Functional Analysis of Environmental DNA-Derived Microviridins Provides New Insights into the Diversity of the Tricyclic Peptide Family

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Microviridins represent a unique family of ribosomally synthesized cage-like depsipeptides from cyanobacteria with potent protease-inhibitory activities. The natural diversity of these peptides is largely unexplored. Here, we describe two methodologies that were developed to functionally characterize cryptic microviridin gene clusters from metagenomic DNA. Environmental samples were collected and enriched from cyanobacterial freshwater blooms of different geographical origins containing predominantly *Microcystis* sp. Microviridins were produced either directly from fosmid clones or after insertion of environmental DNA-derived gene cassettes into a minimal expression platform in *Escherichia coli*. Three novel microviridin variants were isolated and tested against different serine-type proteases. The comparison of the bioactivity profiles of the new congeners allows deduction of further structure-function relationships for microviridins. Moreover, this study provides new insights into microviridin processing and gene cluster organization.

Ribosomally produced and posttranslationally modified peptides (RiPPs) are increasingly recognized as a pharmaceutically promising and structurally versatile source of natural products (1). Cyanobacteria are an exceptionally rich source of such peptides, with a surprisingly wide range of structural features and bioactivities (2–6). One of the unique RiPP families described for the cyanobacterial phylum is the group of microviridins (5, 6). These tricyclic peptides possess an unprecedented cage-like architecture and contain noncanonical lactone and lactam rings (7). Until a biosynthetic pathway was assigned to the peptides in 2008 (5, 6), 10 microviridin variants were discovered through bioactivity-guided screening for protease-inhibitory compounds in cyanobacteria (7–9). Individual microviridin variants show distinct specificities for elastase, chymotrypsin, and trypsin, thereby revealing potential for the treatment of diseases such as lung emphysema, cystic fibrosis, or infections (7–10).

The discovery of the microviridin pathway in the bloom-forming freshwater cyanobacterium *Microcystis* provided an avenue for the heterologous expression of microviridins in *Escherichia coli* (6). Three posttranslational modification enzymes (PTMs) and an ABC transporter (MdnB to MdnE) are needed to modify and process the microviridin prepeptide MdnA in *E. coli* (6). Microviridins were successfully produced by using either a fosmid-based system or a minimal expression platform (6, 11). A PCR survey for microviridin biosynthesis genes in laboratory strains of *Microcystis* and in environmental DNA (eDNA) revealed a widespread occurrence of this pathway (12). Moreover, each of the strains analyzed encoded a novel variant of the 12- to 14-membered peptides (12). From this analysis, one could anticipate a large and unexplored library of natural microviridins in cyanobacterial field strains and culture collections with an as-yet-unknown range of bioactivities.

Whereas genomic mining of natural products is relatively well established for different groups of bacteria and fungi, there are only a few studies using metagenomic libraries for the discovery of biosynthetic genes or the production of small molecules from un-

cultured strains (13, 14). Natural products isolated from eDNA by functional screening include antibacterials and pigments, such as *N*-acylated amino acids, violacein, or indigo (13). More frequently, metagenomic techniques have been used for homology-based screening for specific gene fragments. Pioneering examples are type II ketosynthase genes that belong to the minimal set of genes in aromatic polyketide synthases. The environmental gene cassettes were used to generate novel hybrid polyketides in *Streptomyces* (15). Other studies were aimed at identifying new derivatives of known substances, such as vancomycin-type and teicoplanin-type glycopeptide antibiotics (16). The relatively small size of RiPP gene clusters makes them attractive for heterologous expression from large-insert clone libraries. Indeed, one of the RiPP families, the cyanobactins, was first assigned to a ribosomal pathway after screening of clones of a metagenomic library from a *Prochloron*-ascidian symbiosis consortium (17).

