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Discovery, Production and Modification of Five Novel Lantibiotics Using the Promiscuous Nisin Modification Machinery

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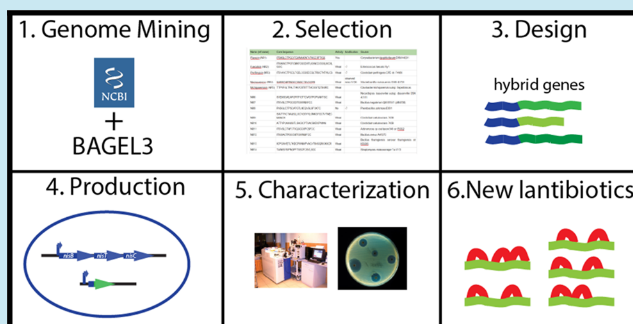
Supporting Information

ABSTRACT: To find the right conditions to isolate natively expressed antimicrobial peptides from a wide range of different microorganisms can be a challenge. Here, we exploited a heterologous expression system to produce and characterize several novel lantibiotics. We identified 54 novel putative class I and class II lantibiotics after inspecting all publicly available prokaryotic genomes using the in-house developed mining tool BAGEL3. The genes encoding these new lantibiotics fused to the nisin leader peptide gene sequence were synthesized, and the constructs were plugged into the nisin expression and modification system. Using this approach 30 peptides could be expressed, 27 of which were dehydrated by NisBC on at least 1 predicted position. Good antimicrobial activity against several pathogenic bacteria could be demonstrated for 5 novel heterologously modified lantibiotics. Lantibiotics from *Corynebacterium lipophiloflavum* DSM 44291 and *Streptococcus agalactiae* ATCC 13813, named flavucin and agalacticin, respectively, were fully modified and displayed high antimicrobial activity. The efficiency of functional expression was significantly enhanced when we made use of the native nisin leader cleavage site, instead of an artificial factor Xa site. Thus, we describe an efficient way for heterologous production of active lantibiotics, facilitating a rapid identification of promising molecules.



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To combat the emerging problem of multidrug resistant pathogenic bacteria, various different novel sources of antibiotic compounds are being investigated. These include secondary metabolites from *Streptomyces* species, derivatized existing antibiotics, but also natural peptides produced by various living organisms.[1] One potential rich source are the so-called lantibiotics. These are ribosomally synthesized peptides consisting of 35−60 amino acids that entail modified residues, which are the result of post-translational modifications by dedicated enzymes. Commonly, the structural gene is encoded in proximity of the genes encoding the modification enzymes.[2] Currently, almost 100 lantibiotics have been described in literature.[3] Using genome mining approaches one can identify lantibiotic gene clusters even in meta-genomic data. Although this can simplify and enhance classic screening approaches by knowing which compounds can potentially be produced by an organism, it still is no guarantee to success. Finding the right conditions at which the lantibiotic is produced can be extremely challenging and some clusters may reside silently in the genome. To overcome this problem, we employ a synthetic biology approach where we use only the coding sequence of the lantibiotic core peptide and combine it with the biosynthesis machinery of the model lantibiotic nisin (see Figure 1). The nisin modification machinery has been shown to have a broad substrate tolerance, modifying diverse (non)lantibiotic peptides when fused to the nisin leader peptide.[4,5] An additional advantage of this approach is that there is no need to work with fastidious or pathogenic strains for production. New lantibiotic genes were identified by *in silico* mining of all public prokaryotic genomes using BAGEL3.[6] 54 promising lantibiotic candidate genes were plugged into the nisin production- and modification system and expressed in *Lactococcus lactis*. This eventually resulted in the actual production of 31 peptides, of which approximately 15% were novel active lantibiotics. Importantly, two peptides showed strong antimicrobial activity at a level comparable to nisin, but with altered target specificities. We further showed that the number of produced peptides increases significantly when the native nisin leader cleavage site is used instead of an artificial factor Xa cleavage site.

RESULTS AND DISCUSSION

Selection of 54 Candidate Class I and II Lantibiotic Gene Clusters for Heterologous Expression Using the Nisin Modification Machinery. BAGEL3 is an automated tool to facilitate the discovery of bacteriocins in genomes, it looks for core sequences and takes the genomic context of these sequences into account. After analyzing the results of

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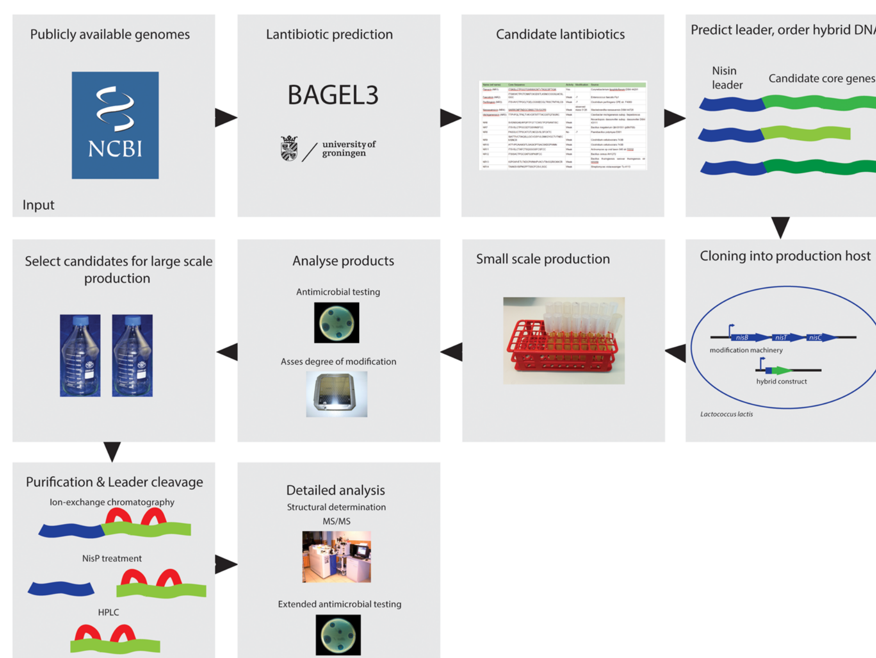

