699835.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2013.04.020.

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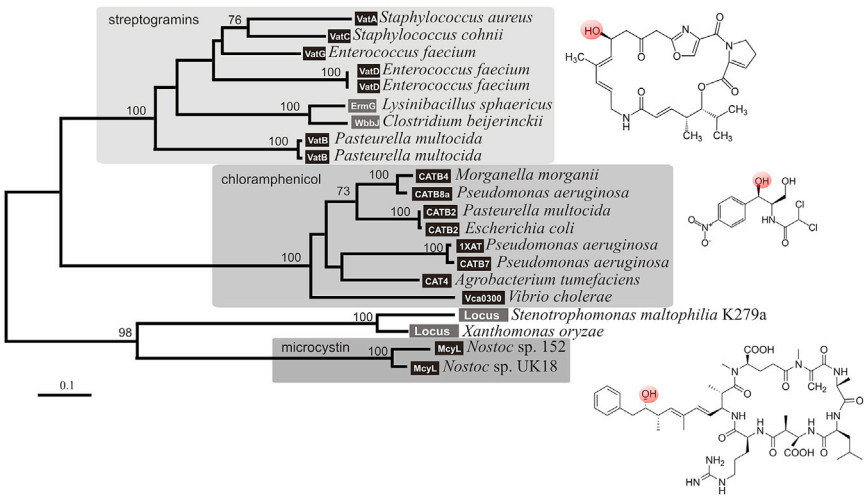

Figure 6. A Maximum-Likelihood Tree of *O*-Acetyltransferases Showing the Phylogenetic Position of McyL

The phylogenetic tree encompasses putative *O*-acetyltransferases from the genomes of a broad range of bacteria as well as enzymes shown to catalyze the CoA-dependent transfer of acetyl to the chloramphenicol and streptogramin A antibiotics. Bootstrap values derived from 1,000 replicates are given at the node. The scale at the bottom of the tree corresponds to 0.1% divergence between sequences.

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