HHS Public Access

Author manuscript

Nat Chem Biol. Author manuscript; available in PMC 2013 September 01.

Published in final edited form as:

Nat Chem Biol. 2013 March ; 9(3): 157–159. doi:10.1038/nchembio.1162.

The Sequence of the Enterococcal Cytolysin Imparts Unusual Lanthionine Stereochemistry

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Abstract

The enterococcal cytolysin is a two-component lantibiotic of unknown structure with hemolytic activity that is important for virulence. We prepared cytolysin by co-expression of each precursor peptide with the synthetase CylM in *E. coli*, and characterized its structure. Surprisingly, cytolysin is the first example of a lantibiotic containing lanthionine and methyllanthionine structures with different stereochemistries in the same peptide, which is determined by the sequence of the substrate peptide.

The enterococcal cytolysin is a two-component lantibiotic made up of the posttranslationally modified peptides $CylL_L''$ and $CylL_S''$. It is produced by many clinical isolates of *Enterococcus faecalis* and is toxic to a broad range of gram positive bacteria[1–3]. The enterococcal cytolysin is a unique lantibiotic in that it has hemolytic activity against eukaryotic cells in addition to its antimicrobial activity[2,4]. The production of cytolysin enhances virulence in infection models and its association with acute patient mortality is supported by epidemiological data[5–7]. Despite the intensive research focused on the biological functions and biosynthesis of the enterococcal cytolysin[8–10], its structure has never been reported.

Like other members in the lantibiotic family[11], each component of the enterococcal cytolysin is initially expressed as a linear precursor peptide ($CylL_L$ and $CylL_S$) consisting of an N-terminal leader peptide and a C-terminal core peptide. The lanthionine synthetase CylM then dehydrates Ser and Thr residues in the core peptide followed by Michael-type addition of the thiols of Cys residues to the resulting dehydroamino acids to generate

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Author Contributions

W.T. and W.A.v.d.D. designed the study. W.T. performed all experiments. W.T. and W.A.v.d.D. analyzed the data and wrote the manuscript.

Additional information

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Competing financial interests

The authors declare no competing financial interests.

lanthionine (Lan) and methyllanthionine (MeLan) structures, respectively[8,12]. CylB then removes most of the leader peptide and secretes the resulting cyclized peptides[13]. Extracellularly, six additional residues are removed from the N-terminus of each peptide by CylA to generate $CylL_L''$ and $CylL_S''$, which make up mature cytolysin[8,14,15].

Intrigued by the unique properties of the enterococcal cytolysin and its contribution to the virulence of *E. faecalis*, we set out to characterize its structure by determining the ring topology and the stereochemistry of its (Me)Lan residues. Because high level production of cytolysin in *E. faecalis* requires contact with target mammalian cells[10], only relatively small amounts of material can be obtained, which probably has contributed to the lack of prior structural studies.

In this work, $CylL_L''$ and $CylL_S''$ were both produced in *E. coli*. First, $cylL_L$ and $cylL_S$ encoding the precursor peptides as well as *cylM* encoding the lanthionine synthetase were synthesized with codon optimization for use in *E. coli*. Each precursor peptide was co-expressed as an N-terminal His_6-tag fusion peptide with untagged CylM from a pRSF-Duet-1 vector, as previously reported for other lantibiotics[16]. After purification by immobilized metal affinity chromatography, the CylM-modified $CylL_L$ and $CylL_S$ exhibited mass losses of approximately 130 Da and 72 Da, respectively (Supplementary Results, Supplementary Fig. 1). The exact mass shift was confirmed by subsequent endoproteinase AspN digest to remove the first 25 and 37 residues of the leader peptides of $CylL_L$ and $CylL_S$, respectively (for the sequence of the leader peptides, see the legend of Fig. 1). Mass spectrometric analysis of the modified $CylL_L$ indicated that the major product had undergone 7 dehydrations, whereas purified CylM-modified $CylL_S$ treated with AspN displayed a mass consistent with 4 dehydrations (Supplementary Fig. 2). These results match the reported masses of $CylL_L''$ and $CylL_S''$, for which 7 and 4 dehydrations were suggested, respectively[15]. To verify the formation of the thioether linkages, tandem mass spectrometry (MS) was employed to analyze the fragmentation pattern of the7-fold dehydrated $CylL_L$ and 4-fold dehydrated $CylL_S$ (Fig. 1a and 1b). Fragmentation was suppressed at stretches of sequence comprising residues 1–5, 14–18, and 34–38 for the core peptide of modified $CylL_L$, suggesting 3 rings were present in these regions. Similarly, suppressed fragmentation was detected between residues 1–5 and 17–21 of modified $CylL_S$, suggesting it had 2 rings spanning these residues. The fragmentation pattern of modified $CylL_L$ also suggested that Thr27 escaped dehydration by CylM. Collectively, these data suggest the structures shown in Fig. 1a and 1b for modified $CylL_L$ and $CylL_S$, respectively.