Figure 1. General overview of the lantibiotic discovery pipeline. All publicly available prokaryotic genomic data is used as input. BAGEL3 is used to predict the presence of lantibiotics. Subsequently a manual selection is made from the prediction. New core peptides are ordered as hybrids (nisin leader with candidate core) and subcloned into the heterologous expression and modification host *L. lactis* NZ9000 (pIL3BTC).[7] In a first round, production is conducted at a small scale and culture supernatants are analyzed after TCA precipitation. On the basis of the analysis, compounds are selected for liter scale production. These are subsequently analyzed in more detail as more and purer material is obtained. Finally, this can be used as an iterative process since the confirmed prediction can be included in BAGEL3 software to improve predictions.

several BAGEL3 runs a selection of 54 candidate lantibiotics was ordered (see Table 1). These 54 candidates were selected based on novelty, the ability to predict the leader cleavage site (*i.e.*, the start of the core sequence) and compatibility with the expression system in terms of additional modification enzymes that could be needed for full modification. The selected candidates showed a high degree of diversity, including candidates from class I (LanBC modified) and class II (LanM modified) lantibiotics and even from two-component lantibiotic clusters. Some of the clusters include the LanD modification enzyme indicating that additional modification is likely necessary for full antimicrobial activity.

Five New Class I Lantibiotics Showed Antimicrobial Activity. Of the 54 candidates, 24 could not be detected after production, either by MALDI-TOF performed on supernatant or TCA precipitated supernatant, by Tricine-SDS-PAGE (using TCA precipitated supernatant) or by antimicrobial activity assay (spot-on-lawn against *Micrococcus flavus*). Of the 31 produced peptides 10 were modified inefficiently (less than 70% of Thr and Ser residues were dehydrated) and 3 were not modified at all (Table 1). Of the 18 produced and modified candidates left, 5 yielded highly active compounds against the strains tested (see Table 2). All of these active peptides are encoded in clusters containing LanBC enzymes (Class I) (Figure 2B). All 5 lantibiotics show activity against *M. flavus* and vancomycin resistant *Enterococcus faecalis* VE14089 (VRE). Four of these lantibiotics are also active against *Bacillus cereus* and *Listeria monocytogenes.* Bagelicin demonstrated the best activity against methicillin-resistant *Staphylococcus aureus* MW2 (MRSA MW2). Maddinglicin expression and purification yielded significantly less material than the other active lantibiotics (data not shown). Notably most active lantibiotics were obtained from constructs containing the original nisin leader peptide. The first 42 candidates were ordered using a tested[8] leader peptide containing a factor Xa cleavage site (Figure 2A top). This only yielded one active lantibiotic, *i.e.*, flavucin. In contrast, 4 of the 13 candidates tested using the native nisin leader (Figure 2A bottom) resulted in active peptides. The adapted leader containing the factor Xa cleavage site was used because we were unsure about the general compatibility with the nisin protease NisP, when the sequence directly after the leader differs from that of nisin. Due to the low success rate we switched to the original nisin leader sequence. The initial screening has its limitations as only one indicator strain was used and modification efficiency, production level and leader cleavage might not be optimal in the tested condition.

Our approach yielded novel lantibiotics originating from various organisms. Flavucin was identified in the genome of *Corynebacterium lipophiloflavum* DSM 44291, which was isolated from a patient with bacterial vaginosis[9] and later sequenced as a part of the human microbiome project. The genome sequence consists of 178 contigs and the one encoding flavucin is only 6172 base pairs long indicating that only relatively short fragments can be sufficient to correctly predict lantibiotics *in silico*. BAGEL3 takes into account the context of the core gene and the larger the contig the more context information is available. This is also a good indication of the minimal sequence length needed to predict post-translationally modified antimicrobial peptides in meta-genomic data. The flavucin amino acid sequence has some similarity to nisin, in particular the N-terminal region. The leader contains a clear DF(D/N)LD box with only one mismatch (Figure 2C). The spacing between the DF(D/N)LD box and the cleavage sites is 13 amino acids, one less than in nisin. Based on homology, the number of dehydrations measured by MS and the N- to C-

Table 1. List of Identified Candidates[a]

