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Post-translational Modification in Microviridin Biosynthesis

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*Cyanobacteria are prolific producers of bioactive natural products that mostly belong to the nonribosomal peptide and polyketide classes. We show here how a linear precursor peptide of microviridin K, a new member of the microviridin class of peptidase inhibitors, is processed to become the mature tricyclic peptidase inhibitor. The microviridin (*mvd*) biosynthetic gene cluster of* P. agardhii *comprises six genes encoding microviridin K, an apparently unexpressed second microviridin, two RimK homologues, an acetyltransferase, and an ABC transporter. We have over-expressed three enzymes of this pathway and have demonstrated their biochemical function in vitro through chemical degradation and mass spectrometry. We show that a prepeptide undergoes post-translational modification through cross-linking by ester and amide bond formation by the RimK homologues MvdD and MvdC, respectively. In silico analysis of the* mvd *gene cluster suggests the potential for widespread occurrence of microviridin-like compounds in a broad range of bacteria.*

Introduction

Products from the nonribosomal peptide synthetase (NRPS) and the polyketide synthase (PKS) pathways dominate the secondary metabolism of the cyanobacteria.[1] *Planktothrix agardhii* NIVA-CYA 126/8 (hereafter referred to as *P. agardhii* CYA126/8) is no exception to this general rule, as the isolation of the [Asp^3]-microcystins-RR and -LR,[2] the aeruginosides 126A and 126B,[3] two new anabaenopeptins, and two new cyanopeptolins has shown.[4]

P. agardhii CYA126/8 is the first example of a cyanobacterium with a reasonably complex secondary metabolism that is reliably amenable to genetic manipulation.[3,5,6] This makes this organism a potentially useful model with which to investigate some of the unique aspects of the biosynthesis of cyanobacterial secondary metabolites. As part of an investigation aimed at the comprehensive analysis of small-molecule peptide natural products in *Planktothrix*, their biosynthesis, and the interactions between the various biosynthetic pathways involved, we set out to establish the chemical structure of the microviridin-type metabolite present in isolate CYA126/8 and to identify its biosynthetic gene cluster. Through a combination of structure determination, reverse genetics, and genomics we have identified the *mvd* gene cluster, which is responsible for microviridin biosynthesis, in *P. agardhii*. We have also established the functions of all but one of the proteins encoded by the *mvd* genes through biochemical analysis in vitro.

Structurally, the microviridins are a family of N-acetylated tri- and tetradecapeptides that are cross-linked through the ω-carboxy groups of glutamate or aspartate residues in positions 2, 3 and 5, respectively. These form an amide bond to the ε-amino group of a conserved lysine in position 9 and ester bonds to the hydroxy groups of the serine and threonine residues at positions 6 and 11, respectively. Such ester and amide bonds through functional groups in the ω-positions in amino acids are common motifs in peptides and depsipeptides biosynthesized by the nonribosomal peptide synthetase (NRPS) route and could indeed be mistaken as hallmarks of this biosynthetic mechanism. Thus, unexpectedly, such crosslinks of ribosomally produced peptides can be introduced through post-translational modifications that are unprecedented, at least in the cyanobacteria.

Microviridins have been reported to be potent inhibitors of chymotrypsin and elastases in vitro and in vivo[7] and are thought to constitute the principal component of the chemical defense against *Daphnia* sp. in the natural habitats of the cyanobacteria.[7] To date they have been isolated and characterized only from the cyanobacteria: specifically the genera *Microcystis*,[8] *Planktothrix*[9] and *Nostoc*.[10] Our data suggest, however, that they might occur more widely than has been appreciated in, for instance, the genus *Anabaena*, but possibly also in the myxobacterium *Sorangium cellulosum* So ce56 and the sphingobacterium *Microscilla marina* ATCC 23134 (Figure S1 in the Supporting Information)

Results and Discussion

We report here how the combination of chemical analysis, in the form of structure determination, and biological techniques, such as reverse genetics and genomics, has led to the identification of the *mvd* gene cluster, which encodes microviridin biosynthesis, in *P. agardhii*. The function of this cluster was

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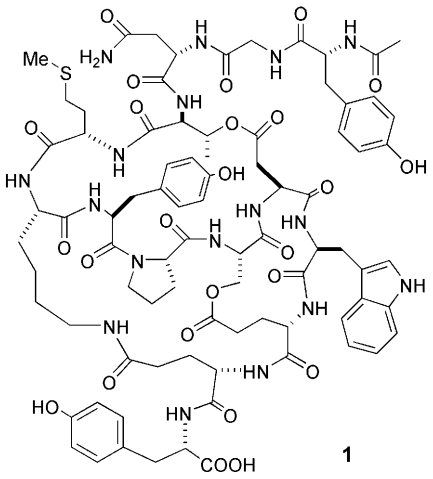
established with the aid of genetic tools and in vitro biochemical assays combined with chemical analysis and chemical degradation. Genomic analysis revealed, unexpectedly for us, that microviridin is a ribosomally encoded peptide rather than an NRPS product. Ribosomally biosynthesized peptides had until recently been known mainly from the lactobacilli[11] and the enterobacteria,[12] but not from cyanobacteria. However, the ribosomal origin of some oxazole-/thiazole-containing secondary metabolites of cyanobacteria has become evident very recently as the result of genomic analyses. At present there are eight examples—all structurally related—of such natural products arising through this biosynthetic mechanism in the cyanobacteria: the patellamides, patellins, and tenucyclamides (collectively called the cyanobactins) of symbiotic *Prochloron* sp.,[13–15] as well as trichamide from *Trichodesmium erythraeum*,[16] and the microcyclamides/aerucyclamides from *Microcystis aeruginosa* PCC 7806.[17,18] These hexa- to octapeptides are biosynthesized in a prepeptide in which they are contained in "cassettes". The appropriate amino acids are then excised from the prepeptide, cyclized head to tail, and oxidized after the oxazoline/thiazoline rings have been formed. All the gene clusters described above have significant similarity to each other and therefore a similar proposed mechanism of elaboration of the natural products.

