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Leader Peptide and a Membrane Protein Scaffold Guide the Biosynthesis of the Tricyclic Peptide Microviridin

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SUMMARY

Microviridins are unique protease inhibitors from bloom-forming cyanobacteria that have both ecological and pharmacological relevance. Their peptide backbones are produced ribosomally, and ATP grasp ligases introduce ω-ester and ω-amide bonds to yield rare cage-like structures. Bioinformatic analysis of the microviridin biosynthesis gene cluster suggests a novel type of processing machinery, which could rationalize the challenging in vivo/in vitro reconstitution of the pathway. In this work, we report the establishment of a minimal expression system for microviridins. Through bioinformatics and mutational analysis of the MdnA leader peptide we identified and characterized a strictly conserved binding motif that is specific for microviridin ligases. Furthermore, we showed that the ABC transporter MdnE is crucial for cyclization and processing of microviridins and demonstrated that MdnE is essential for stability of the microviridin biosynthesis complex.

INTRODUCTION

The last decade has seen a remarkable progress in the discovery of natural products that are ribosomally synthesized and post-translationally modified (Donia et al., 2008; Li et al., 2010; Liao et al., 2009; Meindl et al., 2010). Unlike nonribosomal peptide assembly lines, ribosomal peptide gene clusters are typically small and easily accessible for genetic manipulation. Although limited to 20 canonical amino acids, ribosomal peptides are structurally and functionally diverse featuring various side chain modifications as well as different types of macrocyclization, which can lead to an impressive range of biologically active metabolites (McIntosh et al., 2009).

Microviridins are a family of cyanobacterial tricyclic depsipeptides exhibiting a highly intriguing cage-like architecture (Ishitsuka et al., 1990; Shin et al., 1996). Macrocycles are formed via lactone and lactam linkages between ω-carboxyl groups of aspartate and glutamate and ω-amino and hydroxyl groups of lysine and serine or threonine, respectively (Ziemert et al., 2008). Members of the family are potent inhibitors of serine type proteases (Ishitsuka et al., 1990; Shin et al., 1996). The non-canonical ester and amide bonds were shown to be established by two ATP-grasp ligases via a strictly ordered mechanism (Philmus et al., 2008, 2009). The enzyme MvdD is establishing the larger lactone ring first, followed by the smaller lactone ring. Lactam ring formation requires a lactonized premature microviridin precursor and has been shown to be catalyzed by the enzyme MvdC (Figure 1). Whereas the core of microviridins is highly conserved and conformationally constrained (Philmus et al., 2009), a remarkable natural variability was found in N-terminal and C-terminal amino acid residues (Ziemert et al., 2010). Correctly cyclized and processed microviridin B could be heterologously produced from a fosmid library in *Escherichia coli* (Ziemert et al., 2008).

A common feature of ribosomally synthesized peptides is the presence of N-terminal leader peptides (Oman and van der Donk, 2010). These leaders are essential for the processing and export of their associated peptides. Among the best studied examples is the lantibiotic nisin of *Lactococcus lactis*, a compound widely used as food preservative. The corresponding leader peptide contains a recognition site for the nisin dehydratase NisB and the nisin cyclase NisC (Kluskens et al., 2005; Rink et al., 2007). Additionally, export and processing via NisT and NisP require cooperation of the leader peptide (Kuipers et al., 2004). Leader peptide recognition motifs for posttranslational modification (PTM) enzymes were proposed for a number of further peptides featuring different types of PTMs, i.e., lacticin 481 (Patton et al., 2008) and microcins B17 (Madison et al., 1997) and J25 (Duquesne et al., 2007).

Identification motifs for processing proteases are typically different from motifs for PTM enzymes. Lantibiotics of the group II or nonlantibiotics share a double glycine motif preceding the protease cleavage site that is essential for correct processing (Michiels et al., 2001). Cleavage of peptides containing double glycine motifs is achieved during peptide secretion by the N-terminal C39 protease domains of the cognate peptide transporters (Michiels et al., 2001). A different mode of excision was revealed for cyanobactins where the subtilisin-type protease PatA was shown to recognize specific sequence motifs N-terminal and C-terminal of the core peptide (Donia et al., 2008). Protease cleavage has also been studied for the phalloidin prepeptide in the mushroom *Conocybe alpipes*. Here, a prolyl-endopeptidase is cleaving next to prolines at both ends of the core peptide (Luo et al., 2009).

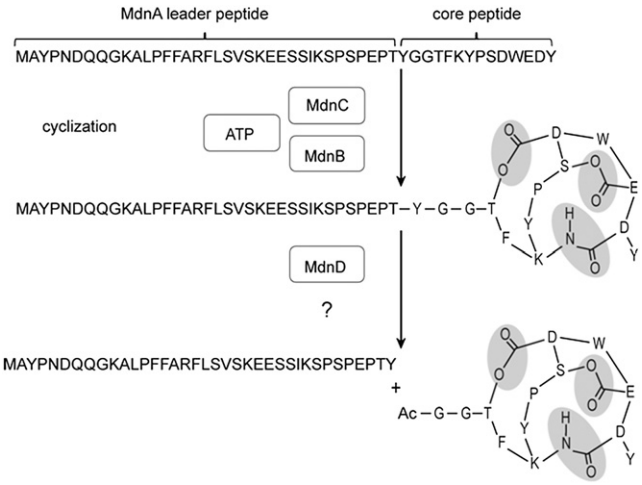

Figure 1. Schematic Representation of Posttranslational Modification and Processing Steps during Microviridin Biosynthesis in *Microcystis*

MdnC is an ortholog of MvdD catalyzing the formation of the two lactone rings (Philmus et al., 2008; Ziemert et al., 2008). MdnB is an ortholog of MvdC catalyzing the formation of the lactam ring (Philmus et al., 2008; Ziemert et al., 2008). MdnD is a GNAT type acetyltransferase catalyzing N-acetylation of the N-terminal amino acid residue (Philmus et al., 2008; Ziemert et al., 2008). Ac, acetyl group.

