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Research Article

Functional Expression and Characterization of the Highly Promiscuous Lanthipeptide Synthetase SyncM, Enabling the Production of Lanthipeptides with a Broad Range of Ring Topologies

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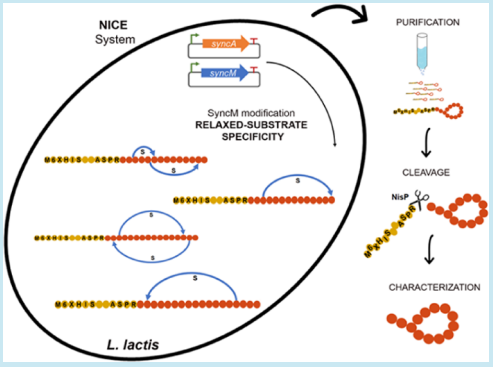
ABSTRACT: Lanthipeptides are ribosomally synthesized and post-translationally modified peptides characterized by the presence of lanthionine rings that provide stability and functionality. Genome mining techniques have shown their huge diversity and potential for the discovery of novel active molecules. However, in many cases, they are not easily produced under laboratory conditions. The heterologous expression of these molecules using well-characterized lanthipeptide biosynthetic enzymes is rising as an alternative system for the design and production of new lanthipeptides with biotechnological or clinical properties. Nevertheless, the substrate-enzyme specificity limits the complete modification of the desired peptides and hence, their full stability and/or biological activity. New low substrate-selective biosynthetic enzymes are therefore necessary for the heterologous production of new-to-nature peptides. Here, we have identified, cloned, and heterologously expressed in *Lactococcus lactis* the most promiscuous lanthipeptide synthetase described to date, i.e., SyncM from the marine cyanobacteria *Synechococcus* MITS9509. We have characterized the functionality of SyncM by the successful expression of 15 out of 18 different SyncA substrates, subsequently determining the dehydration and cyclization processes in six representatives of them. This characterization highlights the very relaxed substrate specificity of SyncM toward its precursors and the ability to catalyze the formation of exceptionally large rings in a variety of topologies. Our results suggest that SyncM could be an attractive enzyme to design and produce a wide variety of new-to-nature lanthipeptides with a broad range of ring topologies.



KEYWORDS: *lanthipeptide, lanthipeptide synthetase, Synechococcus, heterologous expression, lanthipeptide production*

INTRODUCTION

Ribosomally synthesized and post-translationally modified peptides (RiPPS) make up a promising source of bioactive compounds[1] that, in contrast to nonribosomal peptides (e.g. vancomycin, teixobactin, or colistin), do not require multimodular enzyme complexes for their synthesis, which allows the simplification of processes related to their expression, mutation, production, and purification.[2,3] The massive sequencing of bacterial genomes together with genome mining methodologies[4,5] has shown the extraordinary diversity of RiPPs and the broad range of biosynthetic machinery that make this class of natural products an attractive source for "plug-and-play" synthetic biology platform for the creation of novel hybrid RiPPs, combining different modules of enzymes for post-translational modification, processing, and transport.[1,6−9]

Lanthipeptides constitute one of the most studied classes of RiPPs, which are characterized by the presence of thioether cross-links (lanthionine rings). The biosynthesis of lanthipeptides requires a precursor peptide (LanA) with an N-terminal leader and a C-terminal core peptide, which is processed by a specific lanthipeptide dehydratase (LanB) involved in the dehydration of the serine (Ser)/threonine (Thr) residues, and a lanthipeptide cyclase (LanC), catalyzing the lanthionine ring formation by the reaction of cysteine (Cys) residues with the dehydrated amino acids.[10] In class II lanthipeptides, these modifications are carried out by a bifunctional enzyme called LanM.[10] A final step in the maturation of lanthipeptides requires also the activity of a LanT transporter and a LanP protease to release the leader peptide.

Interestingly, this machinery can be used for the heterologous expression of different LanA substrates, with a special focus on lantibiotics,[8,11] a class of lanthipeptides

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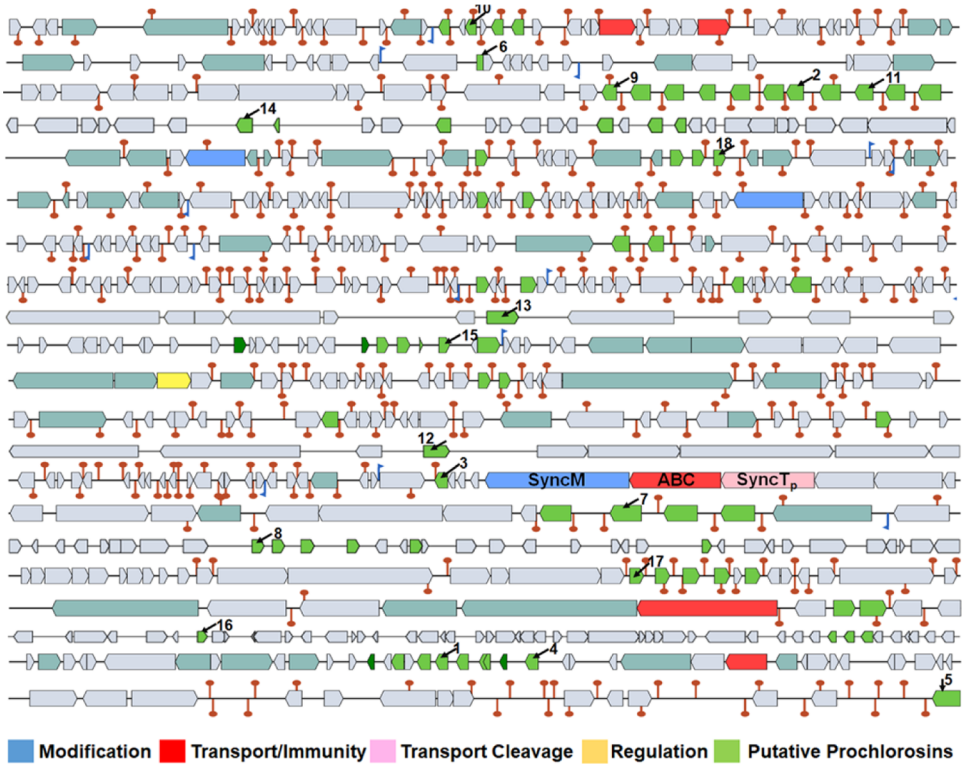

Figure 1. Prochlorosins genome mining with BAGEL4[4] for *Synechococcus* MITS9509. Numbered arrows indicate the diverse positions of selected candidates for heterologous expression in *L. lactis* concerning the processing enzymes.

characterized by the presence of antimicrobial activity, although also lanthipeptides with antifungal, antiviral, morphogenetic, anticonceptive, or antiallodynic functions have been described.[10] Moreover, the lanthipeptide biosynthetic machinery has been used to design new-to-nature lanthipeptides, generating peptides with enhanced activity or stability.[12,13] The heterologous expression of lanthipeptides using the nisin biosynthetic machinery has been broadly used for the heterologous expression of newly designed antimicrobials.[12,14−17] However, the substrate specificity of the synthetase enzymes NisB and NisC limits the production of this type of variant compounds on a large scale. Therefore, new and promiscuous lanthipeptide synthetase enzymes with enhanced properties to fully process a large number of unrelated LanA substrates or with the ability to introduce rings with different topologies could be used for the effective heterologous production of novel bioactive lanthipeptides, either engineered variants or lanthipeptides from different (exotic) origins. In this regard, ProcM-like enzymes constitute a unique class II lanthipeptide modification enzyme family and SyncM is a new example in this group. As a class II lanthipeptide synthetase enzyme, SyncM presents two domains, the N-terminal ATP-dependent dehydratase domain and C-terminal cyclase domain characterized by a very relaxed substrate specificity.[10,18] This characteristic enhances their potential as an alternative system for lanthipeptide bioengineering and production, and therefore, for the future design and heterologous expression of new and potent natural or close-to-nature lanthipeptides with improved or new biological or technological activities.[2,8,13,16,19,20] The first ProcM-like enzyme described was identified in the Gram-negative marine cyanobacterium *Prochlorococcus* MIT9313.[18] This enzyme is able to process up to 29 different substrates, which are named prochlorosins, a group of unusually diverse lanthipeptides[21] found in marine cyanobacterial strains, and with unknown function.[22−25]

In this work, we have characterized the lanthipeptide synthetase SyncM from *Synechococcus* MITS9509, another marine cyanobacterium, with the putative ability to produce at least 79 different prochlorosins using a single ProcM-like enzyme (SyncM), being the most promiscuous lanthipeptide synthetase described to date.[18,22,24] For this purpose, SyncM was cloned and heterologously expressed in *Lactococcus lactis* NZ9000 using the NICE system.[26] Then, the characterization of the enzyme activity was proved successfully by the heterologous expression of 15 out of 18 different prochlorosin precursors with a different structure, dehydration pattern, and ring formation topology, demonstrating that most of the precursors were correctly produced and modified by SyncM.