Name [class]	Core Sequence	Modification	Source
Flavucin (NR1) [1]	ITSKSLCTPGCITGWMMCNTVTKGCSFTIGK	5-9 (9)	*Corynebacterium lipophiloflavum* DSM 44291
NR2 [1]	ITSIKWCTPGTCNNTCKGDSTLKSNCCGGSLMCSLGGC	7 (10)	*Enterococcus faecalis* Fly1
NR3 [1]	ITSVAYCTPGCLTGELCGSSECGLTRSCTNTWLCS	6-7 (11)	*Clostridium perfringens* CPE str. F4969
NR4 [1]	SARRCNPTNDGCSNSCTSVGCPR	(6)	*Stackebrandtia nassauensis* DSM 44728
NR5 [1]	TTPVFGLTFKLTVKVCRTIITTTACGSTQTSGRC	6-8 (12)	*Clavibacter michiganensis* subsp. *sepedonicus*
NR6 [1]	SVENSGAEAPGPITFGTTCWGTPCPSANTISC	(9)	*Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43111
NR7 [1]	ITSVSLCTPGCGDTGSWNSFCC	(7)	*Bacillus megaterium* QM B1551 (pBM700)
NR8 [1]	PASGLICTPSCATGTLNCQVSLSFCKTC	7(8)	*Paenibacillus polymyxa* E681
NR9 [2-2] (NR10)	SIATTIVCTIAQSLLGCVGSYVLGNKGYGCTVTNECMS NCR	4-6 (9)	*Clostridium cellulovorans* 743B
NR10 [2-2] (NR9)	ATTVPCAIAIIGITLSAGICPTSACSKDCPWNN	3-5 (7)	*Clostridium cellulovorans* 743B
NR11 [1]	ITSVSLCTAFCTSQGGGSFCSFCC	(8)	*Actinomyces* sp. oral taxon 848 str F0332
NR12 [1]	ITSISACTPGCGNTGSFNSFCC	(7)	*Bacillus cereus* AH1272
NR13 [2]	IGPGWVETLTKDCPWNMPVACVTIMGQRICKKCR	0 (3)	*Bacillus thuringiensis* serovar *thuringiensis* str. IS5056
NR14 [1]	TAAKSVSIPNGPFTSSCFCSVLSGC	0 (8)	*Streptomyces violaceusniger Tu* 4113
NR15 # [1]	LTSYSLCSFGCGKTGSFNSFCC	3-4 (7)	*Staphylococcus aureus* subsp. *aureus* D139
NR16 # [2]	TANTHCHCYSGKDSCGHGCTITTECPFATLICC	(8)	*Streptococcus agalactiae* FSL S3 014
NR17 [2-2] (NR24)	MFGLSRLLGNNGRWCTITKECMPSCN	0 (4)	*Corynebacterium matruchotii* ATCC 33806
NR18 # [2]	SRWWQGVLPTVSHECRMNSFQHIFTCC	0-2 (5)	*Lactobacillus iners* LactinV 11V1 d contig00006
NR19 [2]	LFTVPGTTTVGHFCTISWECSGFICGK	0-2 (7)	*Rothia dentocariosa* ATCC 17931
NR20 [1]	TLSTYTCGTCAASVCPCHITDATGSACNC	(9)	*Parachlamydia acanthamoebae* UV 7
NR21 [1]	WNTASTNPDCLATGIYDGCEVTGNCNTANHCGNTNNGC QTNGCSNGCGNTQTREIISCYEAC	(12)	*Kordia algicida* GCF 000154725
NR22 [1]	TWGTVVKVSKAICKTGTCIGTISCTNCK	7-8 (8)	*Bacillus cereus* BAG1X1 1
NR23 [1]	TVPTTIFVSRSVCKTLTCICTISCSNCK	7 (10)	*Bacillus thuringiensis* serovar *thuringiensis* str. IS5056
NR24 [2-2] (NR17)	THPSTLIPISIALCPTTRCSRRC	5 (7)	*Corynebacterium matruchotii* ATCC 33806
NR25 [1]	LIRMTDDGCGVTCESACSTTCP	(6)	*Streptomyces clavuligerus* ATCC 27064
NR26 [1]	LIRMTDDGCGTTCETACSTTCP	(7)	*Frankia* sp. CcI3
NR27 [1]	VTSTGCCKN	(3)	*Streptococcus pneumoniae* BS458
NR28 [1]	INLTDDGCGSTCSSPCATNVA	(6)	*Streptomyces zinciresistens* K42
NR29 [1]	IASDGGCGSTCGGNACISSGS	(6)	*Streptomyces coelicolor* A32
NR30 [1]	NLTDDGCKPSCQGSCATNVA	(4)	*Streptomyces zinciresistens* K42
NR31 # [2]	GNGVFHTISHECYYNSWAFIFTCC	3 (4)	*Actinomyces* sp. oral taxon 448 str. F0400
NR32 [2]	GNGVYYTLSHECHMNSWQFIFTCCS	0-3 (5)	*Actinomyces* sp. oral taxon 170
NR33 [2]	AGAGFIKTLTKDCPEVVSQVCGSFFGWVSACKNC	(5)	*Clostridium perfringens* D str. JGS1721
NR34 [2]	VGPGWIATLTKDCPANKPYACITIAGQTICKKCR	3-4 (4)	*Bacillus thuringiensis* IBL 200
NR35# [1]	ITSHLFCSFGCEKTGSFNSFCC	6 #=d0 (6)	*Staphylococcus aureus* D30 contig00429
NR36# [1]	ITSFIGCTPGCGKTGSFNSFCC	6 #=d6 (6)	*Staphylococcus aureus* RF122
NR46 [2]	TAFPGWYSKVIGNRGRVCTVTVECMSVCQ	(5)	*Rothia dentocariosa* ATCC 17931
NR47 [1]	LIRMTDDGCGTTCQSACPNTCPGD	(5)	*Frankia* sp. CcI6
NR48# [1]	FTSVSFCTPGCGETGSFNSFCC	7 #=0 (7)	*Bacillus clausii* KSM-K16
NR49 [2]	GKNGVFKTISHECHMNSWQFLFTCCS	(5)	*Streptococcus equi* subsp. *zooepidemicus* BHS5
NR50 [2]	GKNGAIKTISHECHMNSWQFLFTCCS	2-5 (5)	*Streptococcus equi* subsp. *zooepidemicus* BHS5