A tight complex formation of PTM enzymes, transporters and scaffold proteins has been shown to be important for both in vitro and in vivo synthesis of a number of ribosomal peptides. Reconstitution of microcin B17 was only successful when the two PTM enzymes McbB and McbC cooperated with the scaffolding protein McbD that is the initial recognition site for the peptide substrate (Milne et al., 1999). The lantibiotic nisin is processed by the NisBTC complex including PTM enzymes as well as the corresponding transporter NisT (Kuipers et al., 2004; van den Berg van Saparoea et al., 2008).

Understanding the mechanism of ribosomal peptide synthesis, including the role of leader peptides, mode of action of PTM enzymes, and their cooperation within the enzymatic complex can ultimately facilitate the reprogramming of the pathways in vivo or in vitro (Oman and van der Donk, 2010). This has been successfully demonstrated for various lantibiotic precursor peptides that were extensively manipulated (Kuipers et al., 1996; Levengood et al., 2009; Plat et al., 2011).

Microviridins, however, clearly deviate from the above and represent an entirely new class of ribosomally produced natural products. Leader peptides of microviridin do not contain any known motifs for recognition of PTM enzymes or peptidase cleavage. Thus, we have concluded that microviridins have evolved a novel type of processing machinery.

Here we show that the biosynthesis of the tricyclic peptides is guided by the leader peptide and a membrane protein scaffold. In order to gain insights into that unique mechanism, we have established a minimal in vivo expression system for tricyclic microviridins. We have discovered and characterized the recognition motif for microviridin ligases MdnB and C within the microviridin leader peptide and successfully produced bicyclic microviridin variants. We have further assessed the role of the ABC transporter MdnE for microviridin secretion, stability, and processing of prepeptides. Our data provide insights into the mechanism of microviridin biosynthesis in vivo and lay the groundwork for the manipulation of microviridin type peptides and the use of the ligases in heterologous systems.

RESULTS

Establishment of a Minimal Expression System for Microviridins in *E. coli* and MdnA Transcript Analysis

We have recently reported the production of correctly processed microviridin B from a fosmid library in *E. coli* (Ziemert et al., 2008). However, fosmids are not easily amenable for point mutations. In order to establish a heterologous expression system accessible for manipulations we intended to design a minimal expression system based on the *mdnA–E* genes in *E. coli*. We selected the microviridin L biosynthesis gene cassette from the cyanobacterium *Mycrocystis aeruginosa* NIES843 (Ziemert et al., 2010) as a model. In a first approach, we applied the DUET vector system to express *mdnA*, *mdnB* and *C*, and *mdnD* and *E* from three independent vectors, respectively. However, we could not detect microviridins or any precursor peptides in the resulting recombinant strains (data not shown). We next amplified the *mdnA–E* region including a 169 bp promoter region (representing the intergenic region between *mdnA* and the next annotated ORF upstream) and cloned the fragment directly in the high copy pDrive vector. Again, the resulting strain did not produce any microviridin-type peptides (Figures 2A and 2B). Thus, in parallel, we started investigations into the transcriptional organization of the microviridin biosynthesis gene cluster in the cyanobacterium *M. aeruginosa* using Northern hybridization. The two transcripts of ~450 and 600 nt detected for *mdnA* clearly exceeded the size of the 150 bp coding region for MdnA (see Figure S1 available online). The longer *mdnA* transcript could not be detected in the recombinant strain carrying the *mdnA–E* genes in *E. coli* (Figure S1). We therefore considered the possibility of a longer untranslated leader region for the microviridin biosynthesis gene cluster and cloned the *mdnA–E* cassette with a 714 bp upstream region into the pDrive vector. In contrast to clones carrying the short promoter region these clones produced considerable amounts of microviridin L (**1**), L1 (**2**), L2 (**3**), and L3 (**4**) (Ziemert et al., 2010) and further incompletely processed variants (Figures 2A and 2B). Microviridin L (**1**) and L1 (**2**) were also detected in the original producer *M. aeruginosa* NIES843, **2** typically being the major product (Ziemert et al., 2010).

Identification of the Conserved PFFARFL Region of the Microviridin Leader Peptide as a Recognition Motif for ATP Grasp Ligases

With the stable minimal expression system, we started investigating the role of the microviridin leader peptide by manipulating single amino acid residues. By comparing microviridin prepeptide sequences known from cyanobacteria or present in the microbial genome databases we could detect a strictly conserved PFFARFL motif in the leader peptide (Figure 3). The motif was also found in the leader peptide of marinostatin, a related peptide from *Alteromonas* containing lactone

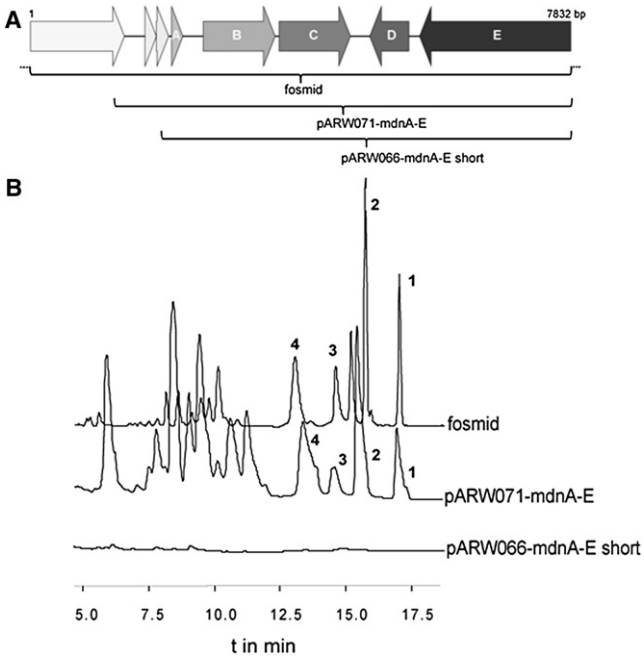

Figure 2. Minimal Expression Construct for Microviridin Biosynthesis in *E. coli*

(A) Schematic representation of microviridin L producing fosmid and minimal constructs containing *mdnA–E* of *M. aeruginosa* NIES843 either with short or long promoter region.