RESULTS

Genome Mining for ProcM-like Enzymes That Can Modify a Large Number of Substrates. Prochlorosins are members of a large family of naturally occurring lanthipeptides, with large sequence variation, modified by a single bifunctional enzyme called ProcM,[22] and produced by marine cyanobacteria such as *Prochlorococcus* sp.[18] To identify the most promiscuous ProcM-like enzyme to be used as a relaxed biosynthetic scaffold in the design and expression of bioactive lanthipeptides, we analyzed similar enzymes using the ProcM synthetase from *Prochlorococcus* MIT9313 as a query in BlastP.[27] Several LanM enzymes across marine bacteria, belonging to cyanobacteria, α-proteobacteria, and δ-proteobacteria (109 hits for organisms), were identified. Based on amino acid similarity and novelty, 18 different marine strains that contain a LanM lanthipeptide synthetase were selected for

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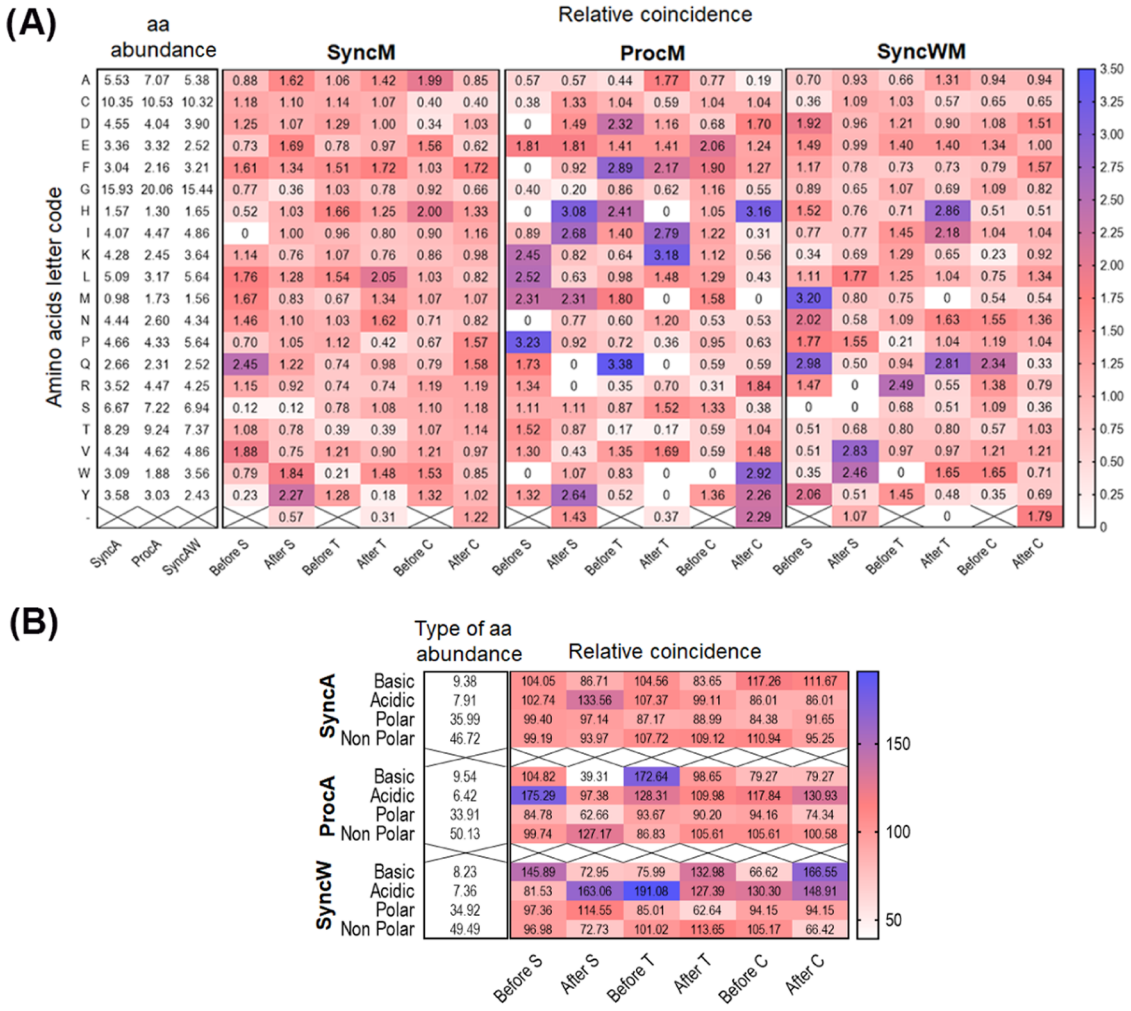

Figure 2. Amino acid profile of prochlorosins from *Synechococcus* MITS9509 (SyncM), *Prochlorococcus* MIT9313 (ProcM), and *Synechococcus* sp UW179A. (A) Amino acid (aa) abundance of 79 SyncA's, 29 ProcA's, and 53 SyncW's is depicted in percentages. Relative coincidence indicates the presence of amino acids at N- or C-terminal positions of Ser, Thr, and Cys of prochlorosin precursors for SyncM, ProcM, and SyncWM, respectively. The values were calculated considering the position and the abundance in the prochlorosin sequences. Values above 1 mean overrepresentation, while values below 1 mean underrepresentation. (B) Summary of the relative presence of amino acids at N- or C-terminal positions of Ser, Thr, and Cys of prochlorosin precursors according to their chemical nature. Values above 100 indicate overrepresentation, while values below 100 indicate underrepresentation.

thorough enzyme and substrate sequence analysis. This analysis resulted in the identification of the "CCG" motif in 15 putative ProcM-like enzymes (Suppl. Table 1, Suppl. Figure 1). This motif has been previously reported[21] as a specific and distinctive characteristic of ProcM-like enzymes. It possesses three zinc-binding sites that have been related to the cyclization step and promiscuity of ProcM.[10,22]

The selected genomes were mined for the presence of putative prochlorosin biosynthetic genes (*procA*) using BAGEL4.[4] These *procA* clusters show genomic flexibility and can be found either in the complete genomic cluster in addition to the processing enzymes, or distributed across the genome in different nonrelated gene clusters[18,24] (Figure 1). The number of precursors found in the selected genomes ranged from 0 to 79 (Suppl. Table 1).[18,24] As reported before in the literature, the leader from this family of RiPPs is highly conserved and has a Gly-Gly cleavage motif,[28] while the C-terminal core part shows very low conservation and an extensive number of ring topologies[18] (e.g., Suppl. Figure 2). Since a higher number of precursors could be related to lower substrate specificity, we selected SyncM from *Synechococcus* sp. MITS9509 as a new class II lanthipeptide synthetase enzyme for cloning and further characterization in a heterologous expression-specialized model organism. In fact, and after an I-TASSER[29,30] structure modeling analysis, the structure template identified by the algorithm was closely related to the LanM enterococcal synthetase CylM[31] that specifically modifies cytolysin S and cytolysin L with a C-score value (−0.01) of the model within the threshold of confidence (−5,2) (Suppl. Figure 3).

Analysis of SyncM Dehydration and Cyclization Amino Acid Tolerance. To explore the putative promiscuity of this enzyme in the dehydration and cyclization activities, we analyzed *in silico* the amino acids present at N- and C-terminal positions of Ser, Thr, and Cys in the 79 putative prochlorosins (called in this work SyncA's) identified in the genome of *Synechococcus* MITS9509. Next, we compared the SyncA's with the 29 putative prochlorosins (ProcA[18]) modified by ProcM[18] and with the 53 putative prochlorisins (SyncW) identified in the genome of *Synechococcus* sp UW179A (Figure 2A,B).

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Table 1. List of SyncA Peptides Selected to Coexpress with SyncM in *L. lactis*[a]

SyncA	Leader peptide	Core peptide	Exp.	Ex. Mass	OD/ED	PR
1	MSEDQLKAFLEKVKSDNSLQEKLKAERVDVVAIAKAAGFSITPDDINSLRENISGKELEAILGG	AGSANPQGGDPWCTWDFPICINHK	++	10727.37	2/2	1
2	MSLEQLKAFLAKVKGDSNLQEKLKAAKSPDEVVSIAEEHGHEFTTDKLSQLSEEELEGVAGG	GCIPFPPYDKNSLLSP	++	9627.83	1/2	1
3	MFVDDIFDQIRYFELVILKSMSKEQLDAFMAKVKTNADLQAKLKDSNSLDDVRGIAKEHGHEIDASAKLSPLSSEELEEVAGG	WCGNACTNWKWSNGAGTGC	-	12006.76	-	-
4	MTQEQLTAFLANAKGNTNLQEQLKAAADTNAVAAIAKEAGFSISDDDLKSAQSKITDEELEGTSGG	EGSWRAGWGPCRVRLSMSPQHKNCRG	++	10530.07	3/3	2
5	MTQEQLKTFLEKVKADTSLQEKLKKAADADEVVAIAKNVGLTISADNLKKAQSELANEELEKVAGG	EQCPLNTGCPLHTGWWCTGVLVDQQPHRSAASNI	++	11528.83	5/5	3
6	MSEEQLKAFLEKVKADNSLQEKLKTAADVDAVLGIANEAGFVISADDVRTEISDSELEGTAGG	GCSFEYGKMGKDKCSR	+++	9186.42	2/2	2
7	MSLEQLKAFLEKVKADTSLQEKLKAAADADAVLAIAKEAGFKISAEDLKNAQVTELSDEELEGVAGG	YQSWDVWDGVGYNC	+	9933.90	1/1	1
8	MSQDQLKAFIEKVKADTSLQENLKAAADVDTALAIAKEAGFLISADDVRTTVSDDELEDAAGGSDDETLTDFGCKDYERY	SDDETLTDFGCKDYERY	+++	9415.45	2/3	1
9	MSEEQLKAFLEKVKGDTSLQEKLKAAADSNAVLAIAKEAGFKISAEDLKTAQVTELSDEELEGAAGG	YLSYDGSERTFGCGGKGCGWVEPPTKIGGGECRDNKLR	+++	11845.81	3/4	2
10	MTQEQLTTFIANAKGNISLQERLKAAADTNAVAAIAKEVGFTISVDDLKRAQSDIPDEVLEGVAGG	ASWSGPMWNTTCIGG	-	9198.53	-	-
11	MSEEQLKAFLDKLKEDTNLQEKLKAAGDSDTFLAIAKKAGFSLSVDDLNQNQSETSEEELEAVAGG	CDIELVSLCSKTHNVLC	-	9770.73	-	-
12	MSEEQLKAFLEKVKDDAILREKLKAAADSDAVVAIAKNVGFAISVDDLQNTQSEMSEEELESVAGG	YYCACTRIPVYNHTN	++	10076.90	2/2	2
13	MSDEQLQFFLAKAKGDTHLQEKLKTAKSAEEVVCLAKEYGCEFTSEKISQLSKEELEHVAGG	CWMNTCWGYGPGTAAAPDWN	+	9854.60	2/2	2
14	MSEEQLKAFLEKVKADTTLQEKLKEAADSDAVLAIAKATGFSITTKDLNSHSQNIPDDELEAGLGG	TDNALNKCTTGCSPSGEVPGYTKPCDNAII	++	10795.24	6/6	3
15	MSLEQLKTFLEKVKGDSNLQEKLKAAKSPEDVIAIAKEHGHKFNSDHLSQLSKEELESVAGG	VTLKYKNWCYCTAL	+	9289.71	2/2	2
16	MTQEQLTAFITNAKGNTSLQERLKVAADTNTVASIAKEAGFSISTDDLNNKAQAEISEGELEGATGG	RIVCVSFGSLFIRRGKLS	+	8704.19	3/3	1
17	MSEEQLKAFLEKVKSDSSLQDKLKAAADVDAALAIAKEAGFTISSDDVRSSVSDDELEGVAAGG	CYNWKSARDY	++	9742.91	1/1	1
18	MSEEQLKAFIEKVQGDDSLQEKLKAAANPDAVVSIAKEAGFMISADDLKEVSEISEEELEGAAGG	SDTNWITEVSKCPWWRRS*	+++	9760.71	4/5	1

[a]Ser and Thr are depicted in green, and Cys is depicted in red. Exp., level of expression observed after induction in *L. lactis*. Ex. Mass, expected mass for the full peptide when all dehydrations are present. OD, observed dehydrations. ED, expected dehydration. PR, putative rings formed. In shadow, peptides selected for further characterizations. [b]Ser C-terminal dehydration could not be confirmed.