The AspN digestion resulted in smaller fragments that allowed tandem MS analysis but did not afford the mature peptides because five residues of the leader peptide remained. To obtain mature $CylL_L''$ and $CylL_S''$, a Glu–1Lys mutation was introduced in $CylL_L$ and $CylL_S$ for subsequent leader peptide removal with trypsin. After co-expression with CylM, His_6-$CylL_L$-E–1K exhibited a mass loss consistent with 7 dehydrations and $CylL_S$-E 1K displayed a mass consistent with loss of 4 water molecules. Tandem MS analysis verified that modified $CylL_L$-E–1K and $CylL_S$-E–1K displayed the same fragmentation patterns as those of the modified wild type peptides. $CylL_L''$ and $CylL_S''$ were successfully obtained by digesting CylM-modified $CylL_L$-E–1K and $CylL_S$-E–1K with trypsin followed by HPLC purification (Supplementary Fig. 3c and 4c).

Although the analysis of the tandem MS fragmentation data suggests that the A rings in both $CylL_L''$ and $CylL_S''$ are formed between Dhb1 and Cys5 (Fig. 1a and 1b), it is difficult to rule out by tandem MS alone that Cys5 is not linked to Dhb2. If this were the case, the final products should have an N-terminal 2-oxobutyryl group as a result of spontaneous hydrolysis of Dhb1, and the final product should display a 1 Da mass increase. To confirm the proposed topology of the A ring, $CylL_L''$ and $CylL_S''$ were analyzed by high resolution electrospray ionization MS[17]. The observed masses of $CylL_L''$ and $CylL_S''$ were 3435.6870 Da and 2031.0260 Da, respectively, consistent with structures containing a MeLan at their N-terminus with calculated masses of 3435.7059 and 2031.0122 (errors of 5.5 ppm and 6.8 ppm, respectively, Supplementary Fig. 3 and 4). The data is inconsistent with 2-oxobutyryl as the N-terminal residue. Therefore, the A rings of $CylL_L''$ and $CylL_S''$ were unambiguously assigned to MeLan residues formed from Cys5 and Dhb1.

Based on the characterization of select lantibiotics in previous studies[18], all the enzymatically formed Lan and MeLan residues in lantibiotics are generally believed to exhibit a "DL" configuration, i.e. the D-configuration at the α-carbon of the former Ser/Thr residue and the L-configuration at the α-carbon of the former Cys residue ((2*S*,6*R*) for Lan and (2*S*,3*S*,6*R*) for MeLan, Fig. 1c, left panel). To confirm the expected stereochemistry of the (Me)Lan residues in $CylL_L''$ and $CylL_S''$, the CylM-modified $CylL_L$ and $CylL_S$ were hydrolyzed in acid, and the amino acids were derivatized and analyzed by gas chromatography-MS (GC/MS) using a column containing a chiral stationary phase[19,20]. Unexpectedly, the MeLan residues in both peptides had the (2*R*,3*R*,6*R*) configuration (Fig. 1c, right panel, 1d, 1f and Supplementary Fig. 5, 6) by comparison with synthetic standards (i.e. both alpha carbons have the L-configuration, hereafter referred to as LL). These findings indicate that the Michael-type addition still occurs with overall *anti* stereochemistry, but with opposite face selectivity for both the attack of the Cys nucleophile onto the Dhb and the protonation of the resulting enolate (Fig. 1c). This observation constitutes the first example of alternative stereochemistry in lantibiotics. Perhaps even more surprising is the finding that the Lan residue in modified $CylL_S$ exhibits the traditional *meso* configuration (DL), and that the two Lan residues in modified $CylL_L$ are present in a 1:1 ratio of DL and LL configurations (Fig. 1e, 1g and Supplementary Fig. 5, 6). These data show that CylM can catalyze the Michael addition with different stereochemistries in a single polypeptide substrate. The 1:1 ratio of DL and LL configurations of the Lan in modified $CylL_L$ could be either a mixture of stereoisomers for both the B and C rings, or it could be a consequence of one ring having the DL configuration and the other ring having the LL configuration. To distinguish between these possibilities, the mutants $CylL_L$-S14T and $CylL_L$-S15T were co-expressed with CylM in *E. coli*, purified, hydrolyzed, derivatized, and analyzed by chiral GC/MS (Supplementary Fig. 7 and 8). The modified $CylL_L$-S14T was found to contain LL-MeLan and only DL-Lan whereas CylM-modified $CylL_L$-S15T exhibited the same GC trace as the modified wild type $CylL_L$ (LL-MeLan, and a 1:1 ratio of LL-Lan and DL-Lan). These results strongly suggest that the B ring of $CylL_L$ is formed between Ser14 and Cys18 and has a LL configuration, whereas the C ring has the DL configuration. With this information, the final structures of $CylL_L''$ and $CylL_S''$ are established (Fig. 2a and 2b).