(B) HPLC profile of microviridins produced from a fosmid and minimal constructs in *E. coli* showing production of microviridin L-L3 (**1–4**) and further false processed variants as in Ziemert et al. (2010). Supported by Figure S1; see also Figure 4 for structures **1–4**.

M. aeruginosa N843	10-ALPFFARFLS-19-TFKYPSDW- 3
M. aeruginosa N298	10-ALPFFARFLS-19-TLKYPSDW- 3
M. aeruginosa MRC	10-ALPFFARFLS-18-TRKYPSDW- 3
M. aeruginosa PCC7806	10-AIPFFARFLS-21-TWKWPSDW- 3
M. aeruginosa K139	10-AIPFFARFLS-21-TWKWPSDW- 2
N. spumigena CCY9414	10-AVPFFARFLA-22-TLKWPSDW- 3
Cyanothece sp. (1)	12-AVPFFARFLE-13-TLKYPSDW- 3
Cyanothece sp. (2)	12-AVPFFARFLE-13-TLKYPSDW- 3
Cyanothece sp. (5)	12-AVPFFARFLE-12-TKKYPSDW- 3
Cyanothece sp. (6)	12-AVPFFARFLE-12-TKKYPSDW- 3
Cyanothece sp. (4)	12-AVPFFARFLE-12-TRKYPSDW- 3
Cyanothece sp. (3)	12-VVPFFARFLE-14-TLKYPSDW- 3
P. agardhii 126/8 (1)	11-AVPFFARFLA-16-TMKYPSDW- 3
P. agardhii 126/8 (2)	11-AVPFFARFLS-18-TFKWPSDW- 3
Cyanothece sp. (7)	12-AVPFFARYLE-21-TLKYPSDS-11
Nostoc sp. PCC7120	12-AVPFFARFLE-22-TRKYPSDC-48
S. cellulosum Soce56	19-AVPFFARFLE-17-TLKYPSDQ-24
M. marina ATCC23134	4-KKPFFAQFLE-31-TMKYPSDA-15
Alteromonas sp. B10-31	2-TTPFFANLLA-34-TMRYPSDS- 9

Figure 3. Features of Microviridin Leader Peptides

Alignment of leader peptides containing the PFFARFL box and the TXKYPSD microviridin core motif from diverse cyanobacteria, *Sorangium cellulosum* and *Microscilla marina*; variable parts of the prepeptide were omitted and are indicated with numbers. The related marinostatin prepeptide of *Alteromonas sp*. B10-38 is shown in the last row. M. aeruginosa, *Microcystis aeruginosa*; N. spumigena, *Nodularia spumigena*; P. agardhii, *Planktothrix agardhii*; M. marina, *Microscilla marina*; S. cellulosum, *Sorangium cellulosum*. Numbers in brackets indicate the number of additional amino acids at the N terminus, between PFFARFL and TXKYPSD core motif and the variable C terminus of the microviridin core peptide, respectively.

crosslinks, but lacking the lactam ring of microviridins. Here, the conserved arginine was exchanged by an asparagine residue (PFFANFL, Figure 3). Because the motif is not common in other ribosomal prepeptides, we presumed that it could serve as a recognition motif for the two ATP grasp type ligases. The marinostatin system contains only one of the ATP grasp ligases (Ziemert et al., 2008). We therefore selected the variant arginine residue as a first target for mutagenesis and changed it into asparagine as in the marinostatin leader peptide. The recombinant strain showed microviridin production patterns that were clearly distinct from the initial clone carrying the intact leader sequence (Figure 4A). Detailed analysis via HPLC and MALDI-TOF mass spectrometry revealed that the strain produced bicyclic microviridins featuring only ω-ester but not ω-amide bonds as in the tricyclic microviridins (Figure 4B; Figures S2 and S3). We next exchanged all single amino acids within the motif (for an overview see Figure 4A). Interestingly, all clones obtained showed similar microviridin profiles, that is, they produce bicyclic microviridins instead of tricyclic microviridins, with major products being bicyclic microviridin L1 (**5**) and L (**6**) (Figure 4B; Figures S2, S3, and S4). These data support the concept that the PFFARFL motif serves as a recognition motif for ATP grasp ligases. The bicyclic microviridins were produced in the same or larger amount as the tricyclic microviridins (Figure S4). The ATP grasp ligase catalyzing ester bond formation, MdnC, was thus fully active. However, the cooperation of the leader peptide with the amide ligase MdnB and/or the cooperation between the two ligases MdnB and C was abolished. As the lactam ring is only formed after lactone ring formation, we did not expect to find peptides with a lactam ring, only. However, as the leader peptides carrying single amino acid mutations in the PFFARFL motif still allowed production of bicyclic microviridins, we constructed a double (PGFLRFL) and a triple mutant (PGFLRAL) of the motif to test whether this could finally also prevent activity of MdnC. Whereas the double mutant could still produce bicyclic microviridins, production of microviridin-type peptides was completely abolished in the triple mutant (Figure 4). We could not detect noncyclized microviridin precursor peptide fragments in these mutants. This is in agreement with our attempts to heterologously express MdnA in absence of the ligases in *E. coli* that failed due to very rapid degradation of the peptide and with knockout studies of MdnB and C that revealed complete degradation of the peptide in absence of both enzymes (Ziemert et al., 2008 and data not shown). We could only avoid that degradation when we fused MdnA to a periplasmatic protein (data not shown). The fact that microviridins are completely degraded in the triple PFFARFL mutant thus indicates that both cyclizations were prevented. We conclude that interaction of both ATP grasp ligases with the PFFARFL motif is essential for posttranslational modification of microviridins.