The present study aimed to establish protocols for metagenomic mining of microviridins. As microviridins from the bloom-forming freshwater cyanobacterium *Microcystis* have been successfully produced in *E. coli* and because of the widespread occurrence of the genus in the field, we considered it to be the lowest-hanging fruit for the establishment of an approach to metagenomic mining of microviridins from eDNA. *Microcystis* is a unicellular cyanobacterium that forms large colonies that can reach diameters typically exceeding 100 μ m (larger colonies are in the low-millimeter range). In summer, *Microcystis* eventually forms a thick scum at

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the surface of lakes, where it often is the dominant phytoplankton type. These features predestine the genus for a metagenomic mining approach. *Microcystis* can be enriched by using nets with an appropriate mesh size and can be separated from planktonic bacteria and smaller picoplankton species.

We have evaluated two heterologous expression approaches for a number of laboratory strains representing various cyanobacterial genera. Functional analysis of the newly identified microviridin congeners revealed new bioactive variants and provides insights into target specificity and processing of microviridins.

MATERIALS AND METHODS

Strains and metagenomes. *Microcystis* blooms were collected in Lake Wannsee by using a Hydro-Bios Kiel net (55- μ m mesh) in July 2010 and in August 2011. Further eDNAs from Volga River, Solonchik River, and Nero Lake, were kindly provided by Sergey I. Sidelev from Demidov State University, Yaroslavl, Russia. *E. coli* strains (Top10, Epi300T1, and XL1-Blue) were obtained from Invitrogen, Epicentre, and Stratagene/Agilent Technologies, respectively.

Isolation of environmental metagenomic DNA. Fifty milliliters of an enriched environmental net sample was centrifuged for 10 min, and the cells were washed in 15 ml Tris-EDTA (TE) buffer, resuspended in 3.5 ml TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer, and further incubated for 1 h at 4°C. Lysozyme was added to 200 μ g/ml, and the mixture was incubated for an additional 1 h at 37°C. Proteinase K was added to a final concentration of 50 μ g/ml, and subsequently, SDS was added to 2% (vol/vol). The suspension was incubated for 1 h at 50°C or until the mixture became clear. The DNA preparation mixture was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1) solutions. DNA present in the aqueous layer was precipitated with 2.5% (vol/vol) cold (−20°C) 96% ethanol, washed with cold 70% ethanol, air dried, resuspended in 500 μ l double-distilled water (ddH₂O), and incubated at 37°C until the genomic DNA completely solubilized. Finally, 2 μ l of RNase A was added, and the DNA was incubated at 37°C for 1 h.

Alternatively, metagenomic DNA was isolated by using a metagenomic DNA isolation kit for water (Epicentre Biotechnologies). The manufacturer's protocol was adapted as follows: 50 ml of environmental sample was centrifuged (4,000 $\times$ g for 10 min), and the pellet was resuspended in 1 to 2 ml of fresh filter wash buffer. Two microliters of Ready-Lyse lysozyme solution and 1 μ l of RNase A were added to the resuspended pellet, and the mixture was mixed and incubated for 30 min at 37°C. Three hundred microliters of Meta-Lysis solution (2 $\times$) and 1 μ l of proteinase K were added to this mixture. The mixture was further incubated for 15 min at 65°C, followed by 5 min on ice. Three hundred fifty microliters of MPC protein precipitation reagent was added to the cell suspension. Cell debris was removed by centrifugation (11,000 $\times$ g for 10 min at 4°C). The supernatant was mixed with isopropanol and centrifuged (11,000 $\times$ g for 10 min at 4°C). The obtained DNA was washed with 500 μ l of ice-cold 70% ethanol and then centrifuged at 4°C for 10 min at 11,000 $\times$ g. DNA was air dried and resuspended in 0.5 to 1.0 ml ddH₂O. To avoid intensive mechanical degradation of the DNA molecules, cut-off edge-rounded pipette tips were used.

Construction of cyanobacterial fosmid libraries. Fosmid libraries from *Microcystis* eDNA were constructed by using the CopyControl fosmid library production kit (Epicentre Biotechnologies) according to the recommendations of the manufacturer.

PCR conditions. Primer pair P216 (5'-GGC GCC ATG AAT TAT CCT AAT AGC GA-3') and P217 (5'-GAC GTC TTA ACT ATC TTC CCA GTC AGA-3') was used to amplify 150 bp of *mdnA* genes by using Dream-Taq polymerase (Fermentas Life Science/Thermo Scientific, St. Leon-Rot, Germany) under the following conditions: an initial denaturation step at 95°C for 3 min followed by 34 cycles of 30 s at 95°C for denaturation, 30 s at 50°C for annealing, and 30 s at 72°C for elongation. A final elongation step at 72°C for 10 min completed the reaction.