Our investigation shows that the microviridins constitute another, structurally distinct group of cyanobacterial metabolites elaborated by the ribosomal biosynthetic mechanism. This had not been our working hypothesis when we began this work, and it was only during the preparation of this manuscript that we became aware of a reference in the secondary literature suggesting a potential ribosomal origin on structural grounds.[19] Moreover, a MvdE homologue in *Microcystis aeruginosa* PCC 7806 had been deposited in GenBank (accession number AM778866.1) and been annotated as a microviridin-type peptide during the *Microcystis* genome project. Lastly, while a draft of this manuscript was under review, the origin of microviridin in ribosomal biosynthesis was verified through heterologous expression in *E. coli*.[20] Thus, while the structural diversity of biologically active small molecules in the cyanobacteria has been noted for some time, our appreciation of the complexity of their biosynthesis as exemplified by the parallel use of the nonribosomal and the ribosomal pathways is still developing.

The presence of a microviridin-like compound in the CYA126/8 isolate of *Planktothrix* had been suggested in a previous MALDI-TOF-MS analysis of a cyanobacterial filament as part of the studies of the microcystin biosynthetic pathway (Figure 5 in ref. [5]). In that analysis a pseudomolecular ion cluster had been evident between *m/z* 1770 and 1808, and was interpreted to be the $[M+H]^+$ and $[M+K]^+$ adducts of a peptide of mass 1769 Da. Microviridins (Table S1), the largest known oligopeptides in the cyanobacteria, fall into that molecular mass range and it therefore appeared reasonable to surmise that this metabolite belonged to this group of compounds. Analysis of an extract of the CYA126/8 isolate by LC-HRESI-TOF-MS indicated the presence, among those of other compounds, of a pseudomolecular ion $[M+H]^+$ at *m/z* 1770.7060, which is in good agreement with the calculated mass for the parent compound suggested by the MALDI-MS analysis mentioned above. This compound was therefore targeted for isolation from cell mass obtained through mass culture by standard natural products chemistry techniques. Interpretation of 1D-TOCSY, gHSQC, gHMBC, and ROESY data (Table S2) led to the assignment of structure **1** to the microviridin from this isolate. The amino acid sequence of **1** is identical with that observed for another microviridin from a *Planktothrix* isolate—microviridin D, which presumably is the methanolysis product of microviridin K.[9]



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With the structure of **1** to hand, we turned to searching for the gene cluster responsible for this peptide. Analysis of the raw, as yet incompletely annotated genome sequence of *P. agardhii* CYA126/8 revealed an insufficient number of adenylation domains to explain the biosynthesis of **1** by a NRPS pathway considering the number of known NRPS-derived metabolites in this isolate. Furthermore, while most NRPS products contain nonproteinogenic amino acids, and/or hydroxy acids, and/or amino acids of D configuration and/or N-methylated amino acids, such structural features are absent in **1**. These observations prompted us to consider a ribosomal origin for microviridin K. In order to detect the putative microviridin gene in *P. agardhii* we therefore constructed hypothetical gene fragments using all possible combinations of codons for the seven N-terminal amino acids present in **1** as suggested by the NMR data. Of the 256 combinations assembled in this way, only one was discovered in the raw genome sequence and this match was unique. Further analysis of the immediate surrounding area revealed that, in addition to the codons for the fourteen amino acids known from the NMR structure of **1**, an additional 102 bp encoding 34 amino acids of a presumable leader sequence were present. It thus appeared that **1** is biosynthesized as a prepeptide, which we named *mvdE,* and requires proteolytic processing as part of the post-translational modification process. The amino acid sequence of MvdE is shown in Figure 1. Downstream from *mvdE* a gene of 153 bp (*mvdF*) encodes a second putative microviridin together with its leader sequence for a total length of 50 amino acids. To

B) MSKNVKVSAPKAVPFFARFLAEQAVEANNSNSAP-YGNTMKYPSDWEEY

Figure 1. A) Organization of microviridin biosynthetic gene cluster from *P. agardhii*. Gray shaded genes are presumed to be outside the *mvd* gene cluster. B) Amino acid sequence of MvdE. The amino acids destined to become microviridin K are underlined.

date we have not found the corresponding microviridin in extracts of *P. agardhii*. The sequence homologues of MvdE and MvdF from available cyanobacterial genomes are shown in Figure S2.

Further analysis of gene sequences upstream from these putative microviridin genes revealed the presence of two genes of 975 (*mvdC*) and 993 bp (*mvdD*). These encode proteins of monoisotopic mass 36840 Da and 37479 Da, respectively, with homology to RimK proteins, a family of carboxylate-amine/thiol ligases.[21] Further upstream, *mvdB* (540 bp) encodes a protein of 19598 Da, which is homologous to acetyltransferases, whereas *mvdA* (1794 bp) encodes a 68712 Da protein with high homology to ABC transporters. Immediately upstream from the latter there is an open reading frame, *orf1*, with homology to *apdE* from the anabaenopeptilide gene cluster.[22] At the 3′ end of the *mvd* cluster, downstream from *mvdF*, there is *orf2*, which is homologous to *apdF*. The *mvd* genes thus appear to be inserted into remnants of an anabaenopeptilide or similar gene cluster. The homologies of Mvd proteins to their nearest homologues in the NCBI database as identified by BLAST search are shown in Table 1. The genes of the recently published microviridin cluster from *M. aeruginosa* NIES-298[20] are all among the top ten hits in the BLAST search, but do not score higher than the entries given in Table 1. Additional alignments are shown in Figures S3–S6.