Mutational Analysis of the C-Terminal Region of the MdnA Leader Peptide

The C-terminal region of the microviridin leader peptide is rich in prolines (Figure 5A). This feature is reminiscent of eukaryotic signaling peptides that consist of an N terminus with positive

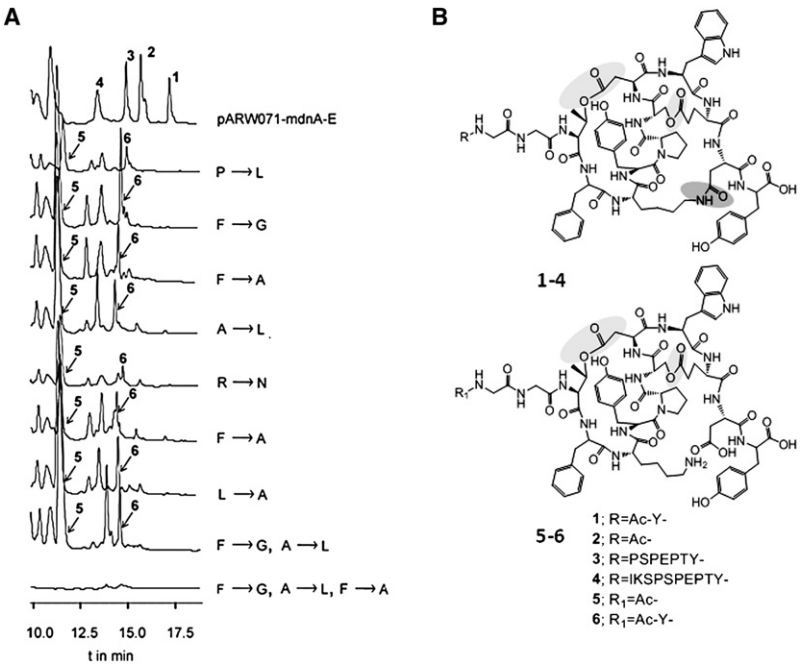

Figure 4. Microviridin Production of PFFARFL Mutants

(A) HPLC profile of single, double, and triple mutants of the PFFARFL motif showing production of bicyclic microviridin L1 (**5**) and L (**6**) instead of tricyclic microviridins (**1–4**) or complete loss of microviridin production. Individual amino acid changes are indicated on the right. Supported by Figure S2, Figure S3, and Figure S4.

(B) Structure of tricyclic microviridins L-L3 (**1–4,** top) and bicyclic microviridin L1 (**5**) and L (**6**) (below). ω-Ester and ω-amide bonds are indicated in light and dark gray, respectively.

net charge, a hydrophobic core region, and a β-turn structure N-terminal to the cleavage site that often contains helix-breaking residues such as proline and glycine (Nothwehr and Gordon, 1989). In addition, specific residues in the C-terminal region of signaling peptides typically play a role in defining the site and efficiency of cleavage. Although these features are not generally conserved in bacterial leader peptides we aimed to assess the impact of the three proline residues for microviridin biosynthesis and processing. First, we exchanged the two prolines at positions −6 and −4 (related to the N terminus of the microviridin core peptide). The resulting recombinant clones were still capable of producing and processing tricyclic microviridins, however, we could also detect significant amounts of bicyclic microviridin L1 (**5**) and L (**6**) (Figure 5B). Only after additionally exchanging the proline at the −2 position, production of tricyclic microviridins was completely abolished. Instead, the strain produced bicyclic microviridin L1 (**5**) and L (**6**) as main metabolites (Figure 5B). These results clearly show that the conformation of the C-terminal part of the leader peptide is crucial for an efficient biosynthesis. In order to get access to the TXKYPSD core region of microviridins (at the C terminus of the prepeptide) and to catalyze cross-links, MdnB and C enzymes that are binding at the PFFARFL region (close to the N terminus of the prepeptide) possibly necessitate a β-turn of the leader peptide. The exchange of the three prolines at the C terminus of the leader peptide had only a minor impact on the cleavage profile of microviridins, thus excluding the possibility of a proline specific endopeptidase.

The Presence of MdnE Is Crucial for Correct Microviridin Processing in *E. coli*

Fosmid clones of the *Microcystis* UOWOCC MRC strain expressing *mdnABC* genes but lacking *mdnD* and *E* genes were shown to produce microviridin J type peptides but were not correctly processed at the N terminus (Ziemert et al., 2008). MdnD is a GNAT-type N-acetyltransferase and MdnE an ABC type transporter composed of a membrane domain and a cytosolic ATP-binding domain. We assumed that the latter protein could play a role in microviridin maturation, in particular as bifunctional transporters catalyzing transport and peptide cleavage are frequently associated with bacteriocin and nonbacteriocin-type peptide systems (Michiels et al., 2001). However, in contrast to the transporter encoded as part of the cryptic microviridin cluster in *Anabaena* PCC7120 that carries an N-terminal C39 peptidase domain (Ziemert et al., 2008), known peptidase signatures could not be detected in MdnE.

Therefore, we were further evaluating the role of MdnE in the minimal *E. coli* expression system established in this study. For this purpose, we have cloned the *mdnA–D* cassette of *M. aeruginosa* NIES843 into the pDrive vector with its long untranslated transcriptional leader. In the system lacking MdnE, microviridin-type peptides were still produced but the peptides were not correctly processed at the N terminus (Figures 6A and 6B). We observed the same phenomenon in three different *E. coli* strains, which suggested that MdnE plays a crucial role in peptide maturation. In addition to the differences in peptide cleavage, we again observed differences in peptide cyclization. All incompletely processed microviridin variants produced by the *mdnA–D* construct were bicyclic. We thus concluded that MdnE is acquired for the assembly of the leader peptide and/or the putative biosynthesis complex. To investigate if MdnE-mediated peptide processing and cyclization is linked to ATP-dependent transport we have exchanged the essential lysine of the Walker A motif in the cytosolic domain with an alanine residue. This replacement would shut down ATP binding and hydrolysis. However, the resulting mutant still produced correctly processed and cyclized microviridins suggesting that the role of MdnE in peptide maturation and cyclization is not linked to transport and does not require ATP hydrolysis (Figure 6B).