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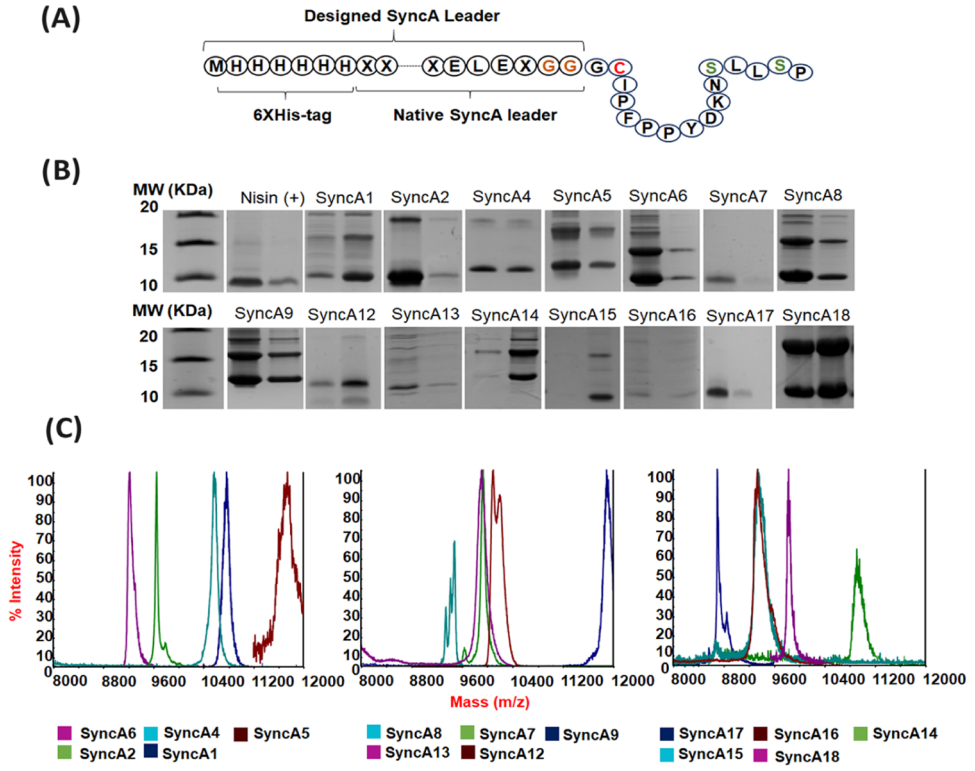

Figure 3. Expression, purification, and identification of new SyncA prochlorosins modified by SyncM. (A) Example of the designed peptides. Each of the peptides was cloned, considering their own leader sequence and adding a 6× histidine tag. (B) Expression of the different peptides. The first two elution fractions of the Tricine-SDS page are shown. Nisin was used as a positive control of induction. (C) MALDI-TOF spectra of purified SyncA's in *Lactococcus lactis* with the NICE system.

Figure 2A shows that, in the case of SyncM, all of the possible amino acids were observed in SyncA precursors at least one time before or after Ser, Thr, and Cys, except Ile in the N-terminal position of Ser. In fact, and despite some amino acids were overrepresented (e.g., Gln or Tyr before and after Ser, respectively, or Leu after Thr, Figure 2A) or underrepresented (e.g., Ser after and before Ser, or some aromatic amino acids before or after Thr, Figure 2) in some positions, the distribution of amino acids before and after the key amino acids involved in the ring formation (Ser, Thr, and Cys) was more homogeneous than ProcM and SyncWM substrates (Figure 2). The amino acid profile of ProcA's was the most restrictive, showing no tolerance to several amino acids (14) at certain positions in some flanking residues (e.g., Asn, His, Phe, or Asp were not found before Ser, neither Gln nor Arg after Ser. His, Met, Gln, Trp, or Tyr were not identified after Thr) and the higher overrepresentation of others (e.g., Pro before a Ser, His after Ser, Gln before a Thr, Ile after Ser and Thr, Gln before Thr, Lys after Thr, or His after a Cys, Figure 2A). In the case of SyncW peptides, the distribution of the amino acids around Ser, Thr or Cys was intermedial, although also less tolerance than for SyncA was observed (Figure 2A). Interestingly and overall, the overrepresentation or underrepresentation of some amino acids at a specific position was not related to the abundance of the amino acid in the ProcA or SyncW peptides, which suggests certain specificities or selectivities. When we consider the chemical nature of the amino acids (Figure 2B), as expected, SyncM substrates have a more homogeneous distribution of flanking residues, where only acidic amino acids show a higher representation after Ser. In contrast, in ProcM substrates, a clear overrepresentation of acidic residues before Ser and basic residues after a Thr occurs, while basic or polar residues were poorly represented after Ser despite a similar abundance of such residues is observed for SyncA and ProcA (Figure 2B). Interestingly, in the case of SyncW, acidic amino acids were overrepresented before Thr and after Ser or Cys, and basic before Ser and after Thr and Cys.

Overall, the amino acid profile analysis and comparison suggest that SyncM could be more tolerable to the positional effect of the amino acids while SyncW has an intermedial tolerance and ProcM could be the most restrictive. This fact could be also related to the lower number of ProcA substrates identified (if less promiscuity implicates less variability in prochlorosins).

To assess the expected SyncM relaxed substrate specificity, we selected 18 different prochlorosins ($SyncA_{1-18}$, Table 1) from the 79 putative lanthipeptides identified in the genome of this bacterium (Figure 1), based on different characteristics, such as the number of putative dehydrations, number of rings, the size of the ring or the diversity in their structure, topology and genomic location. Next, we aimed to modify these prochlorosins by SyncM and to characterize the functionality and specificity of this enzyme in a heterologous expression model organism.

Cloning and Heterologous Coexpression of SyncA and SyncM in *L. lactis*. *E. coli* is one of the most commonly used host organisms for the heterologous production of lanthipeptides,[32] and the marine lanthipeptide prochlorosins are not an exception.[33,34] However, laborious additional purification processes are required due to the frequent accumulation of the produced peptide in inclusion bodies

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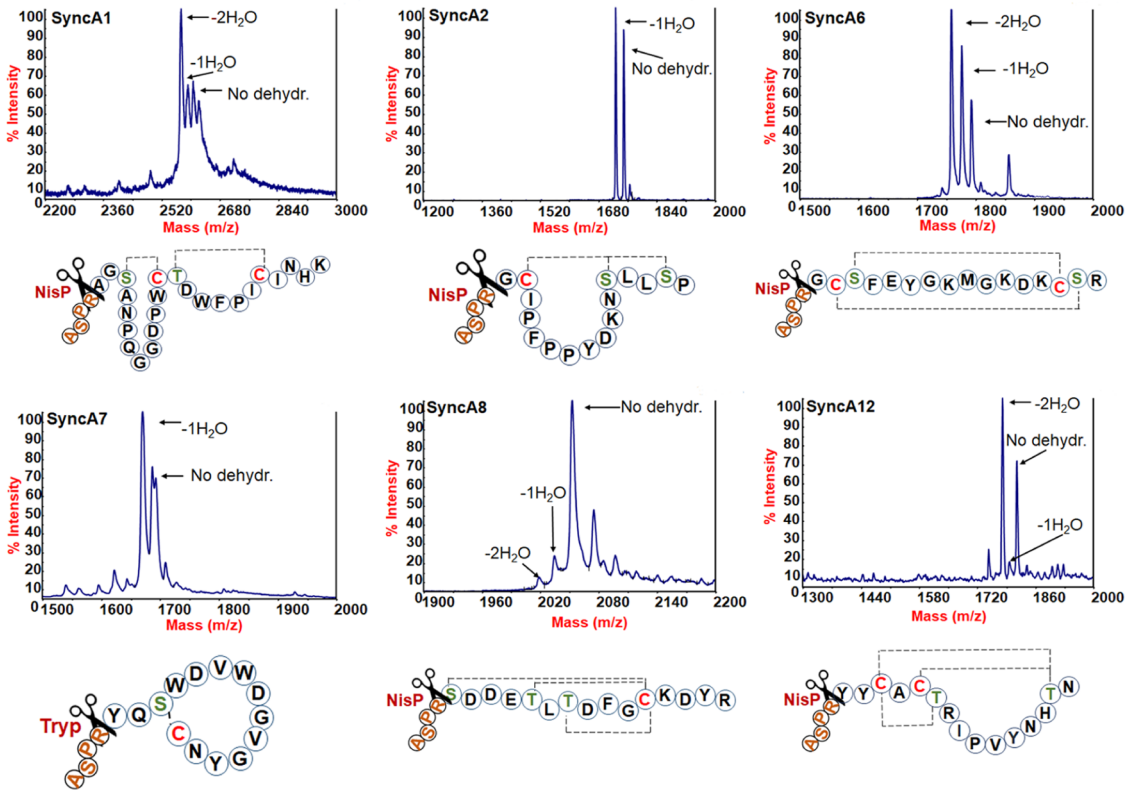

Figure 4. MALDI-TOF for SyncA1, SyncA2, SyncA6, SyncA8, and SyncA12 after NisP leader cleavage and SyncA7 trypsin digestion. The putative structure of the peptides is indicated with the possible rings formed, as well as the dehydration level observed after the MALDI-TOF analysis. Exact ring formation topologies are discussed further in this manuscript.

and the difficulty in the secretion because of the outer membrane. Moreover, the presence of lipopolysaccharide (that also can lead to toxicity) can complicate the downstream applications.[35,36] To avoid these limitations, *L. lactis* offers a well-known, safe, scalable, and broadly used bacterial cell factory platform for the heterologous production of biomedical molecules.[35,37,38] Additionally, this bacterium has been broadly used for lanthipeptide production.[8] Based on these advantages, we used *L. lactis* as a production host to characterize our proposed heterologous SyncM synthetase (Figure 3).[26] Because of the high GC content in the genes of *Synechococcus* strains,[39] a *L. lactis* codon-optimized SyncM synthetic gene was cloned into the plasmid pTLR (Ery^R) under the nisin promoter control (P_{nis}) in the nisin-inducible strain *L. lactis* NZ9000. SyncA substrates with their own leader peptides were also codon-optimized, synthesized, and cloned in the compatible vector pNZ8048 (Cm^R) under the same promoter, and transformed into *L. lactis* NZ9000 pTLR-SyncM, yielding a nisin-dual inducible system.[40] To simplify the purification process, an N-terminal 6xHis-tag was incorporated in each one of the SyncA peptides with its native leader (Figure 3A).