The data clearly demonstrate that LL-MeLan can be formed in the same peptide that also contains Lan with the more common DL-stereochemistry. This highly unusual observation appears to be at odds with the typical high stereochemical fidelity of enzymes. Interestingly, all (Me)Lan residues that exhibited the LL configuration are formed from a DhxDhxXxxXxxCys motif in which Dhx represents Dha or Dhb, and Xxx represents amino acids other than Ser, Thr and Cys (Supplementary Fig. 9). To probe whether this outcome extends to other naturally occurring lantibiotics, we performed searches for the DhxDhxXxxXxxCys motif in documented lantibiotics (Fig. 3a). Several such motifs were found[17,21–23] allowing experimental probing of the hypothesis. We chose Halβ, one subunit of the two-component lantibiotic haloduracin produced by *Bacillus halodurans* C-125[17], as an example. Halβ was obtained by co-expression of the precursor peptide HalA2 with its lanthionine synthetase HalM2 in *E. coli*[16]. To investigate the stereochemistry of the (Me)Lan in the A ring, the HalM2-modified HalA2 was hydrolyzed, and the amino acids were derivatized and analyzed by chiral GC/MS. The data show clearly that the product contained a 1:2 ratio of LL:DL MeLan and only DL-Lan (Fig. 3b, 3c and Supplementary Fig. 10), consistent with the hypothesis because only one of the three MeLan structures in Halβ is generated from a DhxDhxXxxXxxCys motif (the A ring). The assignment of the LL-MeLan to this A ring of Halβ was supported by GC/MS analysis of the HalM2-modified mutant HalA2-C5A (Supplementary Fig. 11), for which the peak for LL-MeLan was reduced to a basal level. These results therefore show that LL-MeLan residues are present in other lantibiotics, and that their formation is not a special property of CylM. The LL stereochemistry is also not caused by expression in *E. coli* because the same results were obtained for Halβ isolated from *B. halodurans* (Supplementary Fig. 12). Analysis of the databases suggest that other lantibiotics that have been previously reported but for which the stereochemistry has not been determined are also likely to contain LL-(Me)Lan residues (Fig. 3a). In turn, these studies also indicate that the sequence of the substrate peptide can determine the stereoselectivity of (Me)Lan formation, which was unanticipated prior to this study and has important implications for lantibiotic engineering. Whether the determinant is the sequence itself or the position of the sequence within the precursor peptide, or both, will require additional studies.

Purified $CylL_L''$ and $CylL_S''$ were tested for their antimicrobial activity against *Lactococcus lactis* HP. Application of $CylL_L''$ and $CylL_S''$ separately did not result in growth inhibition, but when applied together, a strong inhibition zone was observed (Fig. 3d). Furthermore, when the two compounds were placed a short distance from each other, a strong synergistic zone of growth inhibition was only observed between the two spotted samples (Fig. 3d). The hemolytic activity of cytolysin was investigated using rabbit red blood cells (Fig. 3e). $CylL_L''$ and $CylL_S''$ together lead to maximal cell lysis in 90 min at a concentration as low as 200 nM, whereas either $CylL_L''$ or $CylL_S''$ alone induced minimal cell lysis at a concentration of 800 nM. The synergistic anti-microbial and hemolytic activities of the $CylL_L''$ and $CylL_S''$ prepared in *E. coli*, along with the MS characterization, strongly suggests that the cytolysin produced in this work has the same structure as that obtained from the producing strains in previous studies[1,24].

In summary, we have developed a heterologous production system for the enterococcal cytolysin that is strongly linked to virulence, and we unambiguously characterized its structure. Three non-overlapping five-amino acid rings are present in $CylL_L''$ and two such rings are present in $CylL_S''$. The MeLan residues in the A rings of both $CylL_L''$ and $CylL_S''$ and the Lan residue in the B ring of $CylL_L''$ exhibit the LL configuration that is different from all previously characterized lantibiotics. LL-(Me)Lan residues are predicted to also be present in other lantibiotics, and this prediction was confirmed for the A ring of Halβ. These results emphasize the importance of determining the stereochemistry of lantibiotics, which has thus far only been performed for a handful of family members[18]. With a good supply route to the enterococcal cytolysin as well as analogs, the mechanism of its virulence activity can now be further investigated[25].