Primer-pair pCCFos1_fw (5'-GGATGTGCTGCAAGGCGATTAAG TTGG-3') (fosmid flanking; Epicentre) and Miv05 (5'-GGATCCGATG AAAGAATCGCCTAAAGT-3') (*mdnB*-specific primer) was used to amplify a fragment of unknown length from selected fosmid clones under the following conditions: an initial denaturation step at 95°C for 3 min followed by 30 cycles of 30 s at 95°C for denaturation, 30 s at 45°C for annealing, and 10 min at 72°C for elongation. A final elongation step at 72°C for 10 min completed the reaction.

Cut-and-paste of *mdnA* sequences into the minimal expression platform vector pARW081. The minimal microviridin expression platform vector pARW081 (11) was used to introduce *mdnA* sequences from *Microcystis* fosmid eDNA, sequences identified in the Russian environmental samples, and *orf1* from *Microcystis aeruginosa* PCC7941 (18). PCR products of *mdnA*-like sequences were first cloned into pDrive (Qiagen), excised from there by using AatII and EheI (Fermentas), and cloned into appropriately digested and purified pARW081.

Sequencing. Sequencing was carried out commercially at GATC Biotech AG and in the laboratory of Martin Meixner (SMOLBIO) at Humboldt University, Berlin, Germany.

Heterologous production of microviridins from fosmid and the minimal expression platform. Two hundred milliliters of LB medium supplemented with 12.5 μ g/ml chloramphenicol was inoculated with 1.0 ml of an *E. coli* culture grown overnight, and after achieving bacterial growth to an optical density at 600 nm (OD₆₀₀) of 0.8, the fosmids were induced with induction solution (Epicentre Biotechnologies). In general, *E. coli* was cultured for a further 4 h at 37°C with shaking at 220 rpm. Optimization of the cultivation procedure was performed by using different techniques: (i) long cultivation and lower temperatures (16 to 20 h at room temperature [RT] and 30°C), (ii) direct induction of the culture without precultivating the cells, or (iii) the use of EnBase technology (En-Presso; BioSilta) according to the manufacturer's recommendations.

Cryptic microviridins were produced from the minimal expression platform as follows: 400 ml LB medium supplemented with 100 μ g/ml ampicillin was inoculated with 30 ml of a culture grown overnight. Cultures were grown under standard conditions for 5 to 6 h at 37°C.

Extraction of microviridins from *E. coli*. The produced biomass was harvested by centrifugation, and the pellet was resuspended in 5 ml ddH₂O. Cells were lysed by sonication (10 min, pulse of 3 s every 3 s, and 75% amplitude; Sonoplus). The cellular debris was then removed by centrifugation, and the supernatant was loaded onto a Sep-Pak cartridge (Waters Corporation). Before supernatants were loaded, the cartridges were activated with a quick succession of 2 ml of 100% methanol and ddH₂O. The suspension was loaded onto the cartridge, which was washed once with 2 ml 5% methanol, and microviridins were eluted in 2 ml 100% methanol. The extracts were dried in a vacuum, resuspended in 250 μ l 100% methanol, filtered (Acrodisc 4-mm syringe filter and 0.45- μ m nylon membrane; Pall), and analyzed by high-performance liquid chromatography (HPLC).

HPLC analysis of microviridin. A Shimadzu LC-10AD or LC-20AD series solvent delivery system coupled to a Shimadzu UV-visible (UV-Vis) photodiode array detector was used. Chromatographic separation was carried out on a Symmetry Shield C₁₈ reversed-phase HPLC (RP-HPLC) column (4.6 by 100 mm with a 3.5- μ m particle size; Waters) equipped with a Sentry Guard C₁₈ guard column (4.6 by 20 mm with a 5- μ m particle size; Waters). The mobile phase gradient (solvent A [water plus 0.05% trifluoroacetic acid {TFA}] and solvent B [acetonitrile plus 0.05% TFA]) was linear at a flow rate of 1.0 ml/min from 20% to 52% solvent B in 30 min followed by a linear gradient to 100% solvent B in 1 min, followed by a further 2 min under this condition. After postwashing of the column, the concentration of solvent B returned to the initial point of 20% in 3 min, with holding for an additional 5 min for the reequilibration of the column. Injection volumes varied from 10 to 50 μ l. Detection was performed in UV-Vis mode, and microviridins were detected at 210 nm.