The coexpression of the enzyme and peptides was performed by nisin induction, and subsequently, the cells were disrupted by sonication. The peptides were purified by affinity chromatography followed by HPLC. Of the 18 wild-type SyncA peptides selected, 15 were successfully expressed at different levels and purified (Figure 3B, Table 1) with an estimated yield ranging from 0.125 to 31.25 mg/L, similarly to what has been described for heterologously expressed ProcM-modified peptides in *E. coli*.[34] Finally, the peptides were analyzed by MALDI-TOF by observing the corresponding peaks in the expected mass ranges (Figure 3C, Table 1). At this instance, the exact dehydration profile was not determined yet.

To assess the SyncM dehydratase activity and as a preliminary analysis, the expressed SyncA peptides were trypsinized and the fragments analyzed by MALDI-TOF to determine the dehydration profile of the core peptides (Suppl. Tables 2−10). A comparison between the observed dehydrations and the predicted ones is described in Table 1. Strikingly, the trypsin experiment showed dehydrations present in all of the native peptides. The level of dehydration, fully or partially or nondehydrated peptide, varied from SyncA to SyncA. Within the longest peptides, as in SyncA5 and SyncA14, which contain the highest number of expected dehydrations, i.e., 5 and 6, respectively, peaks corresponding to fully and partially dehydrated peptides were found ranging from $-1H_2O$ to $-5H_2O$ (Suppl. Tables 2 and 3, Suppl. Figure 4). Except for peptides SyncA2, with only one dehydrated Ser, SyncA8 with two Ser and/or Thr dehydrations, SyncA9 in which one Ser was not dehydrated (Suppl. Table 4) or SyncA18 for which the dehydration of the C-terminal Ser was not found in the spectra and thus could not be proven unambiguously (because of the limits of the methodology, by which the fragment was lost), all of the Ser/Thr residues of the peptides were found to be dehydrated. Regardless of the number of dehydrations and considering the number of putative rings to be formed (Table 1), in principle all of the rings could be formed (no peptides have more Cys residues than there are Ser/Thr residues).

In summary, our results confirm that *L. lactis* is able to effectively express SyncM and SyncA prochlorosin precursors using the NICE system in *L. lactis* and a high efficiency in the

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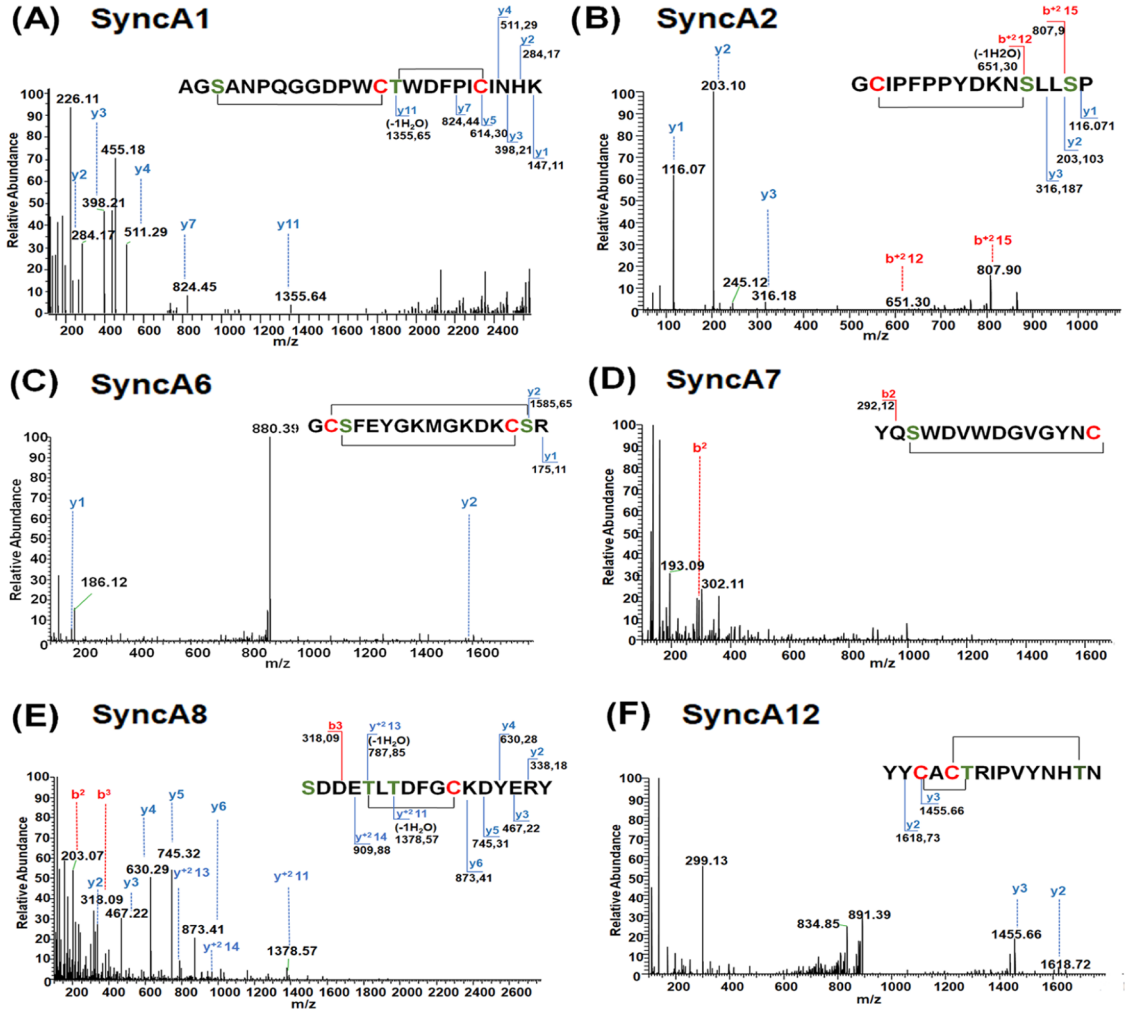

Figure 5. Masses of the LC-MS/MS found for each modified peptide fragmentation pattern of selected peptides. This gives insight into the dehydration and lanthionine ring pattern (black line). (A) SyncA1, (B) SyncA2, (C) SyncA6, (D) SyncA7, (E) SyncA8, (F) SyncA12. Masses of the LC-MS found for each modified peptide are indicated in Suppl. Figure 5. The dotted line indicates secondary ring formation for SyncA1, SyncA6, and SyncA12.

dehydration can be achieved. However, to determine if rings are installed, further analyses are required (see below), so we investigated the processing and SyncM cyclase activity.

Insertion of the NisP Cleavage Site Does Not Hamper the SyncA Maturation. The final activation step of lanthipeptides requires the cleavage of the N-terminal leader from the C-terminal core.[32] The wild-type SyncA's leader has a GG motif[28] for a specific C39 protease found in the $LanT_p$ transporters[24] ($SyncT_p$). To avoid premature activation of the mature peptides and to confirm the heterologous processing of the substrates by SyncM after expression, we engineered the SyncA leader to contain an ASPR cleavage site, which is the natural processing site for NisP that processes nisin precursor, behind the natural −1 G, to guarantee its removal and to obtain mature core peptides. Other commercial proteases have been applied in past studies for the release of prochlorosins.[34] The NisP protease cleavage site has a high substrate tolerance for the cleavage of different antimicrobials heterologously expressed in *L. lactis*, and it is a low-cost candidate protease as it can be induced by nisin and produced in large amounts.[41] To address the ability of NisP to release the leader and to confirm the activity of SyncM, six representative peptides, i.e., SyncA1, SyncA2, SyncA6, SyncA7, SyncA8, and SyncA12 were selected. These representative peptides, containing one or two putative rings, were selected based on their production rate, size, and topology of the putative ring, i.e., to assess whether SyncM can create a large macrocycle, or heterocyclic rings, or rings in N- or C-terminal direction (or both) (Table 1). All of the candidates have a unique dehydration and ring pattern as shown in Figure 4.

After selection, the newly designed peptide genes were cloned into *L. lactis* NZ9000 pTLR-SyncM, nisin-induced, and peptides purified as described before. Finally, purified NisP was added for leader cleavage and the core peptides were analyzed by MALDI-TOF (Figure 4).

As shown in Figure 4, NisP efficiently cleaves almost all peptides, except SyncA7. This lack of cleavage could be due to unknown steric hindrances between the substrate and the protease, (e.g., the effect of the ring size or the topology of the peptide).[14] Taking advantage of the fact that this peptide (SyncA7) does not contain any arginine (R) or lysine (K) within the core, we digested the peptide with trypsin, expecting this protease will cleave just after the ASPR site of NisP.