Inspection of the structure of **1** coupled with the homologies listed in Table 1 readily allowed us to formulate a hypothesis as to the functions of MvdB–D in microviridin biosynthesis. However, the same cannot be said for the *apdE* and *apdF* homologues. These two genes encode a protein containing an *S*-adenosylmethionine (SAM) binding domain and a protein with homology to short-chain dehydrogenases, respectively, of undetermined function in anabaenopeptilide biosynthesis. At present we do not consider these two genes to be part of the *mvd* cluster for two reasons: firstly, *apdE* and *apdF* encode activities involving SAM and oxidation/reduction, respectively, that do not have any obvious function in microviridin biosynthesis, and secondly, only one of the other putative microviridin gene clusters that we have identified from publicly accessible databases includes homologues of these two genes in the immediate vicinity. If they were part of the cluster, then one would expect them to show up in putative *mvd* gene clusters with greater frequency. This is not the case.

The putative enzyme performing the proteolytic processing of the 48-mer to the 14-mer is conspicuously absent from the cluster and its surroundings in *P. agardhii*. The organization of the *mvd* cluster from *P. agardhii* CYA126/8 is depicted in Figure 1. The sequence is deposited in GenBank under the accession number EU438895.

In order to demonstrate that *mvdE* does indeed encode a precursor to **1** and that the genes *mvdB–D* encode the enzymes performing some of the post-translational modifications, we pursued a dual mutagenesis and biochemical characterization strategy. We deleted *mvdE* and *mvdF* using our established technique and replaced these with a chloramphenicol acetyltransferase gene.[5] A deletion strategy was chosen because insertional inactivation constructs of *mvdE* and *mvdF* underwent recombination in *E. coli*; this resulted in the deletion of the insert. After transformation of *P. agardhii* and selection with antibiotic in liquid medium, we obtained cultures deficient in microviridin K production as demonstrated by HPLC and LC-MS (Figure 2B). The biosynthesis of all other known metabolites from this isolate was unaffected in the mutant. Furthermore, PCR analysis with use of primers located outside the homologous recombination area confirmed that the resistance gene had inserted into the genome at the intended position (Figure 2C).

We then heterologously overexpressed MvdC and MvdD in *E. coli* as His_6-tagged fusion proteins. Despite extensive optimization of conditions, the bulk of the protein produced was found in the insoluble fraction. Nonetheless, sufficient pure, soluble protein for the projected in vitro work was obtained after Ni–

Table 1. BLAST homologies of Mvd proteins with closest neighbors.

Protein	Size[a]	Proposed function	Sequence similarity[b]	Identity/similarity
ORF1	265	putative methyltransferase	ApdE *A. circinalis* 90	74%, 83%
MvdA	597	ABC transporter	IPF_2484 *M. aeruginosa* PCC 7806	73%, 87%
MvdB	179	acetyltransferase	MAE_24080 *M. aeruginosa* NIES-843	62%, 76%
MvdC	324	cyclization protein, amide bond forming	Npun02001923 *N. punctiforme* PCC 73102	75%, 88%
MvdD	330	cyclization protein, ester bond forming	Npun02001924 *N. punctiforme* PCC 73102	79%, 90%
MvdE	48	microviridin K prepeptide	MAE_24110 *M. aeruginosa* NIES-843	53%, 70%
MvdF	50	Microviridin 2 prepeptide	IPF_3129 *M. aeruginosa* PCC 7806	51%, 62%
ORF2	245	short chain dehydrogenase	ApdF *A. circinalis* 90	80%, 91%

[a] Amino acids; [b] protein, organism.

agarose chromatography (Figure S7).

The purified enzymes were assayed for cyclization activity with synthetic 48-mer prepeptide as substrate. This peptide is identical in amino acid sequence to MvdE and was prepared by solid-phase peptide synthesis. Incubation of the two over-expressed MvdC and MvdD proteins with peptide substrate (10 μg) in the presence of ATP at pH 8.0 resulted in time-dependent conversion of the 48-mer ($[M+3H]^{3+}$ *m/z* 1795.2007; Figure 3A, $[M]^{+}$ 5382.5784; calcd 5382.5753, 0.5 ppm error) into a slightly less polar compound with concomitant loss of 54 mass units, equivalent to the loss of three water molecules ($[M+3H]^{3+}$; *m/z* 1777.1872, Figure 3B, $[M]^{+}$ 5328.5379; calcd 5328.5435, 1.0 ppm error). In parallel incubations with the same batches of substrate and protein, no conversion was observed in the presence of boiled enzyme or in the absence of either ATP or enzyme. Enzymatic activity was observed only when the 48-mer peptide was used as the substrate, because no cyclization was seen when a linear peptide consisting of the C-terminal 14 amino acids was used as the substrate in parallel reactions. When MvdC was added to the reaction mixture lacking MvdD, no conversion of the starting material was observed. However, when only MvdD was incubated with MvdE under standard conditions, conversion of the starting material was observed, although with concomitant loss of only two water molecules ($[M+3H]^{3+}$; *m/z* 1783.2007, Figure 3C, $[M]^{+}$ 5346.5784; calcd 5346.5541, 4.5 ppm error). This suggested that most likely the two ester bonds were formed first, followed by the amide bond. To support this interpretation, the product of reaction of MvdD

