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Ribosomal Synthesis of Tricyclic Depsipeptides in Bloom-Forming Cyanobacteria**

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In memory of Peter Welzel

Various types of planctonic cyanobacteria such as *Microcystis* frequently form toxic blooms in freshwater lakes (Figure 1 a), leading to a dramatic decrease of biodiversity in these ecosystems.[1,2] Some of the most remarkable cyanobacterial toxins belong to the microviridin family of tricyclic depsipeptides (peptides bearing ester bonds).[3–7] All microviridins feature an unparalleled cage-like architecture and specifically inhibit various proteases. Microviridin J (**2**) affects the molting process of *Daphnia* (Figure 1 c) and is discussed as a feeding deterrent that may partly explain the enormous success of *Microcystis* species in the field (Figure 1 b).[8] On the other hand, microviridin B (**1**) has high therapeutic potential as an elastase inhibitor in the treatment of lung emphysema.[4,5] Thus, knowledge of the molecular basis of microviridin biosynthesis would not only allow monitoring of environment-threatening cyanobacterial blooms but also open the door to engineering valuable new therapeutics. Herein we report that microviridins are synthesized from a ribosomal precursor peptide in *Microcystis* by a unique pathway involving stand-alone ATP-grasp-type ligases for ω-ester and ω-amide bond formation, as well as a specialized transporter peptidase.

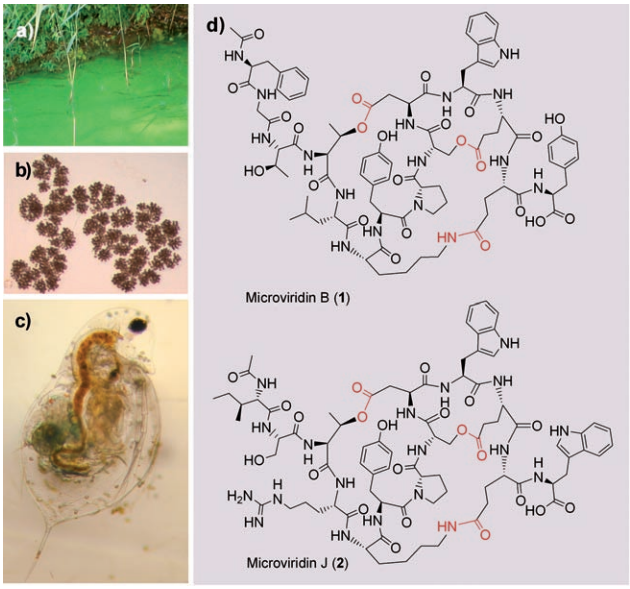

Figure 1. a) Cyanobacterial bloom. b) *Microcystis* colony. c) Daphnia subject to molting disruptant. d) Structures of tricyclic protease inhibitors microviridin B (**1**) and microviridin J (**2**) with ester and ω-amide bonds highlighted in red.

The tricyclic architecture of the microviridins results from intramolecular ω-ester and ω-amide bonds (Figure 1 d). Depsipeptides are generally synthesized by nonribosomal peptide synthetase (NRPS) assembly lines,[9,10] as in the cyanobacterial cyanopeptolin biosynthetic pathway.[11] However, screening microviridin producers for NRPS genes has not yielded candidate genes for microviridin biosynthesis. Because microviridins are solely composed of proteinogenic L-α-amino acids, we hypothesized that they derive from a bacteriocin-like ribosomal biosynthesis,[12] as seen for patellamide[13,14] and related cyanobacterial peptides.[15–17]

By bioinformatic analysis of fully sequenced cyanobacterial genomes for ribosomal prepeptides (http://bioinformatics.biol.rug.nl/websoftware/bagel), we detected a putative peptide precursor gene in strain *Anabaena* PCC7120. The deduced gene product contains three copies of the KYPSD core motif that is characteristic for microviridins (Figure 2). However, no microviridin-like peptides could be detected in extracts of this strain, indicating that this gene locus could be silent. We investigated the flanking regions of the putative microviridin precursor gene in *Anabaena* PCC7120 to identify key features associated with the prepeptide gene that could facilitate the search for corresponding gene clusters in *Microcystis*. We noted two genes in the immediate proximity