SyncM was able to dehydrate the selected peptides with different specificity (Figure 4). In the case of SyncA1, although the fully dehydrated peptide is identified in the vast majority,

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there is also a fraction partially dehydrated. For SyncA2, from the two Ser residues, one is dehydrated; only one ring is expected in this peptide. In contrast, in SyncA6 (two Ser) and SyncA12 (two Thr), the two expected dehydrations in the peptides are found in higher intensity, although there are also some molecules with just one dehydration. In SyncA7, the single Ser is also dehydrated. Finally, for SyncA8 three dehydrations were expected, but only two and one dehydration(s) in the molecules are present. We noted that nonmodified peptide was found in all cases, usually in low or moderate amounts (Figure 4), except for SyncA8. These data suggest that SyncM is heterologously and functionally expressed in *L. lactis* even with the ASPR insertion after the Gly-Gly motif in the C-terminal part of the leader peptide. Further LC-MS/MS analysis was performed to analyze the dehydration pattern and exact ring formation in the cleaved peptides.

Analysis of the Ring Formation by SyncM. SyncM is a LanM enzyme acting as a bifunctional enzyme[10] (Suppl. Figure 3), able to dehydrate and form rings into peptide precursor substrates. Once we established the dehydration pattern of the selected peptides, we investigated the ring formation by SyncM. To this end, the mature peptides were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Figure 5).

The fragmentation analysis (Figure 5) showed the same dehydration pattern as observed with the previous mass spectrometric analyses. Furthermore, different rings formation were confirmed. The ring size of each peptide is counted including the first Ser/Thr to the Cys where it reacts. A NEM reaction (Suppl. Figure 6) is performed in all peptides to support ring formation observed in the LC-MS/MS analysis. Specific characteristics of peptides are discussed further.

Single-ring peptides (SyncA2, SyncA7, and SyncA8) were fully modified by SyncM, with specific characteristics to discuss. In SyncA2, an 11-amino-acid ring is formed between Cys2 and Ser12 (Figure 5B, Suppl. Figure 6B). Interestingly, Ser15 was not dehydrated by SyncM. The rigid backbone of the Pro16[11] could play a role in this specific case. Nonetheless, the correct dehydration of Ser close to Pro or in a similar position has been observed for other SyncA (e.g., SyncA4 or SyncA14, Suppl. Tables 2 and 6). SyncA7 (Figure 5D), also, was successfully modified. Fragmentation analysis indicates that a 12-amino-acid ring was installed in Ser3-Cy14, a finding supported by NEM (Suppl. Figure 6D). Noteworthy, in these two single-ring peptides (SyncA2 and SyncA7), rings were formed in different directions.

For SyncA8, overall, LC-MS/MS (Figure 5E) elucidated that it was the less dehydrated peptide. However, the main dehydrated product corresponded to Thr5 that formed the unique ring with Cys12. Regarding the two-time dehydrated population (Thr5 and Thr7), it was present but in even less proportion. The low dehydration level could be related to the high number (5) of negatively charged amino acid residues present.[11] But, in Figure 2A, we found that negatively charged amino acids are quite regularly present, flanking Ser/Thr, so it is possible that this observation only applies to this case. Moreover, the lack of dehydration of Ser in the first position is also intriguing, since, in the trypsin digestion analysis, peptides with a similar N-core terminal (SyncA14 and SyncA18) their Ser/Thr were dehydrated. Thus, other unknown sterical or topological motives could be involved in the processing, making exact predictions on the action of SyncM more difficult. Interestingly, and despite the scarce dehydration level observed, the NEM reaction showed that the ring was efficiently formed in the $-2H_2O$ (2021 Da) and $-1H_20$ dehydrated (2043 Da) peptide (Suppl. Figure 6E). The preference of SyncM for dehydrating Thr5 is unclear.

For two-ring peptides SyncA1, SyncA6, and SyncA12 LC-MS/MS (Figure 5A,C,F), the analysis showed fully (two rings) and partially modified peptides. This could be similar to ProcM, where once all Ser/Thr are dehydrated, the rings are formed consequently slower in time[42] giving rise to a mixed peptide population. In SyncA1 (Figure 5A), the expected ring formation was found between dehydrated Ser3-Cys13 and Ser14-Cys20. The different ring formation was confirmed with NEM (Suppl. Figure 6A), and fully modified peptide (2578 Da) or a partially modified peptide with two dehydrations and one ring (2704 Da) was present. Differently, for SyncA12 (Figure 5F), our data suggest that SyncM is able to form both rings efficiently. The rings were installed from Cys3 to Thr6 and Cys5-Thr14. The reasons behind this are unclear, but it could be related to the importance of both rings for peptide stability or the small size of the overlapping ring (4 amino acid). This was corroborated with NEM (Suppl. Figure 6F), where the fully modified peptide (1782 Da) is the most abundant, and also we identified that the population of one-ring peptide corresponds to the second ring (Cys5-Thr14).

An outstanding peptide is SyncA6 (Figure 5C). The complexity of this peptide involves two macrocycles with either 12 or 14 amino acids (including the lanthionine), as well as the introduction of overlapping rings in both directions, N- to C- and C- to -N. Strikingly, the two-times charge mass that corresponds to the fully modified peptide (880.39 Da) was found (Figure 5C). However, after the NEM analysis, we observed that the formation of the 12-amino-acid ring (Ser3-Cys14) was the most abundant, which could indicate a C-terminal preference in the ring formation, in this specific substrate (Suppl. Figure 6C). This is opposite to what we observed in SyncA12, also with an overlapping ring, where the one-ring population is formed from N- to C-.

DISCUSSION

The biological introduction of lanthionine rings in biologically active peptides, the engineering of single-ring lanthipeptides, or the design of new close-to-nature antimicrobial RIPPs based on NRPs and/or PKS macrocyclic structures have been discussed as an attractive way to increase the biological activity and/or stability of small peptides and also as a platform for new drug molecules engineering or discovery, escaping from complex chemical reactions and processes.[20,43,44] However, the biological synthesis of these molecules requires usually specific enzymes with low substrate tolerance that render poorly dehydrated/cyclized peptides and therefore less active or stables.

Here and after a BLAST-P analysis, we have cloned, heterologously expressed, and biologically characterized the ClasII LanM synthetase SyncM (Suppl. Figure 3) from the marine cyanobacteria *Synechococcus* sp. MITS9509, which has been identified to be able to process at least 79 different substrates (SyncA) after a gene mining analysis (Figure 1). As far as we know, and considering this, SyncM is probably the most substrate-tolerant LanM enzyme described to date. In fact, previous studies have shown that the presence of certain amino acids either in front of or behind Ser, Thr, and Cys residues can reduce the activity of the enzymes involved in

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Table 2. Strains and Plasmids Use in This Work

strain	characteristic	references
Escherichia coli TOP-10	F-, mcrA, Δ(mrr-hsdRMS-mcrBC),φ80lacZΔM15, ΔlacX74, nupG, recA1, araD139, Δ(ara-leu)7697, galE15, galK16, rpsL(StrR), endA1, λ-	Thermo Fisher Scientific
pUC57-SyncM	Amp^R, *sync*M synthetic gene cloned under P_{nis} promoter	this work
pUC57-SyncA1−2	Amp^R, synthetic genes for *sync*A1 and *sync*A2	this work
pUC57-SyncA3−6	Amp^R, synthetic gene for prochlorosins *sync*A3, *sync*A4, *sync*A5, and *sync*A6	this work
pUC57-SyncA7−10	Amp^R, synthetic gene for prochlorosins *sync*A7, syncA8, *sync*A9, and *sync*A10	this work
pUC57-SyncA11−14	Amp^R, synthetic gene for prochlorosins *sync*A11, *sync*A12, *sync*A13, and *sync*A14	this work
pUC57-SyncA15−18	Amp^R, synthetic gene for prochlorosins *sync*A15, *sync*A16, *sync*A17, and *sync*A18	this work
Lactococus lactis NZ9000	pepN:nisRK	53
pTLR	Ery^R, *E. coli-L. lactis* shuttle vector	Solmeglas, Madrid, Spain[54]
pNZ8048	Cm^R	40
pNZ8048-SyncA (1−18)	Each *syncA* genes cloned in pNZ8048, under P_{nis} promoter	this work
pNZe-NisP8H	Ery^R, Cm^R. NisP producer strain	41
pTLR-SyncM	Ery^R, *sync*M cloned in pTLR under P_{nis} promoter	this work
pTLR-SyncM pNZ8048-SyncA(1−18)	Ery^R, Cm^R, 18 different strains with the different *sync*A genes (1−18) cloned individually under P_{nis} promoter in pNZ8048 in *L. lactis* NZ9000 pTLR-SyncM	this work

dehydration and cyclization, and thus, these amino acid residues are not commonly found at such locations.[11,45] In the case of SyncM and after a positional amino acid *in silico* analysis (Figure 2) considering the key amino acids involved in dehydration and cyclization (Ser, Thr, and Cys), we observed high amino acid tolerance in comparison to other close-related LanM enzymes as ProcM or even other putative SyncM-related enzymes identify in other *Synechococcus* strains as SyncWM (Figure 2).