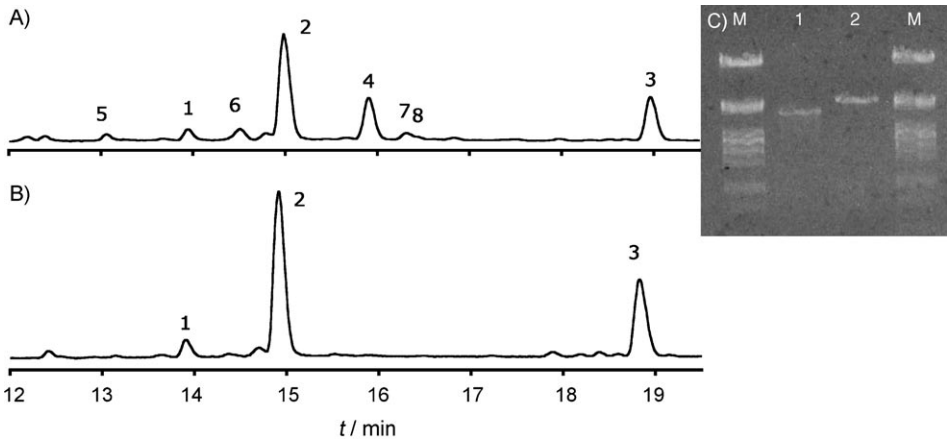


Figure 2. LC-TOF-MS total ion current traces from extracts of *P. agardhii*. A) CYA 126/8-WT, and B) 126/8-ΔMV. Compounds by peak: peak 1: anabaenopeptin 908; peak 2: [Asp^3]-MC-RR; peak 3: anabaenopeptin 915 and [Asp^3]-MC-LR; peak 4: microviridin K (**1**); peak 5: dAc-microviridin K; peak 6: oxidized microviridin K; peak 7: microviridin K + MeOH; peak 8: microviridin K+H_2O. C) PCR products showing the correct insertion of the chloramphenicol acetyltransferase gene during homologous recombination. Lane M: λ/PstI marker; lane 1: PCR product amplified from 126/8-WT genomic DNA; lane 2: PCR product amplified from 126/8-ΔMV genomic DNA.