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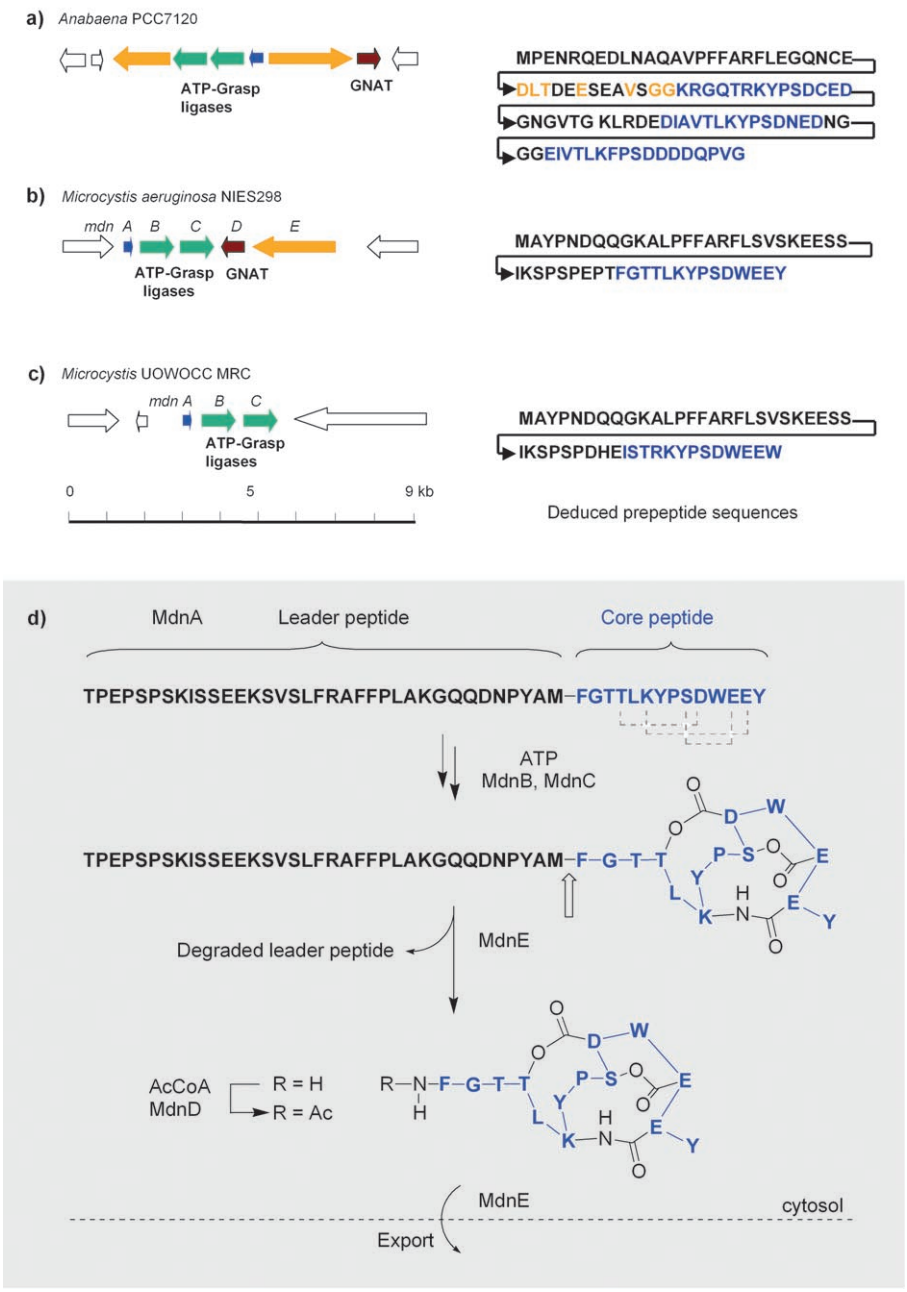

Figure 2. Molecular basis of microviridin biosynthesis and biosynthetic model. Organization of a) a cryptic *Anabaena* PCC7120 gene cluster b) microviridin B (*mdn*) biosynthesis gene cluster from *Microcystis aeruginosa* NIES298, c) microviridin B (*mdn*) biosynthesis gene cluster from *Microcystis* UOWOCC MRC (left), and deduced amino acid sequences of prepeptides (right). Orange: deduced consensus processing site of leader peptide and its putative double-glycine cleavage site; blue: predicted microviridin core regions. Colored arrows indicate corresponding microviridin B pathway genes in different bacteria. d) Model for microviridin biosynthesis involving the cyclization of prepeptide MdnA by grasp ligases MdnB and MdnC prior to cleavage by transporter peptidase MdnD and *N*-acetylation by MdnE (GNAT: GCN5-related *N*-acyltransferase).

that code for as yet uncharacterized enzymes. According to protein motif analyses (http://myhits.isb-sib.ch/cgi-bin/motif_scan), both enzymes show ATP grasp ligase signatures. The diverse family of ATP grasp ligases comprises enzymes such as D-alanine-alanine ligase and glutathione synthetase that catalyze ATP-dependent condensations.[18] We considered the two putative ATP grasp ligases as excellent candidates for microviridin cyclization reactions and employed the putative ligase gene all7011 as a probe for screening genomic DNA fosmid libraries of the microviridin B (**1**) and J (**2**) producing strains *M. aeruginosa* NIES298 and *Microcystis* UOWOCC MRC, respectively. Using this strategy, we have successfully located, cloned, and fully sequenced the putative microviridin (*mdn*) biosynthesis gene loci in both strains (see Figure 2, and Table S1 in the Supporting Information).