Generation of biotin-labeled probes. The Biotin Decalabel DNA labeling kit (Fermentas) was used to generate the biotinylated probes for

screening of the libraries according to the manufacturer's recommendations. The probe was produced from the amplicons generated by PCR using primer pair Miv05 (5'-GGA TCC GAT GAA AGA ATC GCC TAA AGT-3') and Miv06 (5'-GAA TTC TTA GCA TAC TAA AAA ATC AGC GA-3'). The probe was diluted in Church buffer to a final concentration of 300 ng/ml. Before its application onto the membrane, the probe solution was denatured for 10 min at 95°C and directly chilled on ice.

Screening of the fosmid libraries by colony lift, lysis of cells, and DNA fixation on a nylon membrane. Fosmid libraries were carefully transferred onto positively charged nylon membranes (Hybond-N nylon membrane; Amersham), incubated for about 3 to 4 h at 37°C on a fresh agar plate supplemented with induction solution for fosmids, and subsequently incubated for 1 h at 4°C. Membranes were laid onto Whatman 3MM filter paper soaked with denaturation solution and incubated for 15 min at room temperature. Membranes were put onto another Whatman filter paper wetted with neutralization solution and incubated again for 15 min. This step was repeated once more. The membranes were settled onto a filter paper containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and incubated for 10 min. The DNA material was irreversibly cross-linked onto the nylon membrane by UV irradiation for 10 min. A positive control (PCR product used as the template for the preparation of the probe) was applied onto the membrane prior the cross-link.

Membrane hybridization and detection with the biotin chromogenic system. The membranes were prehybridized in 50 ml Church buffer for 2 h at 60°C. The prehybridization solution (Church buffer) was discarded, and biotin-labeled probe solution was added to the membrane. Hybridization was performed at 56°C to 60°C overnight in a PerfectBlot hybridization oven (PqLab). The membranes were washed at RT in the following series: 15 min with 2× washing solution, 15 min with 1× washing solution, and 15 min with 0.5× washing solution. From this point on, the membranes were treated by using a biotin chromogenic detection kit (Fermentas) according to the instructions of the manufacturer. The membranes were photographed under UV light with the ChemiDoc XRS+ molecular imager (Bio-Rad).

MALDI-TOF MS analysis of microviridins. The polypeptides fractionated by analytical reversed-phase HPLC as mentioned above were measured by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Ultraflex; Bruker Daltonics). The structure elucidation of microviridins by PSD (postsorce decay) analysis was previously described (11). Detected monoisotopic ions, which were predicted from the amino acid sequence of the MdnA precursor peptide, were further applied to PSD measurements. The PSD analysis supported the proposed structures by detection of similar fragmentation patterns among microviridin variants (see Fig. S1 to S3 in the supplemental material) (11). These results and the protease-inhibitory activity of microviridins M and N strongly support the tricyclic structure of the new microviridin variants.

Isolation of microviridins M and N. *E. coli* Epi pFos19 (8 liters) and pFos15 (8 liters) cells cultured at 37°C for 5 h were harvested by centrifugation and lyophilized, respectively. The freeze-dried cells were resuspended in 50 ml of methanol and then lysed by sonication (these processes were repeated twice). After the removal of the solvent by evaporation, residues were subjected to an open ODS column (i.e., packed with octadecylsilyl groups; YMC gel, 3 by 12 cm) and eluted by a stepwise solvent system (H₂O, 10% acetonitrile, 80% acetonitrile, and methanol). The membrane-filtered samples were subjected to RP-HPLC (20- by 250-mm, 5-μm Kromasil C₁₈ column with a flow rate of 12 ml/min) using a gradient system of 20% solvent B for 10 min to 100% solvent B in 40 min (solvent A, 0.1% [vol/vol] TFA in H₂O; solvent B, acetonitrile-H₂O [83:17]) to yield microviridins M (0.4 mg) and N (0.1 mg).