On-line Methods

General methods

The genes encoding CylM, $CylL_L$ and $CylL_S$ were synthesized by GeneArt (Invitrogen) with codon usage optimized for *E. coli* expression. All polymerase chain reactions (PCR) were carried out on a C1000™ thermal cycler (Bio-Rad). DNA sequencing was performed by ACGT, Inc. Preparative HPLC was performed using a Waters Delta 600 instrument equipped with appropriate columns. Absorbance of rabbit hemoglobin in 96-well plates was measured with a Synergy™ H4 Microplate Reader (BioTek). GC/MS analysis was performed at the Roy J. Carver Metabolomics Center (UIUC) or Mass Spectrometry Laboratory (School of Chemical Sciences, UIUC) on an Agilent 7890 gas chromatograph (Agilent). LC-ESI-Q/TOF MS analyses were conducted using a Synapt MS system equipped with an Acquity UPLC (Waters). MALDI-TOF MS was carried out on a Voyager-DE-STR (Applied Biosystems). High resolution electrospray ionization (HR-ESI) MS was performed on a Micromass Q-Tof Ultima instrument (Waters).

Materials

All oligonucleotides were purchased from Integrated DNA Technologies and used as received. Restriction endonucleases, DNA polymerases, and T4 DNA ligase were from New England Biolabs. Media components were obtained from Difco Laboratories. Trypsin was purchased from Worthington Biochemical Corporation and other endoproteinases were ordered from Roche Biosciences. Unless specified otherwise, chemicals were purchased from Sigma Aldrich or Fisher Scientific. Defibrinated rabbit blood was ordered from Hemostat Laboratories and used within 10 days of receipt.

Strains and plasmids

The indicator strain, *Lactococcus lactis* HP, and *Bacillus halodurans* C-125 was obtained from American Type Culture Collection. *E. coli* DH5α and *E. coli* BL21 (DE3) cells were used as host for cloning and plasmid propagation, and host for expression, respectively. Co-expression vector pRSFDuet-1 was obtained from Novagen.

Construction of pRSFDuet-1 derivatives for co-expression of CylM and $CylL_L$/$CylL_S$

The gene encoding CylM was first cloned into the multiple cloning site 2 of the pRSFDuet-1 vector (without His-tag) using the *Nde*I and *Kpn*I restriction sites to generate the pRSFDuet-1/CylM-2 vector. The $cylL_L$ and $cylL_S$ genes were then cloned into the multiple cloning site 1 (MCS1) of the pRSFDuet-1/CylM-2 vector (with His-tag) using the *Eco*RI and *Not*I restriction sites to generate pRSFDuet-1/$CylL_L$/CylM-2 and pRSFDuet-1/$CylL_S$/CylM-2 vectors, respectively. Primer sequences are shown in Supplementary Table 1.

Construction of pRSFDuet-1 derivatives for co-expression of CylM and $CylL_L$/$CylL_S$-E 1K, $CylL_L$-S14T, and $CylL_L$-S15T

Site-directed mutagenesis of pRSFDuet-1/$CylL_L$/CylM-2 and pRSFDuet-1/$CylL_S$/CylM-2 constructs was performed by multi-step overlap extension PCR. Negative numbers are used for amino acids in the leader peptide counting backwards from the leader peptide cleavage site. First, the amplification of the 5′ part of $cylL_L$/$cylL_S$ was carried out by 30 cycles of denaturing (95 °C for 10 s), annealing (55 °C for 30 s), and extending (72 °C for 15 s) using the $cylL_L$/$cylL_S$ *Eco*RI FP and an appropriate mutant reverse primer (Supplementary Table 1) to generate a forward megaprimer (FMP). The PCR mixture included *Phusion* DNA polymerase (Finnzymes; 0.04 U/μL), dNTP (2 mM each), primers (1 μM each), and appropriate buffers. In parallel, a PCR reaction using an appropriate mutant forward primer (Supplementary Table 1) and the $cylL_L$/$cylL_S$ *Not*I RP was also performed to produce 3′ fragments (termed reverse megaprimer, RMP). The 5′ FMP fragment and the 3′ RMP fragment were purified by 2% agarose gel and combined in equal amounts (by mol, approximately 20 ng each for a 50 μL PCR reaction) and amplified using the same PCR conditions with $cylL_L$/$cylL_S$ *Eco*RI FP and $cylL_L$/$cylL_S$ *Not*I RP. The resulting PCR products were then cloned into the MCS1 of pRSFDuet-1/CylM-2 to generate pRSFDuet-1/$CylL_L$-E 1K/CylM-2, pRSFDuet-1/$CylL_S$-E 1K/CylM-2, pRSFDuet-1/$CylL_L$-S14T/CylM-2 and pRSFDuet-1/$CylL_L$-S15T/CylM-2.