The microviridin B biosynthesis gene cluster from *M. aeruginosa* NIES298 consists of two genes encoding ATP-grasp-type ligases (MdnB and MdnC) and a gene that codes for a putative peptide precursor (MdnA). The deduced amino acid sequence at the C terminus (FGTTLKYPSDWEEY) matches precisely with the composition of microviridin B (**1**) produced by this strain. Moreover, the cluster contains two further genes that likely code for an ABC transporter (MdnE) and an *N*-acetyltransferase of the GNAT family (MdnD).[19] Since microviridins are commonly acetylated at the N terminus, this finding is in exact agreement with the microviridin structure. Analysis of the microviridin J biosynthesis gene cluster in *Microcystis* UOWOCC MRC revealed the presence of ligase (*mdnB*, *mdnC*) and prepeptide genes (*mdnA*) only (Figure 2). The deduced microviridin J prepeptide sequence (MdnA) is highly similar to the microviridin B precursor except for the C terminus (ISTRKYPSDWEEW), which is characteristic for microviridin J (**2**). It may be noted that the gene cluster isolated from *Microcystis* UOWOCC MRC neither harbors *N*-acetyltransferase nor ABC transporter genes. The two enzymes are likely encoded elsewhere in the genome.

To verify the involvement of the *mdn* gene clusters in microviridin production, we attempted their heterologous expression in *E. coli*. It should be noted that there is to date only a single case of successful production of a cyanobacterial metabolite in a heterologous host described in the literature.[13,14] After initial failures, we succeeded in the heterologous production of microviridin B (**1**) using the fosmid

vectors derived from *Microcystis* NIES298, although only under optimized conditions (see the Supporting Information). The resulting cell extracts were analyzed by reversed-phase HPLC using *E. coli* cells containing the empty vector as a control. Comparison of the metabolic profiles clearly revealed three additional peaks in extracts of cells expressing *mdn* genes (Figure 3a). One of the peaks (**1**) matched the retention time and exact mass of an authentic microviridin B reference isolated from the *Microcystis* cells. The identity of microviridin B and in particular the presence of the correct cyclizations was corroborated with MALDI-TOF spectrometry and NMR spectroscopy (Figures S1–S3, and Table S1 in the Supporting Information). Two further microviridin B variants (**3**, **4**) with higher molecular masses were identified using the MALDI post-source decay (PSD) technique. The fragment pattern clearly indicated that both metabolites derive from the microviridin B peptide precursor and feature the correct ester and amide bonds between side chains (Figures S3 and S4 in the Supporting Information). However, peptides **3** and **4** were not fully processed and contained one and five additional amino acids, respectively, at their N termini.

In a similar approach, microviridin J production in *E. coli* cells expressing *mdn* genes was monitored by HPLC. The comparative analysis with a negative control indicated the production of microviridin-type peptides (**5**–**7**, Figure 3b) and MALDI-TOF and MALDI-PSD spectrometry demonstrated that all peptides are derived from the microviridin J (**2**) precursor and have the correct ester and amide linkages. However, none of the variants was correctly processed: all were either longer (**6**, **7**) or shorter (**5**) at their N termini.

The successful heterologous production of microviridin B in *E. coli* indicates that the cloned *mdn* genes from *M. aeruginosa* NIES298 are sufficent for the processing, cyclization, and *N*-acetylation of microviridin. Although the processing is correctly operating, this seems to be a limiting step for high-yield production of microviridin B in *E. coli*. The most probable candidate for peptide processing is the putative ABC transporter MdnE. The closely related transporter encoded by the cryptic microviridin cluster of *Anabaena* PCC7120 (Alr7014, Figure 2) indeed shows the characteristic signatures of C39-type peptidases (Figure 2a).[20] A dual transporter peptidase function has also been discussed for bacteriocins[21] and the related peptide marinostatin.[22] However, in all cases analyzed previously, the corresponding leader peptides contained a highly conserved double glycine motif, while in *Microcystis*, neither the leader peptide nor the transporter show such characteristic markers for peptide processing. Even so, we found that microviridin is only correctly processed in *E. coli* expressing the entire set of NIES298 *mdnABCDE* genes, and in the absence of *mdnE* only misprocessed variants are observed.

It is worth mentioning that the heterologous production of microviridins (**1** 20, **3** 78, **4** 60, **5** 280, **6** 460, 7 280 $\mu g L^{-1}$) in *E. coli* is at the same range as the wild type titers (**1** 110, **2** 800 $\mu g L^{-1}$); however, *E. coli* grows about 100 times faster than the cyanobacterial strains.