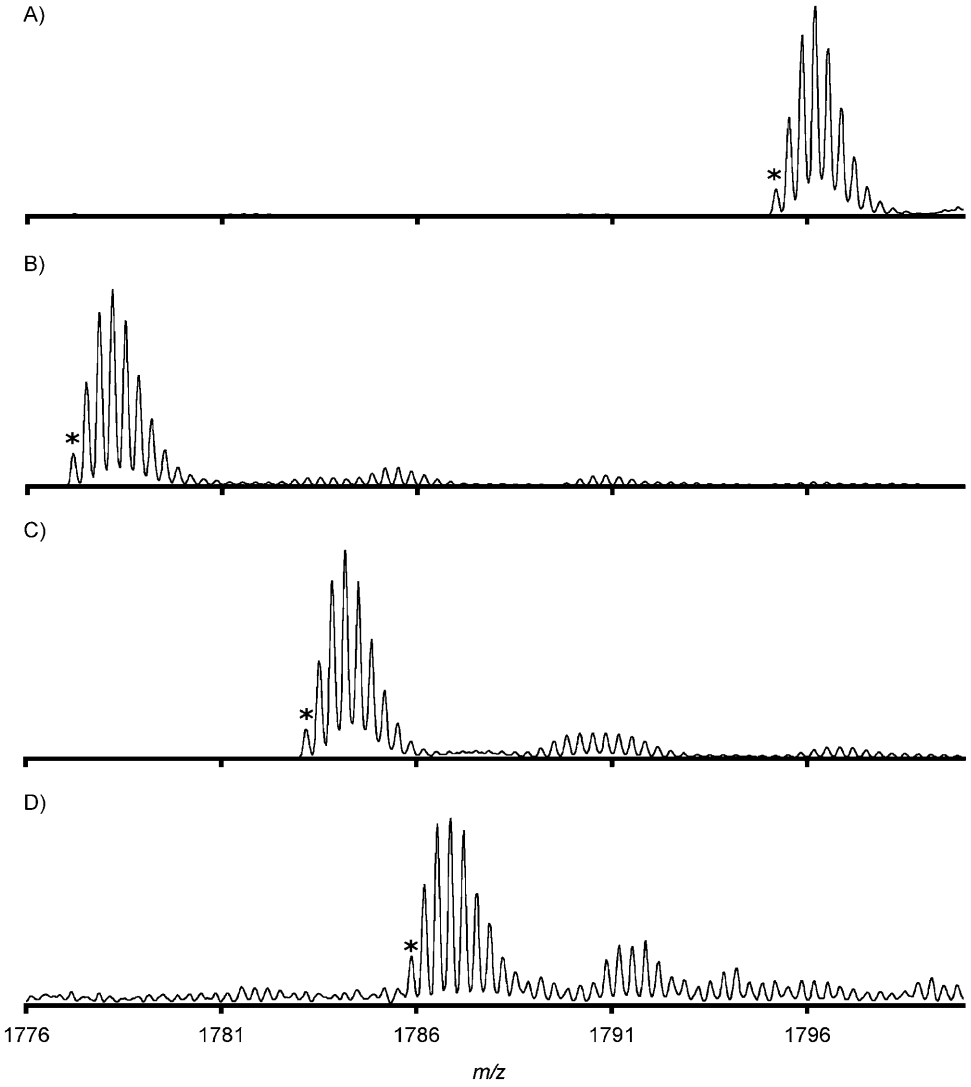


Figure 3. TOF-MS spectra of synthetic MvdE when incubated with: A) no enzyme ($[M+3H]^{3+}$; *m/z* 1795.2007), B) MvdC and MvdD ($[M+3H]^{3+}$; *m/z* 1777.1872), C) MvdD only ($[M+3H]^{3+}$, *m/z* 1783.2007), and D) MvdD followed by addition of $LiBH_4$ in MeOH (room temperature, 15 min, $[M+3H]^{3+}$; *m/z* 1785.8687); * denotes the $[M+3H]^{3+}$ monoisotopic ion peak.

was quenched reductively ($LiBH_4$/MeOH, room temperature, 0.25 h) under conditions that should leave amide bonds unaffected. HRESI-TOF-MS analysis of the reduced reaction mixture showed a shift of 8 Da ($+4H^- + 4H^+$) to higher mass as expected ($[M+3H]^{3+}$; *m/z* 1785.8687, Figure 3D $[M]^+$ 5354.5824; calcd 5354.6173, 6.5 ppm error).

Attempted reductions with $NaBH_4$ in lieu of $LiBH_4$ under otherwise identical conditions did not yield reduced products. This result suggests that MvdD catalyzes ester formation rather than a simple dehydration somewhere in the peptide chain, resulting in dehydroalanine or dehydrobutyrate residues, because $NaBH_4$ readily reduces such N-acyl enamides.[22] This reasoning is also supported by the stoichiometry observed. Reduction of a dehydroamino acid would require one hydride and a proton per double bond and therefore a total of four hydrogens for the reduction of a molecule from which MvdD had cleaved off two equivalents of water. The observed stoichiometry is eight hydrogens per molecule, a requirement that is uniquely met if two carbonyls are reduced with concomitant cleavage of a heterocycle.

The requirement for $LiBH_4$ as a reducing agent strongly supported the interpretation that two ester bonds had been formed by the action of MvdD. However, the location of the ester bonds within the 48-mer did not follow from this experiment. Since the linear 48-mer peptide contains multiple acidic amino acids and multiple serine residues in the leader sequence, the putative cross-links suggested by the reduction experiment could be located there rather than in the portion of the peptide destined to become **1**. The reduction product was therefore next subjected to cyanogen bromide degradation, which selectively cleaves at the carboxy termini of methionine residues. If the putative ester bonds in the 48-mer are located at the positions suggested by the structure of microviridin K, then in the reduction product the third amino acid from the carboxy terminus should be a 5-hydroxynorvaline in lieu of a glutamic acid and the fifth amino acid a homoserine in lieu of an aspartic acid. Thus, comparison by LC-MS of the cyanogen bromide degradation products of the linear 48-mer and of the reduced product of MvdD should show differences within the C-terminal nonapeptides. In the event, treatment in parallel of a sample of the 48-mer prepeptide and of the reduced product of MvdD with cyanogen bromide in aq acetic acid (70%) for 3 h at room temperature, followed by LC-HRESI-TOF-MS analysis, yielded the mass spectra shown in Figure 4. The product of degradation of the uncyclized prepeptide shows the expected mass of $[M+H]^+$ *m/z* 1216.5213 for the C-terminal nonapeptide (calcd 1216.5157, 4.6 ppm error; Figure 4A). The mass of the nonapeptide obtained by degradation of the reduced MvdD product is lower by 28 mass units ($[M+H]^+$; *m/z* 1188.5558, calcd 1188.5499, 5.0 ppm error; Figure 4B), as expected (plus $4H^-$ minus two oxygens) relative to that obtained from the 48-mer prepeptide. This shows conclu-