Expression and purification of modified His_6-$CylL_L$ and His_6-$CylL_S$ precursor peptides

E. coli BL21 (DE3) cells were transformed with the pRSFDuet-1/CylM-2-$CylL_L$ or pRSFDuet-1/$CylL_S$/CylM-2 vectors and plated on an LB plate containing 50 mg/L kanamycin. A single colony was picked and grown in 50 mL of LB with kanamycin at 37 °C for 12 h and the resulting culture was used to inoculate 3 L of LB. Cells were cultured at 37 °C until the OD at 600 nm reached 0.5, cooled and IPTG was added to a final concentration of 0.1 mM. The cells were cultured at 18 °C for another 18 h before harvesting. The cell pellet was resuspended at room temperature in LanA start buffer (20 mM NaH_2PO_4, pH 7.5 at 25 °C, 500 mM NaCl, 0.5 mM imidazole, 20% glycerol) and lysed by sonication. The sample was centrifuged at 23,700×*g* for 30 min and the supernatant was kept. The pellet was then resuspended in LanA buffer 1 (6 M guanidine hydrochloride, 20 mM NaH_2PO_4, pH 7.5 at 25 °C, 500 mM NaCl, 0.5 mM imidazole) and sonicated again. The insoluble portion was removed by centrifugation at 23,700×*g* for 30 min and the soluble portion was kept. Both soluble portions were passed through 0.45-μm syringe filters and the His-tagged modified peptides were purified by immobilized metal affinity chromatography (IMAC) as previously described[26]. The eluted fractions were desalted by preparative reversed phase (RP) HPLC

using a Waters Delta-pak C4 column (15 μm; 300 A; 25 × 100 mm). The desalted peptides were lyophilized and stored at minus;20 °C for future use.

Protease cleavage and purification of core peptides

Modified $CylL_L$ and $CylL_S$ precursor peptides were dissolved in H_2O to a concentration of 3 mg/mL. To 85 μL of peptide solution, 10 μL of 500 mM HEPES buffer (pH 7.5) was added, followed by 5 μL of 0.02 mg/mL AspN protease (for wild type peptides) or 5 mg/mL trypsin (for the E–1K mutants). The protease cleavage reaction mixtures were kept at 25 °C (for wild type peptides) or 37 °C (for the E–1K mutants) for 6 to 16 h, respectively, and were then quenched with 0.5% TFA. The generation of the desired products was confirmed by MALDI-TOF MS and the products were purified by reversed phase (RP) HPLC using a Jupiter proteo C12 column (5 μm; 90 A; 250 × 4.6 mm; Phenomenex). Solvents for RP-HPLC were solvent A (0.1% TFA in water) and solvent B (0.086% TFA in 80% acetonitrile/20% water). The desired core peptides eluted from the column between 50 and 70% solvent B and pure fractions were collected and lyophilized for further usage. Large scale purification was conducted by RP HPLC using a Jupiter proteo C12 semi-preparative column (5 μm; 90 A; 250 × 10.0 mm).

LC-ESI-Q/TOF MS and MSMS analyses

A 5 μL volume of sample (approximately 100 μg/mL) was injected on a Waters Acquity UPLC system equipped with a BEH C8 column (1.7 um, 100 mm × 1.0 mm; Waters). The column was pre-equilibrated in aqueous solvent. The solvents used for LC were: solvent A = 0.1% formic acid in water and solvent B = 0.1% formic in methanol. A solvent gradient of 3%–97% B over 15 min was employed and the fractionated sample was directly subjected to ESI-Q/TOF MS analysis using a Waters Synapt mass spectrometer. The mass spectrometer was calibrated before any sample was injected. Data was acquired in ESI positive mode with the capillary voltage set to 3.0–3.5 kV. The ionization source and desolvation gas were heated to 120 °C and 300 °C, respectively. Cone gas was set to 0 L/h and desolvation gas was set to 600 L/h. The transfer collision energy was set to 4 V for both MS and MSMS analyses. The trap collision energy was set to 6 V for MS analysis. For MSMS analysis, a trap collision energy ramp ranging from 20–40 V was applied on multiply charged parent ions to achieve fragmentation. Suitable trap collision energy was determined by choosing the spectra where both fragment peaks and parent peak could be observed. [Glu^1]-Fibrinopeptide B (Sigma) was directly infused as lock mass with lock spray sampling if desired. The acquired spectra were processed using MaxEnt3 software and analyzed by Protein/Peptide Editor in BioLynx 4.1 (Waters).