Role of MdnE on Microviridin Transport and Stability of the Biosynthesis Complex

We next evaluated the role of ABC transporter protein MdnE for subcellular localization of microviridins and the stability of biosynthetic enzymes. Again, we compared the two recombinant *E. coli* strains harboring either the *mdnA–D* genes or the *mdnA–E* genes and isolated subfractions from cytosol,

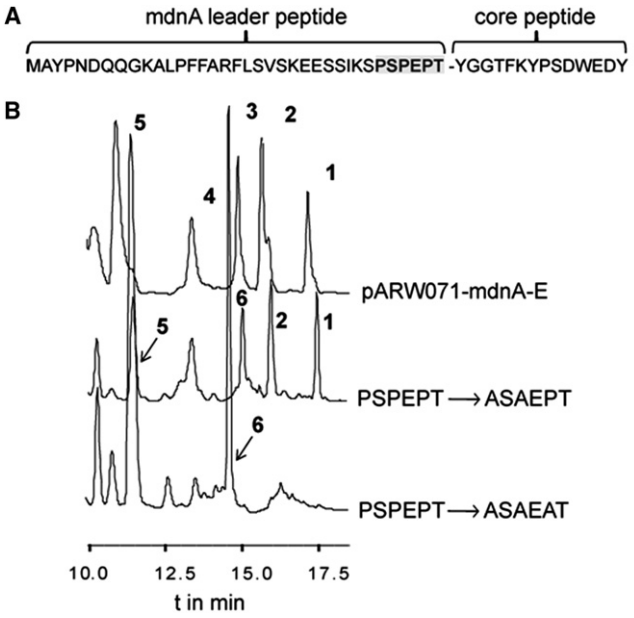

Figure 5. Mutational Analysis of Prolines at the C-Terminus of the Microviridin Leader Peptide

(A) Schematic representation of the MdnA prepeptide highlighting prolines at the C terminus of the leader peptide.

(B) HPLC profile of microviridins from MdnA proline mutants showing production of tricyclic microviridins (**1–4**) and bicyclic variants (**5** and **6**). Individual amino acid changes are shown on the right.

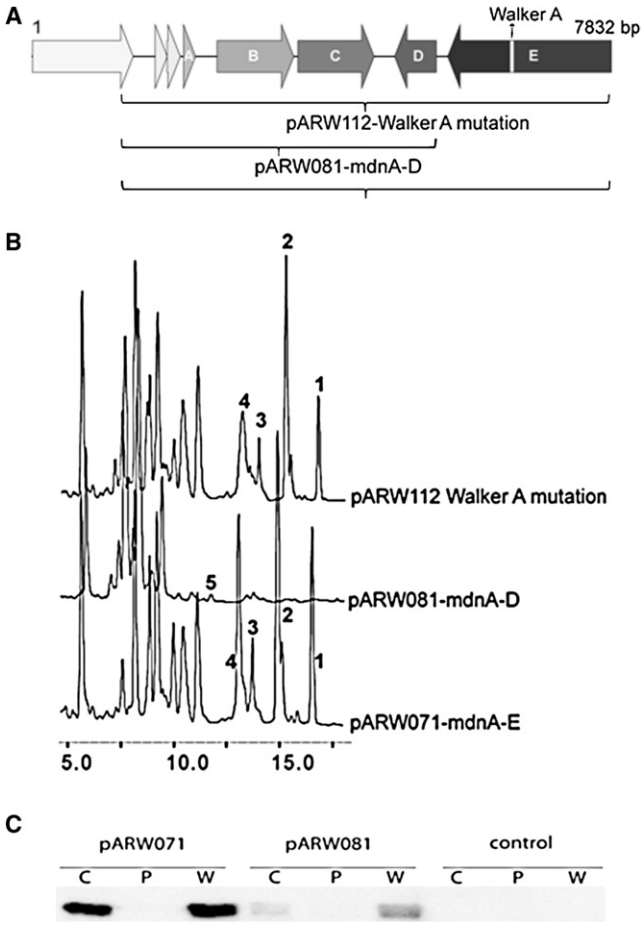

Figure 6. Role of MdnE on Peptide Processing and Stability of the Microviridin Biosynthesis Complex

(A) Schematic representation of *mdnA–D*, *mdnA–E*, and Walker A motif construct.

(B) HPLC profile of *mdnA–E* construct and the Walker A motif mutant showing production of tricyclic microviridins (**1–4**) including correctly processed variants L and L1 (**1** and **2**) and *mdnA–D* construct showing production of bicyclic and incompletely processed variants of microviridin L. Minor amounts of bicyclic microviridin L1 (**5**) are indicated.

(C) Accumulation and subcellular localization of MdnB in absence or presence of MdnE in *E. coli.* C, cytosolic fraction; P, periplasm fraction; W, whole cell fraction.

periplasm, and membrane. Microviridin-type peptides could be detected both in the cytoplasm and the periplasm of *E. coli* (Figure S5). We therefore concluded that microviridin can pass the inner membrane of *E. coli*. However, this phenomenon was independent from the presence of MdnE as we could see similar amounts of microviridin in the periplasm fraction in the strain carrying *mdnA–D* genes, only (Figure S5). We assume that microviridins can potentially cooperate with the Sec transport system in *E. coli.* Bioinformatic analyses for the presence of signal peptides (http://www.cbs.dtu.dk/services /SignalP/) and subcellular localization (http://www.psort.org/) of the peptide produced ambiguous results, thus not excluding the possibility of a Sec-dependent transport for MdnA. Subcellular localization of microviridin was also analyzed in the original producer *M. aeruginosa* NIES298 carrying a complete *mdnA–E* gene cluster. Interestingly, we could not detect microviridin in the periplasm or in the supernatant of the strain but only in the cytoplasm (data not shown). Because the strain is not accessible to genetic manipulation it is currently not feasible to study the role of MdnE in transporting microviridins through the inner membrane of cyanobacteria. Transport in cyanobacteria might nevertheless occur under specific growth conditions not analyzed in this study.