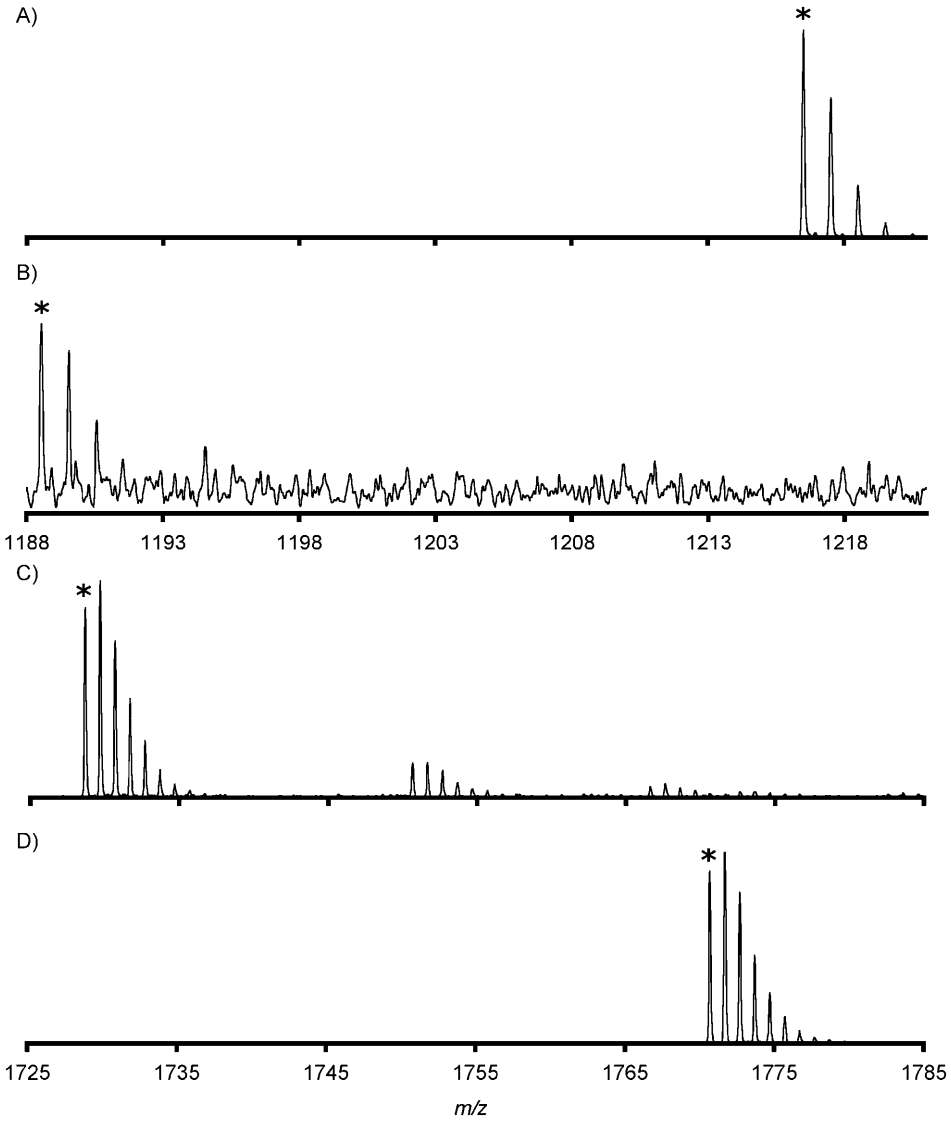


Figure 4. TOF-MS spectra of A) CNBr digest of uncyclized synthetic MvdE ($[M+H]^+$; *m/z* 1216.5213); B) CNBr digest of bicyclic, reduced MvdE ($[M+H]^+$; *m/z* 1188.5558); C) dAc-microviridin K incubated without enzyme ($[M+H]^+$; *m/z* 1728.6866); D) dAc-microviridin K incubated with MvdB $[M+H]^+$; *m/z* 1770.6987; * denotes the $[M+H]^+$ monoisotopic peak.

sively that the sites of the reductions are located within the first nine C-terminal amino acids of the MvdD product.

We next investigated the mechanism by which the ester bonds are introduced. We considered two potential mechanisms: i) formation of an acyl-adenylate intermediate with concomitant release of pyrophosphate, or ii) formation of a carboxylate-phosphate mixed anhydride with release of ADP, followed by release of phosphate upon esterification. In three replicate experiments we incubated MvdE with MvdD until complete conversion was reached as judged by LC-MS. The released phosphate was determined by use of the Malachite green assay. Aliquots of the reaction mixture were removed and quenched by addition of a suspension of charcoal in water. The solids were centrifuged, and the phosphate concentrations in the supernatants were determined. We found that (0.727 ± 0.013) nmol of phosphate were released for every 0.372 nmol of MvdE cyclized. This suggests that MvdD forms the mixed anhydride rather than the acyl-adenylate intermediate and agrees with the mechanism of another RimK homologue, D-alanyl–D-alanine ligase.[24] Given the close sequence homology of MvdC and MvdD it is reasonable to propose that MvdC will follow the same mechanism as MvdD, although this was not rigorously established.

We next expressed MvdB as a His_6-tagged protein in *E. coli*. The protein was obtained in predominantly soluble form and in exceptional yield after Ni-agarose chromatography. The biochemical function as an acetyltransferase suggested during annotation was confirmed by in vitro experiments. During LC-MS screening we had observed a compound with a mass spectrum that suggested that it was the fully cross-linked non-acetylated form of microviridin K. Preparative isolation of this material by HPLC yielded sufficient amounts to be used in a biochemical assay. Incubation of MvdB protein (0.06 μg) with desacetyl microviridin K (5 μg; $[M+H]^+$ *m/z* 1728.6866; calcd 1728.7005, 8 ppm error; Figure 4C) in the presence of acetyl coenzyme A (4 mM) at pH 7.4 for 1 h resulted in complete conversion into a compound that was identical with microviridin K by HPLC and ESI-TOF-HRMS ($[M+H]^+$ *m/z* 1770.6987, calcd 1770.7111, 7.1 ppm error; Figure 4D).

BLASTP analysis of MvdC–D suggests that other cyanobacteria might also possess microviridin biosynthetic gene clusters (Figure 5). Analysis of sequences deposited in the NCBI database suggests that in all but one cluster the protease required to process the cross-linked premicroviridin to the mature compound is missing from the immediate vicinity of the other *mvd* genes, the exception being *Nostoc* sp. PCC 7120, in which it is N-terminally fused to the ABC transporter. Three other species in this analysis—*Nodularia spumigena* CCY 9414 and *Nostoc punctiforme* PCC 73102, as well as *Anabaena variabilis* ATCC 29413—are lacking both the protease as well as the acetyltransferase from their clusters. Microviridin-type metabolites have not been isolated from these organisms, but all known microviridins bear acetyl groups at their N termini. It is therefore likely that the missing acetyltransferase is located elsewhere in the genome rather than missing altogether. Lastly, in *M. aeruginosa* NIES-843 the putative *mvd* cluster comprises the same five genes as in *P. agardhii*, including the acetyltransferase, which is missing in *M. aeruginosa* PCC 7806. In the latter case it is interesting to note that the *mvdE* homologue (IPF_3129) is orphaned on a unique contig when compared with the MvdA, MvdC and MvdD homologues (Figure 5).