To characterize dehydration and cyclization patron *in vitro*, coexpression of SyncM and 18 representative SyncA natural substrates (from which 15 were produced) was performed (Figure 3). Interestingly, the observed dehydration profile *in vitro* supports the overview indicated in Figure 2A that, unlike other LanB dehydratases, SyncM is able to dehydrate Ser/Thr rather independently of the surrounding amino acids, indicating that this enzyme is a good candidate for a promiscuous system for the expression of engineered bioactive lanthipeptides. However, some of the Ser or Thr residues can escape dehydration, as is common in the best-studied lantibiotic nisin, where Ser-29 never gets dehydrated.[46] If there is a preference for dehydration, the current data cannot support a preference for either Ser or Thr as observed for other lanthipeptide dehydratases for which Ser is less readily dehydrated than Thr.[46]

From all dehydration patterns that we observed in the different SyncA's, it is difficult to clearly establish which directionality governs the dehydration in SyncM, as in three specific peptides, nondehydrated Ser was found either at the N- or C- terminal region. This nondirectional order of processing N- to C-terminal directions or vice versa for the dehydrations has also been observed in class II and class III lanthipeptides biosynthetic systems.[10] However, further research is needed to determine this. In the case of ProcM, it has been demonstrated that dehydration occurs from the C- to -N terminus, for ProcA2.8 and ProcA3.3.[10,47] Other class II enzymes like LctM (lacticin 481) and HalM1−2 (haloduracin) strongly support that it has an N- to C-directionality.[10] This implies that Ser/Thr closer to the leader region (N-terminus) would have more probability to be dehydrated than the ones located near the C-terminus.[48] The same observation has been made in the case of class I NisB/NisC-modified peptides.[49]

In the case of cyclization, an important highlight from our results is the ability of SyncM to install 12- or 14-amino-acid ring (Figure 6). Macrocycles are usually not easily installed by lanthipeptide cyclases.[11] From the characterized ProcA substrates modified by ProcM, to date, the largest ring installed is 11 amino acids long (ProcA3.3), with a small inner ring,[25] which is already considered big. Furthermore, a clear pattern in the ring formation could not be identified since the enzyme was able to insert rings either from N- Ser/Thr to Cys- in C-terminus or vice versa, independently of the size.[47] Moreover, cyclization appears to be more effective in single-ring peptides than in peptides with two rings, except for SyncA12. This is in line with the results described for ProcM that its relaxed substrate specificity goes at the cost of a decrease in the efficiency of subsequent ring formations, meaning that after the first ring, the second and with each consecutive further cyclization, the enzyme will slow down the formation of the next.[25,42] However, the determinant for the ring formation by ProcM is different from other class II enzymes, i.e., LanM (HalM2) or class I NisB/NisC that function from N- to C- terminal. Previous studies support the hypothesis that cyclization is probably determined by the sequence of the precursor[10,25] and even a coexisting non-cyclized form and cyclized form with different roles cannot be excluded. A structural NMR analysis of five prochlorosins showed some characteristics that could explain this, although the cyclization process remained elusive.[50] Together these results support a scenario where the different patterns in ring formation observed in our selected peptides by SyncM may also be determined by the peptide conformation, but further work is required to be able to substantiate this idea.

Overall, in this work, we describe the remarkable substrate tolerance of SyncM highlighting its ability to form exceptionally large rings, encompassing up to 14 residues (including the lanthionine), which could be well applicable to engineer single-ring lanthipeptides or close-to-nature lanthipeptides designed with NRPS and/or PKS macrocyclic structures, which have been discussed as an attractive scaffold for drug engineering and discovery.[20,51,52]

CONCLUSIONS

In this work, we aimed to design an efficient heterologous expression system using the novel promiscuous ProcM-like

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Table 3. USER Primers for Cloning of SyncM and SyncM to pTLR and pNZ8048, Respectively[a]

name	sequence
pTLR-user-rv	*AGATCACGAGC**U***CGCGAAAGCTTGTTGTAACTGAAAAAG
pTLR-user-fw	*AAGCATGCGG**U***GTCGACCGATGCCCTTGAGAGCCTTCAAC
Pnis-user-fw	*AGCTCGTGATC**U***AGTCTTATAACTATACTGACAATAGAAAC
LanM-USER-rv	ACCGCATGCT***U***CTGCTTTTTGGCTATCATTCAAGCTTAACACGTG
pNZ-user-rv	*ATGGTGAGTGCC**U***CCTTATAATTTATTTTG
pNZery-user-fw	*AAGCATGCGG**U***CTTTGAACCAAAATTAGAAAACCAAGGCTTG
Proc33-USER-fw	AGGCACTCACCA***U***GAGCCATCATCATCATCATCATCACGAA
SyncA2-USER-fw	AGGCACTCACCA***U***GAGCCATCACCACCATCATCATCATTTAGAACAAC
ProcA-USER-rv	ACCGCATGCT***U***AACACGTGAATTTGTTTAATTGCCATTTCAATTG

[a]Italic letters are the homology between neighboring fragments, and at the 3′ end of the homology region is an uracil residue.

enzyme, SyncM, for the heterologous modification of its precursor peptides, denoted in this study as SyncA's in *L. lactis*. Our results confirm the functionality of SyncM and show the relaxed specificity of this enzyme to dehydrate Ser and Thr in a broad range of substrates, mostly independent of the flanking amino acids. Notably, SyncM was able to introduce rings up to 14 amino acids and in both directions (N-terminal or C-terminal). The high amino acid tolerance (for both dehydration and cyclization) to the surrounding Ser, Thr, and Cys amino acids suggests that the conformation of the peptide, rather than the adjacent residues, could be essential for that function. This study adds SyncM to the biosynthetic tools available for lanthipeptide heterologous production in *Lactococcus lactis*. This is a new promiscuous enzyme with extraordinary flexibility to be used as an alternative to traditional systems for the rational engineering and production of lanthipeptides/lantibiotics, especially those that would need a macrocycles, which might be mimicked with this ring-forming system by the RiPP-based technology.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. *E. coli* TOP-10 was grown in LB (FORMEDIUM, England) medium at 37 °C and 220 rpm shaking. This strain was used for plasmid maintenance of the synthetic genes (GeneScript, Europe). Ampicillin at 100 μg/mL or erythromycin (Sigma-Aldrich) at 250 μg/mL were added when necessary. *Lactococcus lactis* NZ9000 was used for the expression assays. *L. lactis* was grown in M17 (Difco, Le pont de CLaix, France) + 0.5% glucose (GM17) at 30 °C without shaking for genetic manipulation, protein expression, and purification assays. Chloramphenicol and/or erythromycin (Sigma-Aldrich, Darmstadt, Germany) was added at 5 and 10 μg/mL when needed. A list of strains and plasmids is shown in Table 2.

Blast ProcM-Like Enzymes. ProcM-like enzymes were identified using the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). We performed BLAST-P[27] using *Prochlorococcus marinus* MIT9313 as a query sequence. We analyzed all significant hits. To identify putative ProcM-like enzymes and putative precursor prochlorosin sequences, we performed BAGEL4.[4] A ProcM-like enzyme from *Synechococcus* sp. MITS9509 was selected because of the large diversity of its 79 putative prochlorosins productions, possible function, and a future application as a biosynthetic tool for its possible promiscuity.

Molecular Cloning. Six synthetic genes were designed and codon-optimized for *L. lactis* with the GeneSmart tool from GeneScript adding a six-histidine tag at the N-terminus of every precursor prochlorosin. All of the synthetic genes were provided cloned in pUC57 plasmid (Amp^R) by GeneScript and cloned into *E. coli* TOP-10 for their propagation and isolation.

For the cloning of all synthetic genes in their respective plasmid, primers were designed for USER ligation.[55] The SyncM enzyme encoding gene was cloned in the pTLR-plasmid, using the primers pTLR-user-RV and FW to amplify the backbone and Pnis-user-Fw and LanM-user-RV to amplify SyncM together with the P_{nis} promoter. Further, each SyncA was cloned in pNZ8048. For this, the backbone pNZ8048 was amplified, using pNZery-user-fw and pNZ-user-rv (Table 3). Differently, the *syncA* genes were grouped in five different pUC57 plasmids (Table 3). Each pUC57 with a different group of *syncA*' genes was first digested with *Xba*I and *Hin*dIII located between each gene, yielding single-cloned *syncA's*. Subsequently, they were amplified with USER primers. The same reverse (ProcA-rv) for every reaction and different forward (SyncA2-USER-fw and Proc33-USER-fw) primers were used.

PCR and digestion products were purified using the PCR cleaning kit (MN, Duren, Germany). Subsequently, a ligation reaction was performed according to the manufacturer's instruction, using the USER enyme[55] (New England Biolabs, Massachusetts) with a 1:1 ratio of fragment and backbone. Ligations were dialyzed against ultrapure water and transformed into electrocompetent cells of *L. lactis* NZ9000 pTLR-SyncM using a Bio-Rad Gene Pulser (Bio-Rad, Richmond, CA). Preparation of *L. lactis* NZ9000 electrocompetent cells and transformation was performed following Holo and Nes.[56] Finally, all plasmid constructs were confirmed by DNA sequencing (Macrogen Europe, Amsterdam, The Netherlands).

Once all sequences were confirmed, the plasmids containing precursor peptides were isolated and then transformed into the electrocompetent *L. lactis NZ*9000 pTLR_SyncM strain.

Purification of Expressed Peptides. We inoculated 800 mL of GM17 + 5% glucose with a 1/50 dilution from an overnight culture in GM17 of each newly designed strain. When the cultured media reached an OD_{600} of 0.4−0.5, it was induced using 5 ng/mL of nisin and the cells were incubated for 18 h. Then, the cultures were harvested by centrifugation (4 °C, 8000 rpm, 40 min), the supernatant was discarded, the pellet was resuspended in binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 30 mM imidazole, pH 7.4), and cells were lysed by sonication (VibraCell, 30 s ON, 10 s OFF, 75% amplitude). After sonication, the lysates were centrifuged and the peptides were purified from the supernatant through a Ni-NTA agarose (Qiagen) column previously equilibrated with a binding buffer according to the suppliers. Peptides were eluted with an

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elution buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 0.5 M imidazole, pH 7.4). To confirm peptide production, 12 μL of each sample was run in a tricine-gel[57] and positive fractions were purified by a reversed-phase C18 (Phenomenex Aeris 250 × 4.6 mm, 3.6 μm particle size, 100 Å pore size) Agilent Infinity HPLC system. Samples were filtrated through a 0.2 μm filter (Phenomenex) before passing through the HPLC. The column was equilibrated in 5% of solvent B (100% acetonitrile: 0.1% trifluoroacetic acid) and 95% solvent A (ultrapure water plus 0.1% TFA), and then a linear gradient from 5 to 100% of solvent B was applied to elute the peptides. Finally, pure fractions were collected, lyophilized, weighed, resuspended in solvent A, and analyzed by matrix-assisted laser desorption/ionization with a time-of-flight detector (MALDI-TOF).

Peptide mass fingerprinting[58] for protein identification was performed for preliminary assessment of dehydration of expressed wild-type SyncA's. For this, we digested all expressed peptides with trypsin, for later analysis by MALDI-TOF. Finally, we looked for theoretical masses that corresponded to the core peptide (Supporting Information Tables 2−10). The reaction was made as follows: Trypsin (Sigma) was resuspended in 1 mM HCL at a concentration of 1 mg/mL. Lyophilized peptide was dissolved in 100 mM ammonium bicarbonate (Merck), pH 8.5. We added the trypsin solution to the substrate (ratio 1:20) and incubated it for 2 h at 37 °C.