Antimicrobial activity assay of $CylL_L''$ and $CylL_S''$

$CylL_L''$ and $CylL_S''$ were obtained as described above by trypsin cleavage of CylM-modified $CylL_L$ E–1K and $CylL_S$ E–1K followed by HPLC purification. Peptides were dissolved in 2:1 acetonitrile:H_2O to achieve a concentration of 100 μM. The peptide solutions were diluted with phosphate buffered saline (PBS) to prepare a 10 μM solution. *L. lactis* HP cells were grown in GM17 media under anaerobic conditions at 25 °C for 16 h. Agar plates were prepared by combining 20 mL of melted GM17 agar (cooled to 42 °C for 5 min) with 200

μL of dense overnight cell culture. The seeded agar was poured into a sterile 100 mm round dish (VWR) and allowed to solidify at 25 °C for 30 min. Samples were directly spotted on the solidified agar. Plates were incubated at 30 °C for 16 h and the antimicrobial activity was determined by the presence or absence of zones of growth inhibition. A negative control was conducted using solutions with identical make-up but leaving out the peptides.

Hemolytic assay of CylL$_L$″ and CylL$_S$″

A sample of 1 mL of defibrinated rabbit blood was diluted with 20 mL of PBS in a 50 mL conical tube on ice and mixed well by gently inverting the tube. The PBS-diluted blood sample was centrifuged at 1,000×*g* for 5 min at 4 °C and the supernatant containing lysed blood cells and released hemoglobin was discarded. The process was repeated 2 to 4 times until the supernatant was clear. The blood cells were then diluted with PBS to make a 5% solution, which was immediately used to test the hemolytic activity of the peptides. Rabbit blood was used within 10 days after receipt to minimize autolysis. CylL$_L$″ and CylL$_S$″ peptide solutions made as described above were diluted with PBS to prepare a 10 μM solution. To a 96-well plate, 100 μL of 5% washed rabbit blood sample was added to each well followed by the addition of the desired peptide samples or controls. In each sample well, CylL$_L$″ was added and mixed well for 5 min before CylL$_S$″ was added. PBS was used to adjust the final volume to 150 μL. The 96-well plate was kept in a 37 °C incubator to allow the lytic reaction to proceed. At each time point, 20 μL of each reaction mixture was taken out, diluted with 200 μL of fresh PBS and centrifuged at 1,000×*g* for 5 min. The supernatant (120 μL) was transferred to a new well and the absorbance was measured at 415 nm (wave length was optimized for rabbit hemoglobin). The absorbance of prepared blood sample at each time point was analyzed in triplicate and the maximum absorbance was determined by adding 50 μL of 0.1% Triton in PBS to 100 μL of 5% blood sample and using the same analysis procedure.

Construction of pRSFDuet-1 derivatives for co-expression of HalM2 and HalA2 C5A

The gene encoding HalA2 C5A was amplified by PCR from a pET15b/HalA2Xa-C5A vector reported previously[27], and cloned into the multiple cloning site 1 of the pRSFDuet-1/HalM2-2 vector using the *BamH*I and *Hind*III restriction sites to generate the pRSFDuet-1/HalA2-C5A/HalM2-2 vector[16]. Primer sequences used are shown in Supplementary Table 1.

Purification of Halβ from *Bacillus halodurans* C-125

The isolation of Halβ from *Bacillus halodurans* C-125 was performed according to a previously reported procedure[28].

GC/MS analysis

The synthesis of Lan and MeLan standards and the preparation of samples for GC/MS analysis were carried out following a reported procedure published elsewhere[19,20]. The NMR spectra of the synthetic standards matched the literature values.[19] The modified precursor peptides with their leader sequences attached were hydrolyzed and the resulting solutions were dried and directly used for derivatization. The derivatized samples were analyzed by GC/MS using an Agilent 7890 gas chromatograph equipped with a Varian CP-

Chirasil-L-Val fused silica column (25 m × 0.25 mm × 0.15 μm). Samples were dissolved in methanol and introduced to the instrument via a splitless/split (1:20) injection at a flow rate of 1.7 or 2.0 mL/min helium gas. The temperature method used was 160 °C for 5 min, 160 °C to 180 °C at 3 °C/min, and 180 °C for 6 min. The mass spectrometer was operated in simultaneous scan/selected-ion monitoring (SIM) mode, monitoring at the characteristic fragment masses of 365 Da for Lan and 379 Da for MeLan residues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Ms. Xiao Yang for providing the modified HalA2 and Halβ peptides. This work was supported by the National Institutes of Health (GM58822).