We have further assessed how the presence or absence of MdnE affects the localization and stability of the microviridin ligase MdnB. We had previously produced and purified the protein from *E. coli* and generated a specific antibody against the protein. Independent of the presence of MdnE, MdnB was mostly detected in the cytoplasmic fraction of *E. coli* (Figure 6C), however, the loss of MdnE strongly affected the abundance of MdnB. The transcription of the microviridin gene cluster was almost unchanged in the *mdnA–D* construct. These results support the hypothesis that MdnE acts as a scaffolding protein holding and stabilizing the putative microviridin biosynthesis complex at the cytosolic side of the membrane (Figure 6C). Subcellular localization studies in the two cyanobacterial strains *M. aeruginosa* NIES298 and NIES843 revealed the presence of the enzyme in the cytosol, only, although in lower amounts as in the *E. coli* strain carrying the high copy expression plasmid (data not shown).

DISCUSSION

The last decade has revealed the great potential of ribosomal peptides for biosynthetic engineering toward optimized

pharmacological products. Enzymes for posttranslational modification of peptides have evolved as excellent tools to generate libraries of modified and functionalized peptides (Levengood et al., 2009; Li et al., 2010; McIntosh et al., 2009; Oman and van der Donk, 2010). The potential of microviridin biosynthesis for synthetic approaches, however, seems to be limited due to the inflexibility of the enzymes regarding ring size and order of cyclization reactions (Philmus et al., 2009).

The mutational analysis of the microviridin leader peptide performed in this study suggests that the template for the exact timing and conformation is not only encoded within the MdnB and C enzymes, but also defined by their precise collaboration with the leader peptide. Bioinformatic and mutational analyses of the leader peptide strongly supports this hypothesis. Although leader peptides for different ribosomal peptide classes share common general features, e.g., motifs for secretion, recognition of posttranslational enzymes, and processing, they are generally not well conserved in their primary sequence (Oman and van der Donk, 2010). The PFFARFL motif of microviridins, in contrast, is strongly conserved. Exchange of any individual amino acid of the motif not only lowered the efficiency of biosynthesis, but completely disabled the activity of the MdnB enzyme. Similar studies on other leader peptides, such as for lacticin 481 (Patton et al., 2008) and microcin B17 (Cheung et al., 2010) indicated a larger plasticity, where single point mutations usually still allowed complete processing of the peptide.

Our study further indicates that the C-terminal residues of the leader peptide might be crucial for the orientation of the microviridin core peptide toward the PFFARFL-bound MdnB and C enzymes. This phenomenon was specifically observed for the enzyme catalyzing lactam ring formation, MdnB. Efficient lactamization of microviridins may thus not only require recognition and binding of the enzyme to the leader peptide, but a defined orientation of the lactonized (premature) core peptide toward the enzyme. The enzyme catalyzing lactone ring formation, MdnC, seemed unaffected. However, insertion of a five amino acid stretch downstream of the proline at the −2 position completely inhibited the activity of both enzymes, suggesting that also MdnC requires an exact spacing in addition to its binding to the PFFARFL motif (data not shown).

In the second part of our study, we analyzed the role of the ABC transporter MdnE. Our initial analysis revealed that the enzyme is dispensable for macrocyclizations of microviridin in vivo (Ziemert et al., 2008). However, a more detailed analysis in this study could show the microviridin variants produced in the absence of MdnE were not completely processed and thus only bicyclic. The most plausible explanation for this observation is that the lactam ring forming enzyme MdnB requires a defined architecture of the putative prepeptide/enzyme complex, which is dependent on MdnE. This finding is in agreement with the fact that we could not in vitro cyclize the microviridin prepeptide MdnA by the simple assay used by Philmus et al. (2008) for the *Planktothrix* prepeptide MvdE. We assume that the *Microcystis* prepeptide especially requires conformational assistance by the scaffold protein MdnE. The differences between *Planktothrix* and *Microcystis* might be explained by the significant differences in the C-terminal part of the leader peptides. Notably, the activities shown for MvdD and C in vitro were rather low.

Besides its impact on cyclizations, MdnE had a strong influence on the stability of the putative biosynthesis complex, as exemplified by the amount of MdnB in absence or presence of the protein. As MdnE consists of a membrane domain and a cytosolic ATP-binding domain it might be suggested that MdnE serves as a membrane anchor holding the putative microviridin complex together. Apart from the role in microviridin biosynthesis, we could not fully elucidate the impact of MdnE for microviridin transport and cleavage. *E. coli* is not the appropriate host to fully resolve these questions as cleavage of microviridins is rather incomplete in *E. coli*. Future studies will reveal whether a specific *E. coli* protease requiring MdnE assistance is performing the cleavage reaction. The assisting protease could potentially differ in the different types of bacteria. Similarly, transport of microviridins through the plasma membrane seems to differ between cyanobacteria and *E. coli*. In order to understand the role of MdnE on cleavage and transport, we are thus planning to establish a cyanobacterial expression system.

The minimal expression system designed in the present study does not only provide insights into the mechanism of microviridin biosynthesis, but potentially allows the generation of libraries of novel microviridins through point mutations. Such an approach could primarily be based on the known flexibility of the N- and C-terminus of microviridins. Future efforts to expand the biosynthetic repertoire of microviridins toward an alternative ring size or composition have to take the results of our mechanistic study into account. With the generation of a stable bicyclic variant of microviridins we have already gone the first step.

SIGNIFICANCE

Microviridins represent an intriguing family of peptides that specifically inhibit various proteases. The rare cage-like microviridin architecture is formed by two enzymes of the ATP grasp family. In the present study, a minimal expression system was successfully constructed facilitating in vivo manipulations of the microviridin prepeptide and the associated biosynthetic enzymes. Using this methodology, new mechanistic insights into the role of the leader peptide and the ABC transporter MdnE could be gained. Our results show that the strictly conserved PFFARFL motif of the leader peptide is specifically interacting with both microviridin ligases, thereby orchestrating macrocyclization of microviridins. We further show that manipulation of the C terminus of the leader peptide circumvents lactam ring formation of microviridins. Dissection and manipulation of the recognition motif for microviridin ligases can ultimately guide the reprogramming of microviridin biosynthesis. In vivo manipulation further suggests a scaffolding role for the ABC transporter MdnE. The data obtained in our study highlight a novel mechanism for ribosomal peptide maturation and provide the basis for complete reconstitution of microviridin biosynthesis and rational design of new microviridin variants.