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Table 1. continued

Name [class]	Core Sequence	Modification	Source
NR60* [1]	VTSYAFCTPGTCACKEGPTFDSACCSVELTVYYCTR	(9)	*Bacillus cereus* VD022
NR61* [1]	VTSYAFCTPGTCACEDGPTFDSACCSVELTIYNCK	(8)	*Bacillus cereus* VD022
Maddinglicin (NR62*) [1]	ITSKSLCTPGCITGVLMCITQNSCVSCKSCIKC	7-9 (9)	*Clostridium* sp. *maddingley* MBC34 26
NR63* [1]	SWSYCTPGCTSSGGGSGCSHCC	(8)	*Nocardia brasiliensis* ATCC 700358
Bagelicin (NR64)* [1]	VTSISLCTPGCKTGILMTCAIKTATCGCHF	7-8 (8)	*Streptococcus suis* R61
Agalacticin (NR65)* [1]	VTSKSLCTPGCKTGILMTCAIKTATCGCHFG	8 (8)	*Streptococcus agalactiae* ATCC 13813
NR66* [1]	ITSHFLCSFGCGKTGSFNSFCC	(6)	*Staphylococcus aureus* subsp. *aureus* ED133
NR67* [1]	VGSRYLCTPGSCWKLVCFTTTVK	(6)	*Streptococcus pyogenes* MGAS10750
NR68* [1]	ITSVIGCTPGCNPTATLCTSCGFNCTYKAPCK	7 (8)	*Desmospora* sp. 8437
NR69* [1]	TSVMACTPGTCNNKCPNTNWLCSNVCVTKTCWTCA	6 (9)	*Enterococcus faecalis* Fly1
Moraviensicin (NR70)* [1]	ITSKSLCTPGCVTGVLMGCALKTITCNCSVGIGKK	7-8 (8)	*Enterococcus moraviensis* ATCC BAA 383 acOtp
NR71* [1]	ITSVSLCTMGCQGSKLQTVICNWVK	5 (6)	*Enterococcus moraviensis* ATCC BAA 383 acOtp
NR72* [1]	ADSGIICTPTCLTSILNCYTSISHCGPC	5 (8)	*Paenibacillus polymyxa* SC2

[a]* = use of original nisin leader (others contain a Factor Xa cleavage site, Figure 2A), # = also produced with GdmD. Active candidates are highlighted with a light blue background. Modification is the number of dehydrated residues based on the MALDI-TOF mass(es) observed. In between brackets is the total number of serines and threonines in the peptide. In the modification column, # = indicates the result in the production system using GdmD, d indicates decarboxylation observed after MALDI-TOF analysis. If no number is indicated in front of the number in between brackets, no peptide was detected. Cysteines are depicted in red. Serines and threonines are depicted bold.

Table 2. Antimicrobial Activity of Novel Lantibiotics[a]

name	*E. faecalis* VE14089		*B. cereus*		MRSA MW2		*L. monocytogenes*		*M. flavus*	
	trypsin	NisP	trypsin	NisP	trypsin	NisP	trypsin	NisP	trypsin	NisP
Nisin	+	+	+	+	+	+	+	+	+	+
Flavucin* (NR1)	+	−	+/−	−	+/−	−	+	−	+	−
Maddinglicin (NR62)	+/−	−	−	−	−	−	−	−	+	+
Bagelicin (NR64)	+	+	+	+	+	+	+	+	+	+
Agalacticin (NR65)	+	+	+	−	+/−	−	+	−	+	+
Moraviensicin (NR70)	+	+	+	+	−	−	+	−	+	+

[a]* = constructed with a factor Xa leader. + indicates activity, +/− indicates weak activity, − indicates no activity was observed. Every indicator strain has two columns showing results for different enzymes used for leader cleavage (trypsin or NisP). Activity is measured *via* spot on lawn assay and is not quantitative. The leader cleavage enzyme was added *in situ* and in excess.

directionality of the nisin modification machinery,[10] the first three lanthionine rings should be formed as in nisin. The C-terminus is very different. Whereas most similar lantibiotics have two cysteines to form rings four and five, flavucin lacks one cysteine. Flavucin shows the typical charge distribution, with negatively charged residues present in the leader.