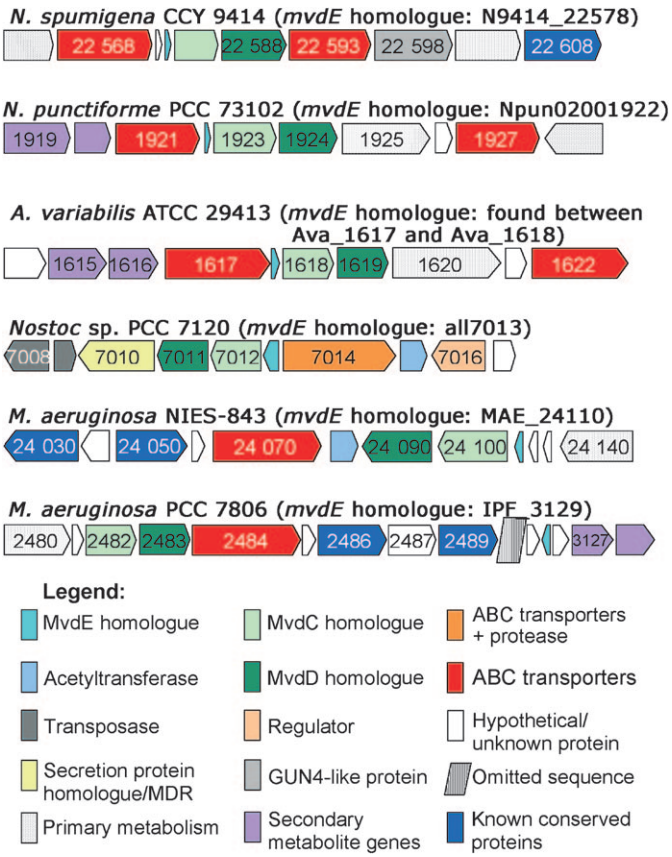


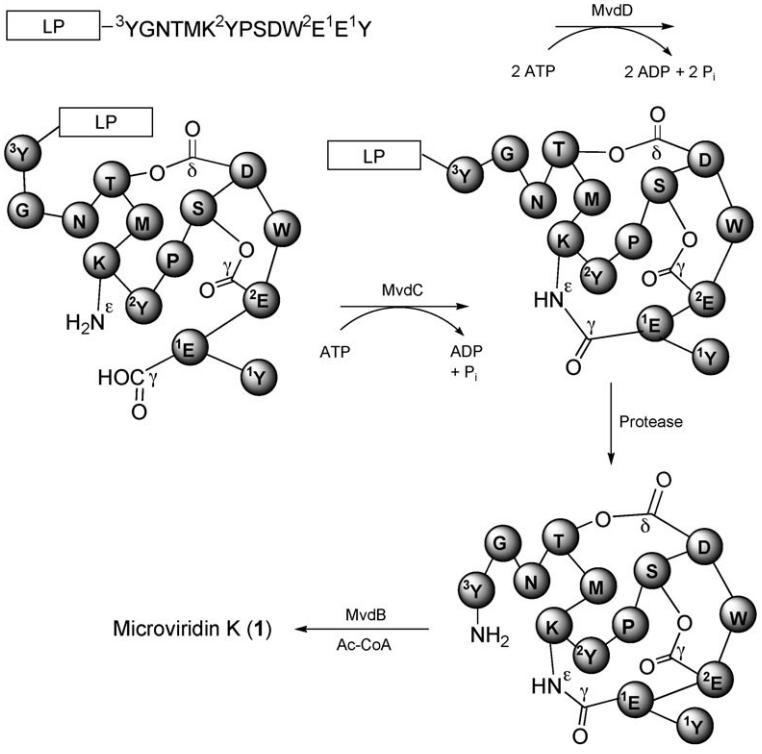
Figure 5. Graphical representation of putative *mvd* biosynthetic clusters in cyanobacterial genome databases obtained from the NCBI database by using the BLAST homology search to MvdD. Block arrows represent open reading frames (ORFs); the gene numbers of the ORFs are indicated in the arrows; genes are not drawn to scale. Gene prefixes are as follows: *N. spumigena*: N9414_XXXXX; *N. punctiforme*: Npun0200XXXX; *A. variabilis*: AvaXXXX; *Nostoc* sp. PCC 7120: allXXXX (transcribed to the left) or alrXXXX (transcribed to the right); *M. aeruginosa* NIES-843: MAE_XXXX; *M. aeruginosa* PCC 7806: IPF_XXXX; where XXXX is the gene number seen in the diagram above.