Protease inhibition assays. Serine protease-inhibitory assays, except those for subtilisin, were carried out according to methods described previously (12). The subtilisin-inhibitory assay was performed according to the modified method of Taichi et al. (19). Thirty microliters of 25 mM phosphate-buffered saline (PBS) buffer (pH 7.0), 50 μl of subtilisin Carls-

berg (10 μg/ml in 25 mM PBS buffer) (catalog number P-8038; Sigma-Aldrich), and 20 μl of sample solution were mixed in a well (96-well plate) and then preincubated at 37°C for 5 min. One hundred microliters of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (0.1 mg/ml in 25 mM PBS buffer) (catalog number S-7388; Sigma-Aldrich) was added to this solution, and enzyme activity was measured at 410 nm after 30 min of incubation at 37°C.

Nucleotide sequence accession numbers. eDNA-derived *mdnA* sequences were submitted to the NCBI database and are available under accession numbers [KF742383](#) to [KF742395](#).

RESULTS

***mdnA* sequences derived from *Microcystis* eDNA.** eDNA from enriched *Microcystis* field samples of Lake Wannsee collected in two consecutive years was used to construct fosmid libraries. As part of the optimization process, two different DNA isolation techniques that differed in their efficiency in cyanobacterial eDNA extraction were compared (Fig. 1) (for details, see Materials and Methods). High-molecular-weight DNA was subsequently used for library production. The estimated amounts of eDNA obtained in the two fosmid libraries were 4.4×10^3 and 3.4×10^2 Mbp (see Table S1 in the supplemental material). Screening of 2,000 clones of both libraries using a combination of hybridization and PCR for the presence of the microviridin amide ligase gene *mdnB* yielded 6 positive clones (Fig. 1 and Table 1). A second PCR targeting the microviridin precursor gene *mdnA* of *Microcystis* also yielded six positive clones; however, only three clones comprised both the *mdnA* and the *mdnB* sequences (Table 1). The *mdnA* gene fragments were sequenced and compared to known microviridin precursor genes. Three microviridin precursor types that differed from the previously characterized microviridin types A to L were identified (Fig. 2). One of the precursor sequences encoding the microviridin type YGGTFKYPSPDWEEY was detected in four different clones, suggesting that the variant was predominant in the Lake Wannsee *Microcystis* consortium in 2010 and 2011 (Fig. 2). Two other clones encoded the microviridin subtypes YSTLKYPSPDWEEY and YNVTLYKYPSPDWEFF. In order to expand the biogeographical diversity of *Microcystis*, we further included eDNA from two Russian water bodies in our survey, namely, Lake Nero and Volga River. As *Microcystis* was only subdominant in the cyanobacterial consortia and due to the limited amount of eDNA, a standard PCR clone library approach was used to assess the *mdnA* sequence diversity in these two samples. Interestingly, the diversity of *mdnA* sequences obtained from the Russian samples was low and yielded only one new microviridin subtype (VWTWKYPSPDWEDS) (Fig. 2). This might be due to the fact that *Microcystis* DNA was generally underrepresented in the samples. Interestingly, Russian MdnA prepeptides differ from most of the previously analyzed sequences in the C-terminal parts of their leader peptides, which are longer than those in other *Microcystis* strains and extremely rich in prolines (Fig. 2).

Production of microviridins from *Microcystis* eDNA. (i) **The fosmid approach.** All *mdnA*- or *mdnB*-positive fosmid clones were subjected to HPLC analysis. One of the nine clones encoding the microviridin type YNVTLYKYPSPDWEFF (pFos19) showed an additional peak (Fig. 3). The sequence information for the MdnA prepeptide was used to confirm the production of a tricyclic but not *N*-acetylated microviridin variant exactly matching the sequence identified via MALDI-TOF/TOF mass spectrometry (Fig. 3; see also Fig. S1 in the supplemental material). Cyclization of the tricyclic core was assessed via PSD spectral analysis. The technique

FIG 1 Experimental flow for *Microcystis* sample enrichment, DNA isolation, fosmid library production, and screening.