The gene encoding agalacticin was identified in the genome of *Streptococcus agalactiae* which is a human and bovine (opportunistic) pathogen that belongs to the so-called Group B *Streptococcus* (GBS). The amino acid sequence is almost identical to that of Bagelicin in which one amino acid is different and the final glycine is lacking. Bagelicin was identified in the genome of *Streptococcus suis* R61, which is a porcine and human multidrug resistant pathogen that was isolated from the lungs of an infected pig in Jiangsu province (China).[11] Both sequences are partly similar to that of nisin and also follow the identical lanthionine bridging pattern, based on homology and the MS/MS results obtained with Agalacticin. The biggest difference compared to nisin is the shortened C-terminus. Also the gene clusters of agalacticin and bagelicin are very similar, containing the same genes in identical order (Figure 2B). The only difference is that in the bagelicin cluster there is an inserted sequence between LanI and the rest of the cluster. The gene clusters contain all the components that have also been identified in the Nisin gene cluster, including modification enzymes (LanBC), a two-component regulatory system (LanRK), two immunity systems (LanFEG, LanI), a transporter (LanT) and leader peptidase (LanP). Agalacticin was produced in a homogeneous state of modification demonstrating perfect compatibility with the nisin modification machinery.

Clostridium sp. *maddingley* was isolated from a brown coal mine in Australia.[12] The amino acid sequence of maddinglicin is the most distinct active new lantibiotic we identified compared to nisin. First of all, it contains 7 cysteines which could result in a C-terminus that contains 4 intertwined rings. The gene cluster contains all components that the nisin cluster entails, except for LanI.

Moraviensicin was identified in *Enterococcus moraviensis* which is a biosafety level 1 organism isolated from a lake in the Czech republic.[13] The amino acid sequence of the core

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Figure 2. Graphical representation of (A) the hybrid design for heterologous expression of the lantibiotic constructs, (B) the source gene clusters encoding the biosynthesis of the new lantibiotics (image created using the genome2d web server http://server.molgenrug.nl) and (C) an ClustalW alignment[14] of several known and new lantibiotics. The ClustalW color scheme: blue = acidic, magenta = basic excluding His, red = small (small + hydrophobic (incl. aromatic excluding Tyr)), green = Hydroxyl + sulfhydryl + amine + Gly. The number at the end indicates the length of the sequence in amino acids.

peptide of moraviensicin deviates at 12 positions when compared to nisin and most of these differences occur at the C-terminus. The lanthionine bridging pattern should be identical to that of nisin based on homology. A striking difference to other gene clusters is the number of copies (5) of the core peptide in the moraviensicin gene cluster.

Purified Flavucin, Agalacticin and Bagelicin Are Potent Antimicrobials against Gram-Positive Bacteria. The three lantibiotics displaying activity against the largest number of bacteria in the test panel (*i.e.*, flavucin, bagelicin and agalacticin; Table 2) were purified in large amount and their MIC value was determined (Table 3). We used pathogenic strains of Gram-positive and Gram-negative bacteria, including multidrug resistant ones. Flavucin (in spite of being a mix of differently modified peptide species, *vide infra*) was particularly active against a vancomycin and avoparcin resistant *Enterococcus faecium*. Bagelicin and agalacticin showed a different potency in spite of their high similarity. While bagelicin displayed activity at a concentration similar to that of the reference lantibiotic nisin, agalacticin showed a low activity. Nisin and flavucin were able to inhibit the growth of the Gram-negative strain *Pseudomonas aeruginosa* at high concentrations (>300 μg/mL) that were not tested for bagelicin and agalacticin.

Table 3. MICs of Nisin, Flavucin, Bagelicin and Agalacticin[a]

MIC (μg/mL)	nisin	flavucin	bagelicin	agalacticin
S. aureus LMG 10147	22	69	40	>85
S. aureus MW2	22	69	20	>85
E. faecalis LMG 08222	5	14	10	>85
E. faecalis LMG 16216	11	34	10	>85
E. faecium LMG 16003	9	4	10	21
L. monocytogenes LMG 10470	11	34	13	85
P. aeruginosa LMG 6395	350	733	>40	>85

[a]The results are the average of three replicates.

The three novel lantibiotics for which the minimal inhibitory sequence was determined displayed a different antimicrobial efficiency. Whereas flavucin was the most potent lantibiotic tested against a drug resistant *E. faecium*, bagelicin was able to inhibit all the Gram-positive strains tested at a low concentration. Surprisingly, agalacticin is less potent than bagelicin although there is only an isoleucin to lysine replacement and an additional glycine at the C-terminus. The addition of an extra positive charge might have a strong impact on the mechanism of action, although this point needs further study.

MS/MS Results Confirm the Homology-Based Lanthionine Ring Pattern of Flavucin and Agalacticin. To verify that the antimicrobial activities we observed originated from the produced lantibiotics, we analyzed all samples by MALDI-TOF (Table 1). MALDI-TOF also gives insight into the degree of modification since the essential dehydration of threonines and serines by NisB can easily be detected (18 Da reduction per dehydration). Of the 30 candidates that yielded a good signal in the MALDI-TOF experiment, 18 showed a high degree of modification (70% dehydration or more). When looking at the degree of dehydration, several peptides produced in our system yield a mix of molecules and some yield one main product. To investigate this phenomenon further we picked

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one active compound that was produced in a homogeneous dehydration state (agalacticin) and one active compound that resulted in a heterogeneous dehydration state (flavucin). Agalacticin and flavucin were produced on a larger scale and HPLC purified for tandem mass spectrometry. Agalacticin production and purification yielded a homogeneous product, containing only the fully dehydrated compound. In contrast, flavucin yielded several fractions containing different degrees of dehydration that could be separated by HPLC. The collision-induced fragmentation yielded several fragments giving insight into the lanthionine bridging pattern (Figure 3). Most observed fragments are in agreement with a bridging pattern derived from homology to lantibiotics with known structures.