To determine the essential enzymes for microviridin cyclization, we reconstituted microviridin formation in vivo. For this purpose we cloned a *mdnABCD* gene cassette and generated mutants lacking individual genes. Heterologous production of the leader peptide MdnA in *E. coli* mutants in the absence of the ATP grasp ligases could not be observed, possibly owing to rapid proteolysis of the leader peptide. Only the co-expression of *mdnA* with the ligase genes *mdnB* and *mdnC* yielded three correctly cyclized variants of microviridin B that only differ in the number of additional *N*-terminal amino acids (**8**–**10**, see Figure S9 in the Supporting Information). These mutational studies as well as the successful production of correctly cyclized microviridins

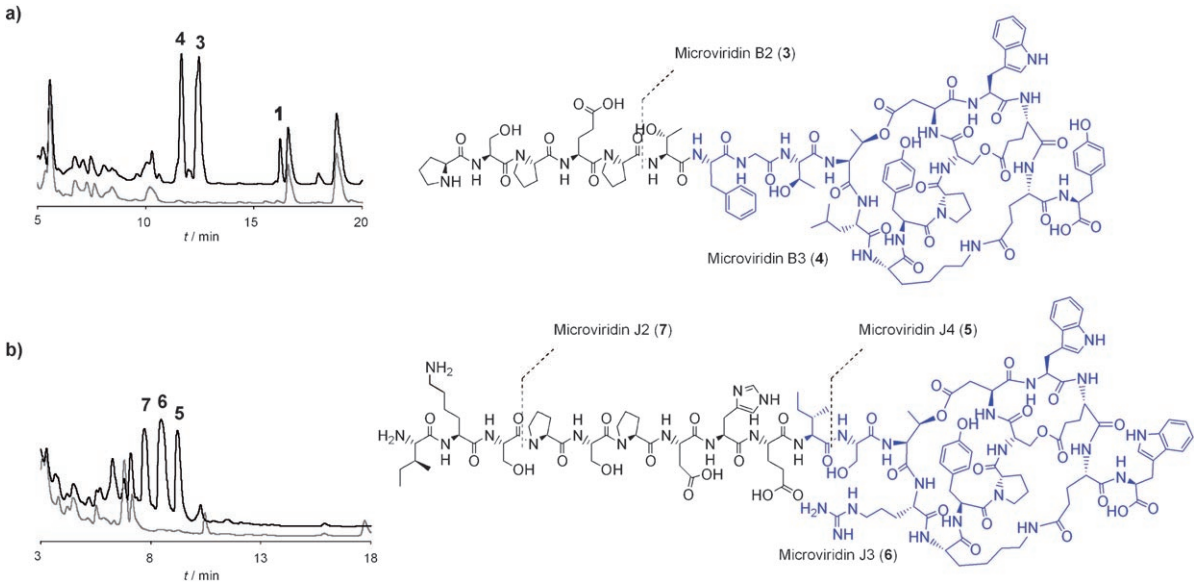

Figure 3. HPLC profiles and structures of microviridins produced by heterologous expression of microviridin B pathway genes *mdnABCDE* (a) and microviridin J pathway genes *mdnABC* (b) in *E. coli*. Extracts from expression strains: black trace; *E. coli* with empty vectors as negative control: gray trace. The microviridin B and J core regions are highlighted in blue.

through expression of the minimal *mdnABCD* cassette in *E. coli* firmly establish that MdnB and MdnC are essential for the formation of three ω-ester and ω-amide bonds. Furthermore, the identification of correctly cyclized microviridin homologues with extended N termini in *E. coli* cells expressing *mdnABCD* provides strong evidence that the putative ATP grasp ligases MdnB and MdnC cyclize the intact, full-length leader peptide prior to cleavage. Future biochemical studies will reveal the specific requirements of MdnB and MdnC and the timing of ring closures.

In summary, we have identified, cloned, and sequenced two unprecedented cyanobacterial gene clusters that code for the biosynthesis of ecologically and therapeutically important selective protease inhibitors, microviridin B (**1**) and J (**2**). The successful heterologous production of these tricyclic depsipeptides in *E. coli* demonstrates for the first time that microviridins derive from a ribosomal peptide precursor. Reconstitution of the pathway in vivo and identification of microviridin variants (**3**–**11**) shows that the leader peptide is processed by a transporter peptidase and cyclized by two cyclases related to ATP grasp ligases. The utilization of these novel biocatalysts for depsipeptide formation from ribosomally produced peptides represents a new addition to the various known natural strategies for cyclopeptide biosynthesis[23–25] and sets the ground for engineering microviridin variants.

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