EXPERIMENTAL PROCEDURES

Cultivation of Bacterial Strains and DNA Isolation

M. aeruginosa NIES843 and NIES298 were obtained from the National Institute for Environmental Studies (NIES). MRD UOWOCC was kindly provided by Prof. Brett Neilan from the University of New South Wales,

Australia. Cultures were grown under permanent low light conditions at 23°C in BG-11 medium. DNA of cyanobacterial lab strains was isolated as described previously (Franche and Damerval, 1988).

Strains of *E. coli* (Top10, Epi300T1, XL-I Blue, BL21, XL10) were obtained from Invitrogen, Epicentre, and Stratagene/Agilent Technologies, respectively. Cultures were grown under standard conditions at 37°C and 240 rpm in LB-medium with appropriate antibiotics if necessary. Plasmids isolation was carried out using a QIAprep Spin Miniprep Kit.

Sequencing

Sequencing was carried out commercially in the lab of Dr. Martin Meixner (SMOLBIO) at Humboldt University, Berlin, Germany.

Preparation of Total RNA from Bacterial Cultures

Harvesting of bacterial cell material was performed by pelleting 20–50 ml bacterial cultures for 10 min at 4000 rpm and 4°C. RNA isolation from bacterial cell suspensions was conducted following the Invitrogen Trizol RNA Isolation Kit with a modified homogenization step at 65°C for 20 min at constant shaking after freezing the Trizol-cell-mix in liquid nitrogen.

Northern Hybridization

Prior to Northern blotting RNA agarose gel electrophoresis (50–80 V) was carried out in a 1.5% (w/v) agarose gel supplemented with 3.5% formaldehyde in MEN-buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA pH 8.0) system. Twenty micrograms of total RNA were run. Size separated RNA was transferred to a Hybond N^+ nylon membrane by standard capillary blotting (Southern, 1975). Northern hybridization and detection followed the recommendations of the used Fermentas Biotin Chromogenic Detection/Biotin DecaLable DNA Labeling Kits. Probes against the microviridin precursor gene *mdnA* were designed and hybridized in Church-buffer (0.342 M Na_2HPO_4; 0.158 M NaH_2PO_4; 7% [w/v] SDS; 1 mM EDTA) at 64°C for 10–16 hr following standard protocols.

Heterologous Peptide Synthesis

DUET Vector System

The genes *mdnA*, *mdnB*, *mdnC*, *mdnD*, and *mdnE* from *M. aeruginosa* NIES298 were amplified by Taq polymerase (QIAGEN, Germany) and first cloned into pDrive cloning vector (QIAGEN). Gene products were excised with the appropriate restriction enzymes to be cloned into compatible Duet vectors (Novagen, Madison, WI) with individual cloning sites and T7 promotors for every single gene, resulting in petDuet-1-mdnA, pACYCDuet-1-mdnB/C, pCOLADUET-1-mdnD/E. *E. coli* BL21 with combined Duet vectors has been grown at 37°C until OD_{600} = 0.6. Cultured cells were induced with IPTG to a final concentration of 1 mM and again grown for 3 hr. Cultured cells were harvested and microviridins were purified as described below.

Minimal Expression Platform for Microviridins

The microviridin biosynthetic gene cluster has been amplified from genomic DNA of *M. aeruginosa* NIES843 using Taq DNA Polymerase (QIAGEN) and three different pairs of primers. Primer pair P166: 5′-GGCCGGATTAGTGCT TATGA-3′, P167: 5′-AGGAATTCGCCCTGAGAGTC-3′ amplified the genes *mdnA–E* with a short promotor region of 169 bases. The PCR product was cloned into pDrive cloning vector (QIAGEN, Germany), yielding in the 9.9 kb plasmid pARW066. Analogously, the genes *mdnA–E* including a long promotor region of 714 bases were amplified using primers P173: (5′-TTGCCTTGGGTTTAGTGGAA-3′) and P167. The resulting 10.4 kb plasmid was named pARW071. Primer pair P173 and P97: (5′-gaattcTCAGCAAACCC TACTTAATTTCC-3′) amplified the microviridin cassette from *mdnA–D*, lacking *mdnE*, but carrying the same 714 bp long promotor region as described before. The resulting 7.8 kb plasmid was named pARW081. All variants of the minimal microviridin expression platform were transformed into different strains of *E. coli* and analyzed for their ability to generate microviridin-like peptides.

Mutagenesis of the Microviridin Gene Cluster

Mutations in microviridin expression platform vectors were introduced using the QuikChange II XL Site-Directed Mutagenesis Kit from Stratagene/Agilent Technologies following the manufacturer's instructions. Primers utilized for site directed mutagenesis and subsequent sequencing are listed in Table S1.

Isolation of Microviridins and HPLC Conditions

Four hundred milliliters of *E. coli* Top10 or XL-1 Blue respectively, containing pARW066, pARW071, or pARW081 were cultured at 37°C, 240 rpm for 5–10 hr in LB-medium and cultured cells were harvested by centrifugation at 4°C, 6000 × *g* for 10 min. The precipitated cells were washed once with Tris-HCl, pH 7.4 and resuspended in deionized water. Cell lysis was achieved by sonication. The samples were centrifuged again and purified on C18-Sep-Pak cartridges (Waters, Germany). Before elution with 100% methanol, cartridges were washed once with 5% aqueous methanol (v/v). Eluted compounds were dried in vacuo, and resuspended in 50% aqueous methanol (v/v) and filtered over Aerodisc 4 mm syringe filters (Pall, Germany). Extracts were analyzed by a Shimadzu HPLC system and separated on a Symmetrie-Shield RP18 (3.5 μm, 4.6 × 100 mm; Waters) column. Water containing 0.05% TFA as solvent A and acetonitrile containing 0.05% TFA as solvent B were used as a mobile phase with gradient conditions as follows: flow rate 1 ml/min, 1 min of loading to 20% solvent B, a linear gradient to 52% solvent B within 30 min, followed by a linear gradient to 100% solvent B in 1 min and kept for 2 more min, before returning to 20% solvent B in 3 min, holding for 5 min. Microviridin L isolated from a recombinant *E. coli* was used as a standard. All samples were analyzed by mass spectrometry.