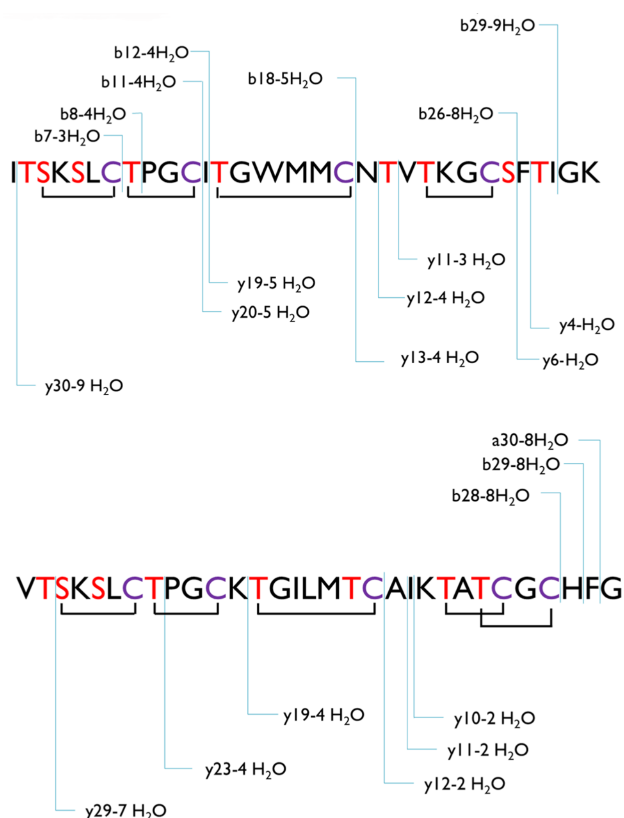

Figure 3. MS/MS results of flavucin (top) (9 times dehydrated variant only) and agalacticin (bottom) (8 times dehydrated). Indicated are the observed fragments after collision induced dissociation. The fragmentation gives insight into the lanthionine bridging pattern. Indicated with black lines are the bridging pattern based on homology to known lantibiotics. For the MS/MS pattern of the 6 times dehydrated flavucin, see the Supporting Information.

Specific Antimicrobial Activity of Flavucin Depends on Its Dehydration Degree and Its Integrity. Producing flavucin using NisBTC resulted in a mix of different dehydration states (observed by MALDI-TOF) (Figure 4). It is unclear what causes this phenomenon, one explanation could be partial incompatibility with the nisin modification system employed in this study although the system has been described to have a broad substrate tolerance.[4,15] Additionally the modified leader (factor Xa cleavage site) could influence the modification efficiency.[8] Alternatively these variants could be naturally occurring, as even nisin's Ser33 partially escapes dehydration in nature. The competitive advantage of producing a battery of compounds could be that these variants have different specific antimicrobial spectra. To investigate this, the different dehydration states were partially separated by HPLC and the concentrations were determined using BCA protein assay (Bio Rad). Equal volumes of equal concentration were spotted on different indicator strains (Figure 4). Nisin performs best in all cases. Unexpectedly the truncated five times dehydrated flavucin (consisting of residue 1−23) showed the highest activity against *B. cereus*, *E. faecalis* and *G. vaginalis*. This truncated flavucin is most likely an artifact from the leader cleavage with trypsin. If the modification takes place directionally from the N-terminus (as one would expect based on the literature[10]) and only 5 residues are dehydrated then lysine 23 could not be inside a lathionine ring making trypsin cleavage likely. The 7−8 times dehydrated entity performs better against *M. flavus* and *S. aureus* than the 7−9 times dehydrated entity. Unfortunately we could not identify any growth conditions in the source organism *C. lipophiloflavum* that yielded detectable antimicrobial activity (data not shown).

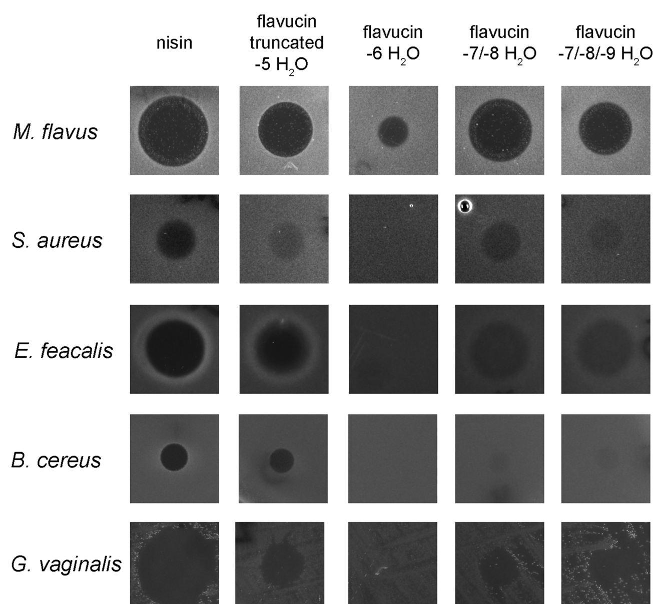

Figure 4. Comparison of the antimicrobial activity of different HPLC fractions of Flavucin compared to HPLC grade Nisin. All samples are spotted (4 μL) at a concentration of 0.5 mg/mL. On the left the indicator strains are indicated. On the top the composition of the HPLC fraction is indicated (based on MS results), different fractions show different degree of dehydration. The truncated flavucin consists of the first 23 residues of flavucin and shows 5 dehydrations (enough to form the first three rings).