was originally developed for heterologously produced microviridin B and confirmed by ^1H nuclear magnetic resonance (NMR) (6). The newly produced microviridin was designated microviridin M and represents the first microviridin successfully produced from eDNA (Fig. 3). Microviridin M was shown to be a moderate inhibitor of the protease activity of elastase and subtilisin (50% inhibitory concentration [IC_{50}] values of 2.9 and 6.9 μM , respectively) (Table 2) and to be inactive against trypsin and chymotrypsin (IC_{50} of $>34 \mu\text{M}$). None of the other *mdnA*- or *mdnB*-carrying fosmids produced microviridin.

One of the problems associated with the heterologous production of microviridins in *E. coli* is the accumulation of falsely processed variants that contain additional amino acids at the N ter-

minus. The Fos19 clone, however, predominantly produced the correctly processed variant. A closer inspection of the *mdnA* precursor sequence revealed differences in the two C-terminal amino acids of the leader peptide (PT→RE) (Fig. 2) that may improve processing efficiency in *E. coli*. The heterologous expression approach with eDNA-derived fosmids thus not only can yield a new and highly active variant but also provides further lessons for the improvement of the efficiency of production of microviridins in *E. coli*.

(ii) **The minimal platform approach.** Although the direct production of microviridins from fosmids was generally successful, we aimed at further improving the production success rate. We therefore established a second technique for the production of microviridins from eDNA. Our recently constructed minimal microviridin expression platform (11) was modified to allow the introduction of *mdnA* cassettes from eDNA by a simple cut-and-paste technique (Fig. 4A). The *mdnA* gene cassette encoding the unusual microviridin variant YSTLKYPDWEEY (pFos15) (Fig. 2) was selected to prove the principle. Introduction of the gene in the minimal expression platform resulted in a number of new peaks in our HPLC analysis (Fig. 4B). Mass spectrometry revealed the production of a correctly processed tricyclic and *N*-acetylated microviridin variant along with falsely processed and bicyclic variants (Fig. 4B; see also Fig. S2 in the supplemental material). The new microviridin type was designated microviridin N. The yield of production was low due to the division into several completely and incompletely processed microviridin variants (here named microviridins N1 to N5). Bioactivity assays could be performed only for the acetylated (Ac) bicyclic microviridin N3 type (Ac-YS TLKYPDWEEY), which was isolated in the largest amounts. In comparison with other microviridins, the bicyclic field microviridin derivative only moderately inhibits the enzymatic activity of

TABLE 1 Characterization of fosmids from the metagenomic library of a *Microcystis* species bloom collected in Lake Wannsee

Yr	Fosmid	PCR screening result for presence of:		Length of fosmid-end PCR product (kb) ^a
		<i>mdnA</i>	<i>mdnB</i>	
2010	pFos10	+	+	~300
	pFos11	—	+	ND
	pFos12	+	—	ND
	pFos13	+	—	ND
	pFos14	—	+	~1,400
	pFos15	+	+	~400
2011	pFos17	+	—	ND
	pFos18	—	+	ND
	pFos19	+	+	ND

^a For fosmid-end PCR, fosmid-flanking primers were combined with an *mdnB*-specific primer. ND, no specific PCR product detected.

FIG 2 Sequence diversity and sequence logos of eDNA-derived microviridin prepeptide sequences. The PFFARFL region of the leader peptide that is essential for posttranslational modifications and the conserved core peptide region are highlighted by white and gray boxes, respectively. The *MdnA* prepeptide sequences for microviridin L-producing strain NIES843 and microviridin B-producing strain NIES298 are shown for comparison. pFos sequences were sequenced from fosmid clones. pRus sequences were obtained from an *mdnA* clone library from Russian samples.