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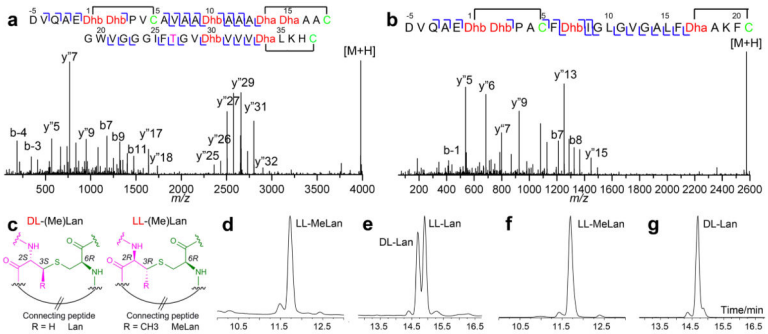

Figure 1. Structural analysis of CylM-modified $CylL_L$ and $CylL_S$

(a) Fragmentation pattern of the $CylL_L$ core peptide modified by CylM in *E. coli*. **(b)** Fragmentation pattern of the CylM-modified $CylL_S$ core peptide. Both core peptides contain five residues at their N terminus that remain from the leader peptides after AspN cleavage. The b7 fragment ions observed for both peptides are attributed to in-ring fragmentation. Similar fragmentation has been observed in the A rings of nisin and geobacillin II[16,23]. **(c)** Canonical (DL, left) and unusual (LL, right) stereochemistries of (Me)Lan residues in previously characterized lantibiotics and in cytolysin investigated in this work, respectively. GC-MS traces of **(d)** hydrolyzed and derivatized MeLan residues from CylM-modified $CylL_L$, (e) hydrolyzed and derivatized Lan residues from CylM-modified $CylL_L$, (f) hydrolyzed and derivatized MeLan residues from CylM-modified $CylL_S$, and (g) hydrolyzed and derivatized Lan residues from CylM-modified $CylL_S$. For co-injection traces with synthetic (Me)Lan standards, see Supplementary Fig. 5 and 6. Dha, dehydroalanine; Dhb, dehydrobutyrine. The sequences of the leader peptides of $CylL_L$ and $CylL_S$ are MENLSVVPSFEELSVEEMEAIQGSGDVQAE and MLNKENQENYSNKLELVGPSFEELSLEEMEAIQGSGDVQAE, respectively. The leader peptide residues remaining after cleavage with AspN are underlined.

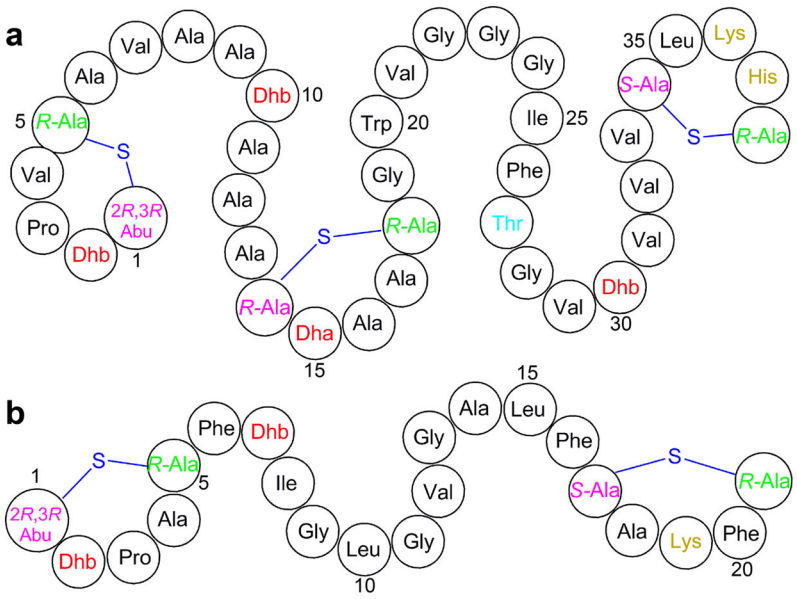

Figure 2. Structure of cytolysin determined in this work

(a) $CylL_L''$. **(b)** $CylL_S''$. Abu, α-aminobutyric acid. Overall, these peptides are highly hydrophobic except for the positively charged N-termini and residues (Lys and His) near the C-terminus (marked in dark yellow). Production of cytolysin in *E. coli* allows investigation of the importance of these residues for the unique hemolytic activity of this lantibiotic.