CONCLUSIONS

Demonstrating that an *in silico* identified lantibiotic has antimicrobial activity can be challenging. If the sequence originates from an isolated bacterium that can be grown in the lab, one can obtain the source organism and start screening different production conditions. If production is achieved, which is not always trivial, one has to screen for target organisms. Especially the first step can be very challenging since the numerous environmental conditions in which production might occur in nature are impossible to recreate in the lab. If the lantibiotic is discovered in meta-genomic data and the source organism is not available this approach is impossible. Using the heterologous modification approach applied here, helps to overcome the first hurdle, as production and

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Table 4

strains	characteristic	purpose	reference
Lactococcus lactis NZ9000	*pepN::nisRK*	expression host	[16]
Listeria monocytogenes LMG 10470		indicator strain	LMG
Staphylococcus aureus MW2	methicillin resistant	indicator strain	Lab collection
S. aureus LMG 10147		indicator strain	LMG
Enterococcus faecalis VE14089		indicator strain	Lab collection
E. faecalis LMG 08222		indicator strain	LMG
E. faecalis LMG 26216	*vanB*	indicator strain	LMG
Enterococcus faecium LMG 16003	vancomycin, avoparcin resistant	indicator strain	LMG
Bacillus cereus ATCC 14579		indicator strain	Lab collection
Pseudomonas aeruginosa LMG 6395		indicator strain	LMG
Gardnerella vaginalis ATCC 14018		indicator strain	DSM
Listeria monocytogenes		indicator strain	Lab collection
Micrococcus flavus		indicator strain	Lab collection
plasmids	**characteristic**	**purpose**	**reference**
pNZE-NRx	EryR. PnisA nisin leader-candidate. X denotes the number of the candidate	production of new lantibiotics	this study
pNZE-NRDx	EryR, gdmD PnisA nisin leader-candidate. X denotes the number of the candidate	production of new lantibiotics	this study
pNZE-empty	EryR	cloning of PnisA nisin leader-candidate hybrid gene	[17]
pNZE-gdmD	EryR, gdmD decarboxylase (from *Staphylococcus gallinarum Tü 3928*)	cloning of PnisA nisin leader-candidate hybrid gene	[17]
pNZ-NisP-8H	CmR	protease production	[18]
pIL3BTC	CmR	NisBTC (originating from *L. lactis*) production	[7]

modification conditions become a standardized and controlled process.

Eventually we managed to produce 5 new lantibiotics, including flavucin, which we did not manage to produce using the natural producer. The selection of an appropriate leader peptide is of great importance as was shown by the increased success rate when the WT leader was used. Changing the N-terminus of lantibiotics can be very detrimental for the antimicrobial activity. Therefore, it is of utmost importance to correctly predict the end of the candidate leader peptide/the start of the candidate core peptide. Some of the candidates we selected might be silent clusters that have deteriorated in time resulting in inactive lantibiotics. Also the lantibiotic might be active against an organism that was not part of our target panel. Alternatively, additional modifications that take place in the original biosynthesis might be essential for activity. Finally, in some cases the production level reached was not sufficient to show activity in the first screening round.

The approaches as the one employed here can help to extend the number of natural products that can be isolated and characterized. The type of challenges faced in this approach are very different from those faced in classic approaches. The biggest advantage of our approach is the scalability, enabling the screening of more than 50 potential candidates from many different source organisms, by just using the digital information on their structural genes. This has until now yielded 5 new antimicrobial compounds, that in the future can be further tested to assess potential use in (veterinary) medicine.

MATERIALS AND METHODS

Strains and Media. The strains and plasmids used in this study are listed in Table 4. *S. aureus* and *E. faecalis* were grown in M-17 (Oxoid) supplemented with 0.5% glucose (GM-17) at 37 °C. *L. lactis* was grown at 30 °C in GM-17. *B. cereus* was grown in LB (Supelco) at 30 °C. *G. vaginalis* was grown in TSB (Oxoid) supplemented with 3% defibrinated sheep blood at 37 °C.

Cloning and Expression of Hybrid Genes. For the expression of the new candidates a two plasmid expression system using pIL3BTC (for modification (NisBC) and tansport (NisT)) and a pNZ plasmid containing the candidate gene. The hybrid genes encoding the nisin leader and the candidate core were ordered from Life Technologies as a fragment encompassing the nisin inducible promoter PnisA and the hybrid nisin leader-core peptide. The core peptide was codon optimized for *L. lactis*. The construct was subcloned into pNZE-empty using *Hin*dIII and *Bgl*II (Thermo Scientific) to yield pNZE-NRx (when applicable the constructs were also subcloned into pNZE-gdmD to yield pNZE-NRDx). The constructs were checked by sequencing (Macrogen). The pNZE-NR(D)x plasmids containing the hybrid candidate genes were cloned into *L. lactis* NZ9000 containing pIL3BTC to yield the producer strains. To induce the expression of the nisin controlled hybrid genes and the modification genes 5 ng/mL of nisin was added at an OD_{600} 0.4−0.6. For small scale production lantibiotics were precipitated from the supernatant by TCA precipitation which was performed according to Sambrook *et al.*[19]

***In Silico* Prediction and Selection.** BAGEL3[6] was run using all publicly available prokaryotic genomes as input. Results were limited to only contain clusters containing a PF05147 pfam domain (indicative of LanM or LanC). From this list 54 candidates were selected manually based on completeness of the gene cluster, predictability of the leader cleavage site and novelty.