chymotrypsin and trypsin (IC_{50} of $>30 \mu M$), whereas its inhibitory potential against elastase and subtilisin was at least 10-fold higher (IC_{50} s of 2.5 and 2.0 μM , respectively). We next evaluated whether the same technique can be applied to *mdnA* fragments from the Russian eDNA library. One representative *mdnA* fragment encoding the microviridin VWTWKWPSDWEDS was selected and inserted into the microviridin expression platform. Again, HPLC analysis of the respective clone revealed a number of additional peaks. Mass spectrometric analysis revealed that the dominant variants were only bicyclic and not correctly processed. The tryptophan residue at position 2 may hamper microviridin processing and cyclization in *E. coli*. We did, however, detect some minor tricyclic variants of the peptide (see Fig. S3 in the supple-

mental material). The new peptide was designated microviridin O. The yield of production was too low for bioactivity analysis. Taken together, these examples show that production of microviridins from *Microcystis* eDNA either by a fosmid-based technique or on a minimal expression platform is generally possible. The efficiency of production and processing significantly varies depending on the sequences of both the core peptide and the leader peptide part of the prepeptide.

Analysis of fosmid clones containing *mdnB* but lacking *mdnA*. An unexpected finding of this study was the frequent occurrence of fosmid clones lacking either *mdnA* or *mdnB* (Table 1). This could be due to the fact that some of the fosmid clones contain only partial *mdn* gene clusters at the end of fosmid inserts. To

FIG 3 (A and B) HPLC (A) and mass spectrometric (B) analyses of pFos19 producing the novel microviridin variant microviridin M. (C) Structure of microviridin M. The β , γ -ester and ϵ -amide bonds between amino acid side chains are highlighted in gray. For further analytical confirmation, see Fig. S1 in the supplemental material. mAU, milli-absorbance units; u.a., arbitrary units.

TABLE 2 Bioactivity profiles of newly characterized microviridins M and N3 compared to those of previously analyzed microviridin types B and L

Microviridin type	Sequence	IC ₅₀ (μM)				Reference
		Elastase	Chymotrypsin	Trypsin	Subtilisin	
B	Ac-FGTTLKYPDSWEEY	2.6×10^{-2}	1.5	34	ND	9
L	Ac-GGTFKYPSDWEDY	>58	42	58	8.7	11
M	VTLYKYPDSWEEF	2.9	>34	>34	6.9	This study
N3 ^a	Ac-YSTLYKYPDSWEEY	2.5	>30	>30	2.0	This study

^a Bicyclic derivative.

ascertain the physical localization of the *mdnB* gene, a PCR approach combining a fosmid-flanking primer with an *mdnB*-specific primer was developed. Indeed, in three cases, *mdnB* was localized toward the end of a fosmid insert (at distances of 300, 400, and 1,400 bp) (Table 1), thus explaining the physical absence of *mdnA*. An alternative explanation for the lack of *mdnA* in *mdnB*-positive clones could be an *in trans* localization of *mdnA* at a different position in the genome. To get an overview of the physical organization of *mdn* gene clusters in *Microcystis*, we assessed the 14 available complete *Microcystis* genome sequences in the NCBI database (18). To our surprise, the *mdnA* gene was absent in three strains containing the *mdnB* to *mdnE* gene cluster (Fig. 5). In

only one of the three cases was the *mdnA* gene localized at a different position in the genome (PCC7806) (Fig. 5); in two other strains, the *mdnA* gene was completely lacking. This raised the question of whether *Microcystis* genomes frequently carry truncated nonfunctional *mdn* gene clusters or whether the Mdn proteins can collaborate with an alternative precursor. A closer inspection of the *mdn* locus revealed the presence of a novel uncharacterized open reading frame (*orf1*) replacing the *mdnA* position in the three strains (Fig. 5). One strain, *M. aeruginosa* PCC9808, contained both *orf1* and *mdnA* upstream of the *mdnB* to *mdnE* gene cluster. *Orf1* encodes a protein of 73 amino acids (aa) sharing neither the characteristic PFARFFL motif in the

FIG 4 (A) Experimental flow for the production of eDNA-derived microviridins via the minimal expression platform. (B) HPLC profile of the pFos15-derived clone showing the production of differently processed microviridin N variants. (C) Structure of microviridins N1 to N5. For mass spectrometric confirmation, see Fig. S2 in the supplemental material.