Mass Spectrometry. Each sample (1 μL) was spotted on the target and dried at room temperature. Then, 0.5 μL of α-cyano-4-hydroxycinnamic acid matrix (3 mg/mL) was spotted over the samples. Once the samples were dried, the mass spectrometry analysis was performed using a 4800 plus MALDI/TOF analyzer (Applied Biosystems) operated in MS linear mid-mass positive mode.

NisP Purification and Leader Cleavage. For leader peptide release, the NisP protease was used. NisP was purified following Montalbán-López et al.[41] protocol using *L. lactis* pNZe-NisP8H as a producer strain and HisTrap excel column for purification according to suppliers (GE Healthcare). For the leader cleavage, the peptides were freeze-dried and resuspended in 50 mM ammonium acetate (Sigma-Aldrich) adjusted to pH 6.5. An appropriate amount of NisP was added and incubated at 37 °C for 2 h.[2] Finally, the digestion was analyzed by MALDI-TOF as described above.

Trypsin digestion was performed for the release of the SyncA7 core peptide and for a peptide mass fingerprint for the rest of the WT peptides.

LC-MS/MS. Online chromatography of peptides was performed with an Ultimate 3000 nano-HPLC system (Thermo Fisher Scientific) coupled online to a Q-Exactive-Plus mass spectrometer with a NanoFlex source (Thermo Fisher Scientific) equipped with a stainless steel emitter. Samples were loaded onto a 5 mm × 300 μm i.d. trapping microcolumn packed with PepMAP100 5 μm particles (Dionex) in 0.1% FA at the flow rate of 20 μL/min. After loading and washing for 3 min, the peptides were forward-flush eluted onto a 50 cm × 75 μm i.d. nanocolumn, packed with Acclaim C18 PepMAP100 2 mm particles (Dionex). The column temperature was 40 °C. The following mobile phase gradient was delivered at the flow rate of 300 nL/min: 2−85% solvent B in 60 min; 85% solvent B during 5 min, and back to 2% solvent B in 1 min and held at 2% solvent B for 15 min. Solvent A was 100:0 H_2O/acetonitrile (v/v) with 0.1% formic acid, and solvent B was 0:100 H_2O/acetonitrile (v/v) with 0.1% formic acid.

MS data were acquired using a data-dependent top-10 method dynamically choosing the most abundant not-yet-sequenced precursor ions from the survey scans (300−2000 Th) with a dynamic exclusion of 5 s. Sequencing was performed via higher-energy collisional dissociation fragmentation with a target value of 1×10^4 ions determined with predictive automatic gain control. Isolation of precursors was performed with a window of 2 Da. Survey scans were acquired at a resolution of 70 000 at m/z 200. Resolution for HCD spectra was set to 17 500 at m/z 200 with a maximum ion injection time of 100 ms. Normalized collision energy was set at 28. Furthermore, the S-lens RF level was set at 60 and the capillary temperature was set at 250 °C. Precursor ions with single, unassigned, or five and higher charge states were excluded from fragmentation selection.

NEM Reaction. NEM reactions were performed to test whether the rings were formed. *N*-Ethylmaleimide will bind to free Cys adding a mass of 125.13 Da, in case no ring is present. The reaction involved first dissolving the peptide in reaction buffer (0.1 M phosphate, 0.15 M sodium chloride; pH 7.2) with a minimum of 1 mg/mL final concentration, followed by adding a 10-fold molar excess of NEM previously dissolved in water (100 mM). After 2 h of incubation, we dialyzed the blocked protein to remove excess of NEM at room temperature and masses were analyzed by MALDI-TOF.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00224.

> Putative ProcM-like enzymes, MALDI-TOF analysis after trypsin digestion of SyncA's, alignment of ProcM-like enzyme highlighting the "CCG" motif, sequence logo of putative prochlorosins, SyncM model, SyncA5 and SyncA14 MALDI-TOF of dehydration analysis, LC-MS of selected SyncA's, and MALDI-TOF spectrum of the NEM reaction (PDF)

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Author Contributions

O.P.K. conceived and directed the research and corrected the manuscript. P.A.O. designed, performed, and analyzed experiments. P.A.O. wrote the manuscript and prepared the figures. R.C. helped in the design of the experiments, provided daily supervision, performed the *in silico* analysis of the amino acid profile of prochlorosins, and wrote parts of the manuscript. M.I. and J.L. performed and analyzed experiments.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Montalbán-López, M.; Zhou, L.; Buivydas, A.; van Heel, A. J.; Kuipers, O. P. Increasing the success rate of lantibiotic drug discovery by synthetic biology. *Expert Opin. Drug Discovery* **2012**, *7*, 695−709.
(2) Zhao, X.; Li, Z.; Kuipers, O. P. Mimicry of a non-ribosomally produced antimicrobial, brevicidine, by ribosomal synthesis and post-translational modification. *Cell Chem. Biol.* **2020**, *27*, 1262−1271 e1264.
(3) Ortega, M. A.; van der Donk, W. A. New Insights into the biosynthetic logic of ribosomally synthesized and post-translationally modified peptide natural products. *Cell Chem. Biol.* **2016**, *23*, 31−44.
(4) van Heel, A. J.; de Jong, A.; Song, C.; Viel, J. H.; Kok, J.; Kuipers, O. P. BAGEL4: a user-friendly web server to thoroughly mine RiPPs and bacteriocins. *Nucleic Acids Res.* **2018**, *46*, W278−W281.
(5) Weber, T.; Blin, K.; Duddela, S.; Krug, D.; Kim, H. U.; Bruccoleri, R.; Lee, S. Y.; Fischbach, M. A.; Müller, R.; Wohlleben, W.; Breitling, R.; Takano, E.; Medema, M. H. antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res.* **2015**, *43*, W237−W243.
(6) Montalbán-López, M.; Scott, T. A.; Ramesh, S.; Rahman, I. R.; van Heel, A. J.; Viel, J. H.; Bandarian, V.; Dittmann, E.; Genilloud, O.; Goto, Y.; Grande Burgos, M. J.; Hill, C.; Kim, S.; Koehnke, J.; Latham, J. A.; Link, A. J.; Martinez, B.; Nair, S. K.; Nicolet, Y.; Rebuffat, S.; Sahl, H. G.; Sareen, D.; Schmidt, E. W.; Schmitt, L.; Severinov, K.; Sussmuth, R. D.; Truman, A. W.; Wang, H.; Weng, J. K.; van Wezel, G. P.; Zhang, Q.; Zhong, J.; Piel, J.; Mitchell, D. A.; Kuipers, O. P.; van der Donk, W. A. New developments in RiPP discovery, enzymology and engineering. *Nat. Prod. Rep.* **2021**, *38*, 130−239.
(7) van Heel, A. J.; Mu, D.; Montalban-Lopez, M.; Hendriks, D.; Kuipers, O. P. Designing and producing modified, new-to-nature peptides with antimicrobial activity by use of a combination of various lantibiotic modification enzymes. *ACS Synth. Biol.* **2013**, *2*, 397−404.
(8) Montalbán-López, M.; van Heel, A. J.; Kuipers, O. P. Employing the promiscuity of lantibiotic biosynthetic machineries to produce novel antimicrobials. *FEMS Microbiol. Rev.* **2017**, *41*, 5−18.
(9) Ruijne, F.; Kuipers, O. P. Combinatorial biosynthesis for the generation of new-to-nature peptide antimicrobials. *Biochem. Soc. Trans.* **2021**, *49*, 203.
(10) Repka, L. M.; Chekan, J. R.; Nair, S. K.; van der Donk, W. A. Mechanistic understanding of lanthipeptide biosynthetic enzymes. *Chem. Rev.* **2017**, *117*, 5457−5520.
(11) Rink, R.; Kuipers, A.; de Boef, E.; Leenhouts, K. J.; Driessen, A. J. M.; Moll, G. N.; Kuipers, O. P. Lantibiotic structures as guidelines for the design of peptides that can be modified by lantibiotic enzymes. *Biochemistry* **2005**, *44*, 8873−8882.
(12) Li, Q.; Montalban-Lopez, M.; Kuipers, O. P. Feasability of introducing a thioether ring in Vasopressin by *nisBTC* Co-expression in *Lactococcus lactis*. *Front. Microbiol.* **2019**, *10*, 1109.
(13) Kluskens, L. D.; Kuipers, A.; Rink, R.; de Boef, E.; Fekken, S.; Driessen, A. J. M.; Kuipers, O. P.; Moll, G. N. Post-translational modification of therapeutic peptides by NisB, the dehydratase of the lantibiotic nisin. *Biochemistry* **2005**, *44*, 12827−12834.
(14) Cebrián, R.; Macia-Valero, A.; Jati, A. P.; Kuipers, O. P. Design and expression of specific hybrid lantibiotics active against pathogenic *Clostridium* spp. *Front. Microbiol.* **2019**, *10*, 2154.
(15) Schmitt, S.; Montalbán-López, M.; Peterhoff, D.; Deng, J.; Wagner, R.; Held, M.; Kuipers, O. P.; Panke, S. Analysis of modular bioengineered antimicrobial lanthipeptides at nanoliter scale. *Nat. Chem. Biol.* **2019**, *15*, 437−443.
(16) Moll, G. N.; Kuipers, A.; Rink, R.; Bosma, T.; de Vries, L.; Namsolleck, P. Biosynthesis of lanthionine-constrained agonists of G protein-coupled receptors. *Biochem. Soc. Trans.* **2020**, *48*, 2195−2203.
(17) van Heel, A. J.; Kloosterman, T. G.; Montalban-Lopez, M.; Deng, J.; Plat, A.; Baudu, B.; Hendriks, D.; Moll, G. N.; Kuipers, O. P. Discovery, production and modification of five novel lantibiotics using the promiscuous nisin modification machinery. *ACS Synth. Biol.* **2016**, *5*, 1146−1154.
(18) Li, B.; Sher, D.; Kelly, L.; Shi, Y.; Huang, K.; Knerr, P. J.; Joewono, I.; Rusch, D.; Chisholm, S. W.; van der Donk, W. A. Catalytic promiscuity in the biosynthesis of cyclic peptide secondary metabolites in planktonic marine cyanobacteria. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 10430−10435.
(19) Karbalaei-Heidari, H. R.; Budisa, N. Combating antimicrobial resistance with new-to-nature lanthipeptides created by genetic code expansion. *Front. Microbiol.* **2020**, *11*, No. 590522.
(20) Yang, X.; Lennard, K. R.; He, C.; Walker, M. C.; Ball, A. T.; Doigneaux, C.; Tavassoli, A.; van der Donk, W. A. A lanthipeptide library used to identify a protein-protein interaction inhibitor. *Nat. Chem. Biol.* **2018**, *14*, 375−380.
(21) Zhang, Q.; Yang, X.; Wang, H.; van der Donk, W. A. High divergence of the precursor peptides in combinatorial lanthipeptide biosynthesis. *ACS Chem. Biol.* **2014**, *9*, 2686−2694.
(22) Yu, Y.; Mukherjee, S.; van der Donk, W. A. Product formation by the promiscuous lanthipeptide synthetase ProcM is under kinetic control. *J. Am. Chem. Soc.* **2015**, *137*, 5140−5148.
(23) Tang, W.; van der Donk, W. A. Structural characterization of four prochlorosins: A novel class of lantipeptides produced by planktonic marine cyanobacteria. *Biochemistry* **2012**, *51*, 4271−4279.
(24) Cubillos-Ruiz, A.; Berta-Thompson, J. W.; Becker, J. W.; van der Donk, W. A.; Chisholm, S. W. Evolutionary radiation of lanthipeptides in marine cyanobacteria. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E5424−E5433.
(25) Le, T.; van der Donk, W. A. Mechanisms and evolution of diversity-generating RiPP biosynthesis. *Trends Chem.* **2021**, *3*, 266−278.
(26) Mierau, I.; Leij, P.; van Swam, I.; Blommestein, B.; Floris, E.; Mond, J.; Smid, E. J. Industrial-scale production and purification of a heterologous protein in *Lactococcus lactis* using the nisin-controlled gene expression system NICE: The case of lysostaphin. *Microb. Cell Fact.* **2005**, *4*, 15.
(27) Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403−410.
(28) Haft, D. H.; Basu, M. K.; Mitchell, D. A. Expansion of ribosomally produced natural products: a nitrile hydratase- and Nif11-related precursor family. *BMC Biol.* **2010**, *8*, 70.
(29) Yang, J.; Zhang, Y. I-TASSER server: new development for protein structure and function predictions. *Nucleic Acids Res.* **2015**, *43*, W174−181.
(30) Zhang, C.; Freddolino, P. L.; Zhang, Y. COFACTOR: improved protein function prediction by combining structure, sequence and protein-protein interaction information. *Nucleic Acids Res.* **2017**, *45*, W291−W299.