Liter Scale Lantibiotic Production and Purification. Lantibiotics were purified essentially as described previously,[17] with an additional trypsin cleavage step after the elution from the 5 mL HiTrap SP-Sepharose (GE Healthcare) column. The pH was adjusted from 4 to ~6.5 using sodium hydroxide. Trypsin (Sigma-Aldrich) solution 1 mg/mL was added in a 1:100 ratio to the elution fractions and the mix was incubated for 3 h at 37 °C. Subsequently, the lantibiotic containing fractions were desalted with an open spherical C18 versaflash (Supelco) column. The column was washed with 5 CV of

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washing buffer (Milli-Q with 0.1% TFA) and subsequently eluted with a gradient (33%, 66%, 100%) of elution buffer (2:1 2-propanol and acetonitrile with 0.1% TFA). The lantibiotic containing elution fractions from the open C18 column were freeze-dried and purified in a C12 Jupiter 4 μm Proteo 90 Å, LC Column 250 × 10 mm (Phenomenex, USA) using an agilent 1260 HPLC system.[20] The lantibiotic was lyophilized (Labconco, USA) and stored as powder until further use. Concentrations of protein solutions were determined using BCA protein assay (Sigma-Aldrich Cat.No. BCA1) according to the manufacturer's instructions.

Antimicrobial Activity Assays. To assess the antimicrobial activity on plates, the appropriate medium containing 1.5% (w/v) agar was cooled down to ∼45 °C after which 1% (v/v) of an overnight cultured strain was added and mixed. Plates were poured from this mix using 13 mL per plate. Once the plates were solidified they were dried for ∼30 min at 37 °C. 4 μL of the lantibiotic dilutions were pipetted on the plates after which the plates were transferred to the appropriate stove for incubation overnight. The MIC assay was performed according to Wiegand *et al.*[21]

MALDI-TOF. A 1 μL sample of the HPLC purified fractions were spotted and dried on the target. Subsequently, 1 μL of matrix solution (4 mg/mL α-cyano-4-hydroxycinnamic acid from Sigma-Aldrich dissolved in 50% acetonitrile and 0.1% trifluoroacetic acid) was spotted on top of the sample. A Voyager DE PRO matrix-assisted laser desorption ionization−time-of-flight (MALDI-TOF) mass spectrometer (Applied Biosystems) was used to obtain mass spectra. Data were analyzed with "Data Explorer" software version 4.0.0.0 (Applied Biosystems).

MS/MS. To gain insight in the lanthionine bridging pattern we performed MS/MS. The samples were analyzed by nLC-MS/MS on an Ultimate 3000 system (Dionex, Amsterdam, The Netherlands) interfaced online with a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Peptides were loaded onto a trapping micro column (5 mm × 300 μm i.d.) packed with C18 PepMAP100 particles (5 μm, Dionex) in 0.1% formic acid at a flow rate of 20 μL min^{-1}. After loading and washing for 2 min, the peptides were forward-flush eluted onto a nanocolumn (15 cm × 75 μm i.d.) packed with C18 PepMAP100 particles (3 μm, Dionex). The following mobile phase gradient was executed at a flow rate of 300 nL min^{-1}:10−90% of solvent B in 10 min; 90% B for 10 min, and back to 10% B in 1 min. Solvent A was 100:0 H2O−acetonitrile (v/v) with 0.1% formic acid, and solvent B was 10:90 H2O−acetonitrile (v/v) with 0.1% formic acid. Peptides were infused in the mass spectrometer *via* a dynamic nanospray probe (Thermo Electron Corp.) with a stainless steel emitter (Proxeon, Odense, DK). The typical spray voltage was 1.6 kV with no sheath and auxiliary gas flow; the ion transfer tube temperature was at 200 °C. The mass spectrometer was operated in data-dependent mode. Automated gain control (AGC) was set to 1E6 charges for full scan FTMS and 8000 charges for MS/MS at the linear ion trap analyzer. The Data Dependent Acquisition (DDA) cycle consisted of the survey scan within m/z 400−1700 at the orbitrap analyzer with target mass resolution of 60 000 (full width half-maximum at m/z 400) followed by MS/MS fragmentation of the most intense precursor ions under the relative collision energy of 35% in the linear trap and measured at a resolution of 7500 in the orbitrap. Singly and doubly charged ions were excluded from MS/MS experiments. The ion selection threshold for triggering MS/MS experiments was set to 5000 counts. An activation q of 0.25 and an activation time of 30 ms were applied.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00033.

MS/MS pattern of the 6 times dehydrated flavucin (PDF)

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Notes

The authors declare no competing financial interest.

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