Recent work on the biosynthesis of microviridin in *M. aeruginosa* through heterologous expression in *E. coli* suggested that the protease that removes the leader peptide is integrated into the ABC transporter.[20] In our view this hypothesis is incorrect for several reasons. Firstly, the ABC transporter identified in that work—MdnE—has the highest homology to ABC transporters found in other putative *mvd* gene clusters as well as others found in NRPS systems (NcpC, *Nostoc* sp. ATCC 53789, 76% similarity; NosG, *Nostoc* sp. GSV224, 77% similarity; McnF, *Microcystis* sp. NIVA-CYA 172/5, 75% similarity). None of the products of these NRPS systems are known to require processing by a peptidase, and the associated ABC transporter is therefore most unlikely to have an integrated peptidase. Secondly, the ABC transporter of the *mvd* cluster from *Nostoc* sp. PCC 7120, which has the peptidase fused to the N terminus, is unique in its length in relation to all other *mvd* ABC transport-

ers analyzed (Figure S3), and the N-terminal 110 to 135 amino acids align well with peptidases. As shown in Figure S3, MdnE does not align well with this "long" transporter and is therefore unlikely to contain a protease activity. Thirdly, we obtained truncations similar to those observed by Ziemert et al.[20] when we attempted to express MvdE in *E. coli* as a SUMO fusion protein. Since in our case the bacterium had not been transformed with the *mvdA* gene from *P. agardhii*, which encodes the ABC transporter, the transporter cannot be responsible for the observed proteolytic degradation of the sumoylated MvdE. A broad specificity peptidase endogenous to *E. coli* is the more likely candidate, which could also explain the range of products observed during heterologous expression.

Conclusions

We have established the ribosomal origin of the protease inhibitor microviridin K in *Planktothrix agardhii* CYA126/8 and have biochemically demonstrated three of the four post-translational modifications required to convert the linear prepeptide MvdE into microviridin K. On the basis of the experiments discussed in this manuscript we can summarize the sequence of steps to microviridin K (Scheme 1). The requirement for the cross-linking to precede cleavage by the peptidase stems from the observation that a peptide lacking the leader sequence is not a competent substrate for MvdD. MvdC can introduce the amide bond only once the ester bonds have been formed. It remains to be seen whether two ester bonds are required before a cross-linked or partially cross-linked MvdE is a competent substrate for MvdC. The final step must be the acetylation as the tyrosine nitrogen of the substrate is liberated through action of the peptidase that at this time remains elusive.



Scheme 1. Sequence of steps in microviridin biosynthesis.

Experimental Section

General: NMR spectra were recorded by using a Varian Inova instrument at 500 MHz (^{1}H) and 125 MHz (^{13}C) in a 3 mm probe (either Shigemi tubes, Shigemi Inc., Allison Park, PA, USA, or standard tubes). Residual protiated solvent was used as chemical shift standard. HRESI-TOF-MS spectra were recorded by using an Agilent 6100 TOF instrument fitted with an Agilent 1100 solvent delivery system with standard operating parameters (350 °C drying gas temperature, 10 L min^{-1} drying gas, 275.8 kPa nebulizer gas, fragmenter 225 V, skimmer 60 V, OctRFV 250 V, capillary 3000 V). The spectra were analyzed with Analyst QS software v1.1 (Applied Biosystems). Optical rotations were recorded with a JASCO DIP-700 polarimeter.

Cyclization of MvdE: Reaction mixtures contained Tris (1 M, pH 8.0, 5 μL), ATP (0.1 M, 2.5 μL), $MgCl_2$ (0.1 M, 5 μL), MvdC (1.2 mg mL^{-1}, 2.5 μL), MvdD (1.2 mg mL^{-1}, 2.5 μL), KCl (0.5 M, 5 μL), and prepeptide (1 mg mL^{-1}, 10 μL) in a final volume of 50 μL. The reaction was incubated at 37 °C without shaking for 16 h. An aliquot (15 μL) of the reaction mixture was analyzed on an Eclipse XDB-C18 column (5 μm, 4.6 mm × 150 mm, Agilent Technologies, Santa Clara, CA, USA). The following linear gradient was used to separate the prepeptide from other reaction components: aq ACN (10%)+formic acid (0.1%) to aq ACN (50%)+formic acid (0.1%) over 30 min at 0.7 mL min^{-1} flow rate. The prepeptide eluted at 19.6 min and the tricyclic prepeptide at 20.5 min, while the 14-mer eluted at 16.5 min. Mass spectrometry was performed by ESI-TOF-MS.

Reduction of cyclized peptides: The reaction was set up analogously to that above, except that MvdC was omitted from the reaction mixture. $LiBH_4$ (4.5 mg) in methanol (250 μL) was added to the reaction mixture (25 μL), which was stirred for 15 min at room temperature. Glacial acetic acid (approximately 25 μL) was added to quench the excess $LiBH_4$, and the mixture was concentrated to dryness under a stream of nitrogen. The resulting solid was dissolved in doubly distilled H_2O (30 μL) and 20 μL was analyzed by LC-MS as described above.

CNBr digest of the reduced product of MvdD: The residue from a fully reduced and dried reaction mixture (vide supra) was dissolved in aq acetic acid (70%, 100 μL), and a crystal of CNBr was added. The mixture was incubated at 21 °C for 3 h in the dark and was then concentrated to dryness. The residue was dissolved in MeOH (25 μL) and diluted with doubly distilled H_2O (25 μL), and 20 μL of the resulting mixture were analyzed by ESI-TOF-MS as described above.

Malachite green assay: Cyclization of synthetic MvdE was performed as described above. An aliquot (10 μL) of the reaction mixture was combined with distilled H_2O (30 μL) and activated charcoal (2.5%, *w/v*; 15 μL) in distilled H_2O. The charcoal was mixed and then centrifuged in a tabletop centrifuge (14000 rpm, 5 min). A sample (50 μL) was removed from each mixture and placed in a well of a 96-well plate, Green reagent (Calbiochem, Cat. No. KP8404, 100 μL) was added, and the system was mixed thoroughly by gentle pipetting. The color was allowed to develop until absorbance stopped increasing (~45 min). The A_{620} was then determined with a Multis-

kan MCC/340 spectrophotometer (ThermoLabsystems, Waltham, MA, USA). The amount of phosphate released was determined from a standard curve recorded concurrently.

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