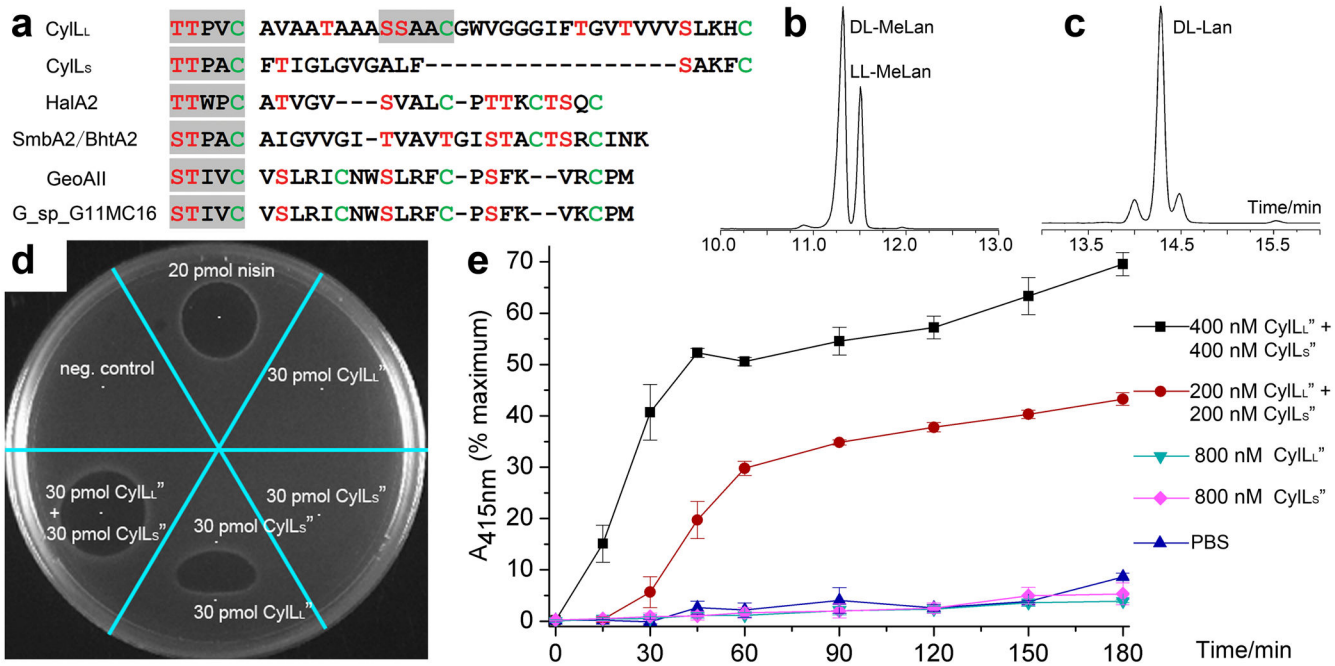

Figure 3. Alignment of 7 lantibiotic core peptides, stereochemical analysis of Halβ, and bioactivity of CylL$_L$″ and CylL$_S$″

(a) Alignment of lantibiotic core peptides that are transformed to intermediates with the DhxDhxXxxXxxCys motif highlighted in grey. The amino acid sequences of SmbA2 and BhtA2 are identical, but it is unclear whether they are modified into the same structure. **(b)** GC-MS traces of hydrolyzed and derivatized MeLan residues from HalM2-modified HalA2. **(c)** GC-MS traces of hydrolyzed and derivatized Lan residues from HalM2-modified HalA2. For co-injection traces with synthetic (Me)Lan standards, see Supplementary Fig. 10. **(d)** Antimicrobial activity assay of CylL$_L$″ and CylL$_S$″ against *L. lactis* HP. The amounts of compounds applied are indicated (scale bar 1 cm). **(e)** Hemolytic assay of CylL$_L$″ and CylL$_S$″ against rabbit red blood cells. At each time point, the hemoglobin released from the lysed cells was quantified in triplicate by measuring the absorbance of the supernatant at 415 nm. Error bars indicate the standard deviation of the three separated experiments.