https://doi.org/10.1021/acssynbio.1c00224
ACS Synth. Biol. 2021, 10, 2579−2591

(31) Dong, S. H.; Tang, W.; Lukk, T.; Yu, Y.; Nair, S. K.; van der Donk, W. A. The enterococcal cytolysin synthetase has an unanticipated lipid kinase fold. *Elife* **2015**, *4*, 5387.
(32) Zhang, Y.; Chen, M.; Bruner, S. D.; Ding, Y. Heterologous production of microbial ribosomally synthesized and post-translationally modified peptides. *Front. Microbiol.* **2018**, *9*, 1801.
(33) Bobeica, S. C.; van der Donk, W. A. The enzymology of prochlorosin Biosynthesis. *Methods Enzymol.* **2018**, *604*, 165−203.
(34) Shi, Y.; Yang, X.; Garg, N.; van der Donk, W. A. Production of lantipeptides in *Escherichia coli*. *J. Am. Chem. Soc.* **2011**, *133*, 2338−2341.
(35) van Tilburg, A. Y.; Cao, H.; van der Meulen, S. B.; Solopova, A.; Kuipers, O. P. Metabolic engineering and synthetic biology employing *Lactococcus lactis* and *Bacillus subtilis* cell factories. *Curr. Opin. Biotechnol.* **2019**, *59*, 1−7.
(36) Le Loir, Y.; Azevedo, V.; Oliveira, S. C.; Freitas, D. A.; Miyoshi, A.; Bermudez-Humaran, L. G.; Nouaille, S.; Ribeiro, L. A.; Leclercq, S.; Gabriel, J. E.; Guimaraes, V. D.; Oliveira, M. N.; Charlier, C.; Gautier, M.; Langella, P. Protein secretion in *Lactococcus lactis*: an efficient way to increase the overall heterologous protein production. *Microb. Cell Fact.* **2005**, *4*, 2.
(37) Song, A. A.; In, L. L. A.; Lim, S. H. E.; Rahim, R. A. A review on *Lactococcus lactis*: from food to factory. *Microb. Cell Fact.* **2017**, *16*, No. 55.
(38) Morello, E.; Bermúdez-Humarán, L. G.; Llull, D.; Solé, V.; Miraglio, N.; Langella, P.; Poquet, I. *Lactococcus lactis*, an efficient cell factory for recombinant protein production and secretion. *J. Mol. Microbiol. Biotechnol.* **2008**, *14*, 48−58.
(39) Lee, M. D.; Ahlgren, N. A.; Kling, J. D.; Walworth, N. G.; Rocap, G.; Saito, M. A.; Hutchins, D. A.; Webb, E. A. Marine *Synechococcus* isolates representing globally abundant genomic lineages demonstrate a unique evolutionary path of genome reduction without a decrease in GC content. *Environ. Microbiol.* **2019**, *21*, 1677−1686.
(40) Kuipers, O. P.; de Ruyter, P. G. G. A.; Kleerebezem, M.; de Vos, W. M. Quorum sensing-controlled gene expression in lactic acid bacteria. *J. Biotechnol.* **1998**, *64*, 15−21.
(41) Montalbán-López, M.; Deng, J.; van Heel, A. J.; Kuipers, O. P. Specificity and application of the lantibiotic protease NisP. *Front. Microbiol.* **2018**, *9*, 160.
(42) Thibodeaux, C. J.; Ha, T.; van der Donk, W. A. A price to pay for relaxed substrate specificity: a comparative kinetic analysis of the class II lanthipeptide synthetases ProcM and HalM2. *J. Am. Chem. Soc.* **2014**, *136*, 17513−17529.
(43) Driggers, E. M.; Hale, S. P.; Lee, J.; Terrett, N. K. The exploration of macrocycles for drug discovery an underexploited structural class. *Nat. Rev. Drug Discovery* **2008**, *7*, 608−624.
(44) Ongey, E. L.; Neubauer, P. Lanthipeptides: chemical synthesis versus in vivo biosynthesis as tools for pharmaceutical production. *Microb. Cell Fact.* **2016**, *15*, No. 97.
(45) Zhao, X.; Cebrian, R.; Fu, Y.; Rink, R.; Bosma, T.; Moll, G. N.; Kuipers, O. P. High-Throughput screening for substrate specificity-adapted mutants of the nisin dehydratase NisB. *ACS Synth. Biol.* **2020**, *9*, 1468−1478.
(46) Lubelski, J.; Rink, R.; Khusainov, R.; Moll, G. N.; Kuipers, O. P. Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin. *Cell. Mol. Life Sci.* **2008**, *65*, 455−476.
(47) Mukherjee, S.; van der Donk, W. A. Mechanistic studies on the substrate-tolerant lanthipeptide synthetase ProcM. *J. Am. Chem. Soc.* **2014**, *136*, 10450−10459.
(48) Lee, M. V.; Ihnken, L. A. F.; You, Y. O.; McClerren, A. L.; Donk, W. A. vd.; Kelleher, N. L. Distributive and directional behavior of lantibiotic synthetases revealed by high-resolution tandem mass spectrometry. *J. Am. Chem. Soc.* **2009**, *131*, 12258−12264.
(49) Lagedroste, M.; Reiners, J.; Knospe, C. V.; Smits, S. H. J.; Schmitt, L. A structural view on the maturation of lanthipeptides. *Front. Microbiol.* **2020**, *11*, No. 1183.
(50) Bobeica, S. C.; Zhu, L.; Acedo, J. Z.; Tang, W.; van der Donk, W. A. Structural determinants of macrocyclization in substrate-controlled lanthipeptide biosynthetic pathways. *Chem* **2020**, *11*, 12854−12870.
(51) Driggers, E. M.; Hale, S. P.; Lee, J.; Terrett, N. K. The exploration of macrocycles for drug discovery an underexploited structural class. *Nat. Rev. Drug Discovery* **2008**, *7*, 608−624.
(52) Ongey, E. L.; Neubauer, P. Lanthipeptides: chemical synthesis versus in vivo biosynthesis as tools for pharmaceutical production. *Microb. Cell Fact.* **2016**, *15*, No. 97.
(53) Kuipers, O. P.; de Ruyter, P. G.; Kleerebezem, M.; de Vos, W. M. Controlled overproduction of proteins by lactic acid bacteria. *Trends Biotechnol.* **1997**, *15*, 135−140.
(54) García-Cayuela, T.; de Cadinanos, L. P.; Mohedano, M. L.; de Palencia, P. F.; Boden, D.; Wells, J.; Pelaez, C.; Lopez, P.; Requena, T. Fluorescent protein vectors for promoter analysis in lactic acid bacteria and *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **2012**, *96*, 171−181.
(55) Geu-Flores, F.; Nour-Eldin, H. H.; Nielsen, M. T.; Halkier, B. A. USER fusion: a rapid and efficient method for simultaneous fusion and cloning of multiple PCR products. *Nucleic Acids Res.* **2007**, *35*, e55.
(56) Holo, H.; Nes, I. F. Transformation of *Lactococcus* by electroporation. *Methods Mol. Biol.* **1995**, *47*, 195−199.
(57) Schägger, H. Tricine−SDS-PAGE. *Nat. Protoc.* **2006**, *1*, 16−22.
(58) Pappin, D. J. C.; Hojrup, P.; Bleasby, A. J. Rapid identification of proteins by peptide-mass fingerprinting. *Curr. Biol.* **1993**, *3*, 327−332.

https://doi.org/10.1021/acssynbio.1c00224
ACS Synth. Biol. 2021, 10, 2579−2591