FIG 5 (A) Schematic representation of cryptic microviridin gene clusters lacking the precursor gene *mdnA* but encoding the uncharacterized Orf1 at a similar position. (B) Comparison of Orf1 sequences from four laboratory strains and a metagenomic fosmid clone.

leader peptide of MdnA nor the TXKYPSD core sequence of the microviridin core peptide (Fig. 5). The Orf1 protein sequence does not show similarity to characterized proteins in the database. Nevertheless, the C-terminal part of the peptide contains glutamate, threonine, serine, and lysine residues, thus not excluding the possibility that Orf1 could represent an alternative prepeptide for lactone and lactam ring formation via MdnC and MdnB. Next, the fosmid clones were tested for the presence of *orf1*. Indeed, *orf1* was detected upstream of *mdnB* in one of the *mdnA*-lacking fosmids (fosmid 11) (Fig. 5). The fosmid clone was subjected to a careful reanalysis to assess the possibility of the production of an alternative peptide; however, no candidate peak could be detected by HPLC and mass spectrometric analyses. Next, the *orf1* gene cassette was inserted into the minimal expression platform via the cut-and-paste mechanism (Fig. 4A). Subsequent HPLC analysis did now show an additional peptide peak. The heterologous production approach thus could not confirm the hypothesis that Orf1 replaces MdnA as a prepeptide in some of the *Microcystis* strains.

DISCUSSION

The present study exemplifies possibilities and challenges for the metagenomic mining of microviridin-type peptides from bloom-forming *Microcystis*. We have established two different heterolo-

gous production approaches based on entirely different vectors and insert sizes. Our analysis was motivated by the enormous sequence diversity observed for *mdnA* prepeptide sequences in a recent field survey (12). During their long evolutionary history, microviridins have gone through a diversification-and-selection process, the analysis of which can help one to understand the traits and constraints of the microviridin RiPP system. If proteases are the natural targets of microviridins, one can speculate that nature has already optimized highly active variants. Although information on evolutionary diversification can be deduced directly from the sequence, bioactivity assessment ultimately requires the production of metabolites. The two microviridin variants for which a bioactivity profile could be obtained in the present study (tricyclic YNVTLKYPSPDWEED and bicyclic YSTLKYPSPDWEED) show surprisingly similar inhibitory potentials toward the four different serine-type proteases tested (Table 2). Both microviridin types inhibit elastase and subtilisin in low-micromolar concentrations. One can therefore draw some conclusions about the impact of structural features on bioactivity. The lactam ring that differentiates the tricyclic from the bicyclic structure is apparently not essential for bioactivity. The length and amino acid composition of the N-terminal side chain also seem less important for protease specificity. The widespread variant YNVTLKYPSPDWEED is, how-

ever, less active against elastase than some of the above-described variants, e.g., microviridins B and G (Table 2). One may therefore speculate that the microviridins were rather optimized against other serine protease targets in their environmental context. Protease inhibitors seem to play a role in balancing cyanobacterium-herbivore interactions. *Daphnia*, for example, possesses a large number of trypsin- and chymotrypsin-type enzymes in their guts (representing 80% of the catalytic activity) (20).

In the present study, we have analyzed relatively small data sets, i.e., 4,000 fosmid clones and two small *mdnA* clone libraries. Our methodological study nevertheless provides the basis for the analysis of a larger number of eDNA-derived microviridin variants in the future. The combination of one of the two cloning techniques with a screening technique, e.g., based on a colorimetric protease assay, would allow translating the metagenome mining approach into a high-throughput approach. Each of the microviridin production systems used in this study has advantages and disadvantages. The fosmid approach provides information on gene cluster organization. The physical localization of the *mdnA* gene toward the *mdnB* to *mdnE* gene cluster is unchanged. The minimal microviridin production platform, on the other hand, allows analysis of *mdnA* sequences from partial *mdn* gene clusters and from PCR libraries. Although the PCR cloning vector used has significantly higher copy numbers than the fosmid system, the yield of microviridin production was not increased. Instead, microviridins were divided into many incompletely and completely processed variants. This could be due to the fact that protease processing in *E. coli* is slower than cyclic peptide production. Presumably, in cyanobacteria, a specific protease that is not present in *E. coli* is required for leader peptide cleavage. The technique to be selected for any future metagenomic mining of microviridins may thus depend on the individual question to be addressed and the quality of eDNA available.

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