MALDI-TOF MS Analysis

Obtained samples were measured by Bruker Ultraflextreme as described previously (Ziemert et al., 2008).

Purification of Bicyclic Microviridin L and Variants

Five liters of *E. coli* carrying pARW071 with a mutation in the PFFARFL region of MdnA were grown at 37°C for 10 hr were harvested by centrifugation (4000 × *g* for 10 min). The precipitated cells were resuspended in 300 ml of methanol and then lysed by sonication. The methanol extracts were separated by centrifugation (8000 × *g* for 10 min). After the removal of solvent by evaporation, residues were dissolved in acetonitrile/H_2O (1:9) and subjected to ODS column (5.0 × 10.0 cm, YMC GEL ODS-A 60-S50) using eluents (10%, 40%, 80% aqueous acetonitrile, and methanol). The membrane filtered samples were subjected to RP-HPLC (YMC-Pack ODS SH-343-5, 20 × 250 mm, 5 μm, 120 Å, flow rate 12 ml/min) using a gradient system 20% solvent B for 10 min, to 100% solvent B in 40 min (solvent A; 0.1% TFA in H_2O, solvent B; acetonitrile/H_2O 83:17) to yield bicyclic microviridin L1 (1.5 mg) and bicyclic microviridin L (0.4 mg).

Detection of Microviridins from Periplasmic, Cytoplasmic, Membrane, and Total Cell Extracts of *E. coli* and Cyanobacteria

Detection from E. coli Cultures

Four hundred milliliters of *E. coli* Top10, carrying pARW071 and pARW081 as well as plain Top10 cells were grown under standard conditions for 5–7 hr. Cells were harvested by centrifugation at 4000 × *g* for 20 min. The pellet was resuspended in 30 mM Tris-HCl containing 20% sucrose, pH 8 at 80 ml per gram wet weight. Cells were kept on ice and EDTA was added to a final concentration of 1 mM. Cells were kept for 10 min on ice under slightly agitation, followed by centrifugation for 20 min, 8000 × g at 4°C. The pellet was resuspended in the same volume of ice cold 5 mM $MgSO_4$ and stirred for 10 min on ice. The cell suspension was again centrifuged as before. The resulting supernatant was the osmotic shock fluid containing the periplasmic proteins. This fraction was collected and the pellet was washed again with Tris-HCl. 5 ml of deionized water were added to resuspend the pellet. Cell lysis was achieved by sonication. Cells were centrifuged again and the supernatant was collected, representing the cytoplasmic protein fraction. The remaining pellet, representing the membrane fraction, was washed once with water. Resuspension was achieved by the addition of 5 ml methanol and a pulse of sonication. The supernatant was collected. For the comparison of subcellular localization of microviridin in periplasmic, cytoplasmic and membrane extract with the total cell extract, 400 ml of *E. coli* cells were centrifuged at 4000 × *g* for 20 min, the pellet was washed once with Tris-HCl, pH 7.4, centrifuged again and resuspended in 5 ml of deionized water. Sonication was used to achieve a complete cell lysis. Centrifugation was repeated and the clear supernatant comprised the total cell extract. Microviridins were purified from all supernatant fractions and analyzed by HPLC as described above and verified by mass spectrometry.

Detection of Microviridins from Cyanobacterial Cultures

Three hundred milliliters of *M. aeruginosa* NIES298 and NIES843 (OD_{750} = 0.342), respectively, were harvested by centrifugation (6000 × *g*, 10 min, 4°C). Periplasmic proteins were isolated similar to the method described by Fulda et al. (1999) using wash and plasmolysis buffer with 2 mM NaCl.

The osmotic shock supernatant, representing the periplasmic fraction, was collected and the remaining cell pellet was washed once with water. One milliliter of water and the same volume of glass beads were added to the pellet and the mixture were placed in liquid nitrogen. Cells were thawed in a water bath of 42°C. Cells were disrupted using a Retsch mixer mill for 10 min. The freeze, thaw, and disruption procedure was repeated three times. After centrifugation the supernatant containing the cytoplasmic fraction was collected and the pellet was washed again with water, before 4 ml of methanol were added to extract the membrane fraction. Microviridins from isolated membrane fraction together with cytoplasmic and periplasmic fraction were purified on C18 Sep-Pak cartridges as described above.

Total cell extracts were made from 300 ml of an identical culture using the same procedure as described for the cytosolic fraction. Microviridins were purified by C18-Sep-Pak cartridges and injected into RP-HPLC as described above and verified by mass spectrometry.

Subcellular Localization of Microviridin Ligase MdnB in E. coli and Cyanobacteria

Fifty micrograms of protein from periplasmic, cytoplasmic, membrane, and total cell extract from above mentioned 400 ml samples of *E. coli* have been used in SDS-PAGE. Protein content was measured by the Bradford method (Bradford, 1976). SDS gels were stained with GelCode Blue safe Protein stain (Thermo Scientific) and alternately destained three times with 50% acetonitrile (20 min) and rehydrated in water (20 min), incubated for 30 min in 2% SDS followed by incubation for 30 min in SDS/Tris-glycine running buffer. Proteins were transferred to a Hybond-C membrane (GE Healthcare) for immunodetection. The membrane was probed with a previously generated polyclonal anti-MdnB antibody from rabbit in TBS-T (500 mM Tris, 150 mM NaCl, 1% Tween 20, pH 7.5) as primary and anti-rabbit IgG Horseradish Peroxidase conjugate (Sigma) in TBS-T as secondary antibody. Visualization was performed using SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford) and a Lumi-Imager (Boehringer).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at doi:10.1016/j.chembiol.2011.09.011.

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