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Structure and Biosynthesis of Carnolysin, a Homologue of Enterococcal Cytolysin with D-Amino Acids

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Supporting Information

ABSTRACT: Lantibiotics are a group of highly post-translationally modified bacterial antimicrobial peptides characterized by the presence of the thioether-containing amino acids lanthionine and methyllanthionine. *Carnobacterium maltaromaticum* C2 was found to produce a two-component lantibiotic homologous to enterococcal cytolysin. Through tandem mass spectrometry and NMR spectroscopy, the post-translational modifications of carnolysin were established, and the topologies of the lanthionine and methyllanthionine rings were determined. Chiral GC-MS analysis revealed that, like cytolysin, carnolysin contained lanthionine and methyllanthionine residues of unusual stereochemistry. Carnolysin, unlike cytolysin, was shown to contain D-alanine and unprecedented D-aminobutyrate derived from serine and threonine, respectively. Carnolysin was heterologously expressed in *Escherichia coli*, demonstrating that reductase CrnJ is involved in the formation of the D-amino acids.

Bacteriocins are a group of ribosomally synthesized antimicrobial peptides produced by bacteria.[1] The lantibiotics, or lanthipeptides, are a major group of highly post-translationally modified bacteriocins. Lantibiotics are characterized by the presence of the thioether-bridged amino acids lanthionine (Lan) and methyllanthionine (MeLan) (Figure 1A).[1] During lantibiotic maturation, select serine and threonine residues are dehydrated, forming dehydroalanine (Dha) and dehydrobutyrine (Dhb) respectively. Then, a cysteine thiol undergoes an enzyme-catalyzed Michael addition onto the β-position of Dha or Dhb, thereby forming Lan or MeLan.

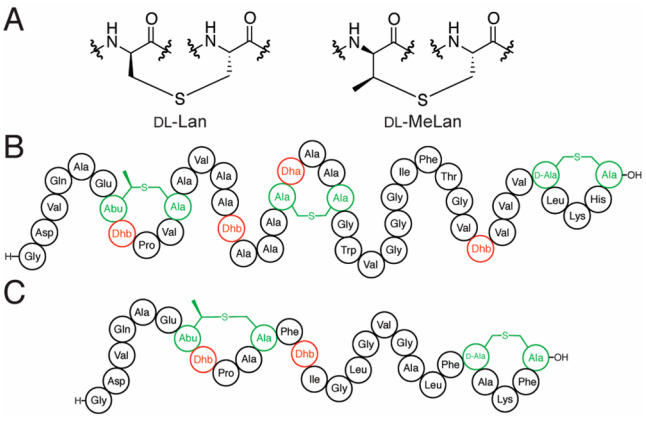

Figure 1. Structures of (A) DL-lanthionine and DL-methyllanthionine, (B) cytolysin L_L', and (C) cytolysin L_S'.[4] Lan/MeLan indicated in green, Dha/Dhb in red.

Enterococcal cytolysin, a virulence factor produced by strains of *Enterococcus faecalis*, exhibits both hemolytic and antibacterial activity.[2] Structurally, cytolysin is a lantibiotic consisting of two components: $CylL_L''$ and $CylL_S''$ (Figure 1B, 1C).[2−4] Cytolysin maturation begins with the formation of Dha/Dhb and Lan/MeLan residues by lantibiotic synthetase CylM. During export, the protease domain of transporter CylB removes a portion of the leader sequence, forming singly cleaved $CylL_L'$ and $CylL_S'$. Then, extracellular protease CylA removes a secondary leader sequence, affording the doubly cleaved fully active $CylL_L''$ and $CylL_S''$.[3]

Until very recently, only the DL-Lan and DL-MeLan stereoisomers had been found in lantibiotics.[4] However, stereochemical analysis of enterococcal cytolysin revealed the presence of unusual LL-Lan and LL-MeLan stereoisomers in certain positions, and the more typical DL-Lan stereoisomer in others (Figure 1B, 1C).[4a] The LL-Lan and LL-MeLan rings all contained a Dha/Dhb as the N-terminal residue within the ring. It was suggested that this caused the enzymatic machinery to affect the cysteine trajectory during Lan/MeLan formation.[4a]

Previously, we described the isolation of carnolysin A1′ and A2′, a two-component lantibiotic produced by *Carnobacterium maltaromaticum* C2.[5] The carnolysin biosynthetic genes were homologous to those associated with cytolysin.[2] Furthermore, the isolated carnolysins CrnA1′ and CrnA2′ resembled singly cleaved cytolysins $CylL_L'$ and $CylL_S'$, respectively. However, the doubly cleaved peptides (CrnA1″ and CrnA2″) were not detected in the culture supernatant. Further, no antimicrobial activity was observed for CrnA1′ and CrnA2′. We now describe our characterization of the structure, biosynthesis, and biological activity of carnolysin.

Isolated CrnA1′ was first characterized by tandem mass spectrometry (Figure 2A). As with other lantibiotics, fragmentation was not observed in regions contained within Lan and MeLan rings.[6] The Lan/MeLan topology of CrnA1′ appeared similar to $CylL_L''$, bearing an N-terminal MeLan ring, a central Lan ring, and a C-terminal Lan ring. Furthermore, the MS/MS data unexpectedly suggested the presence of four alanine residues in positions genetically encoded as serine residues.

To resolve the structural ambiguities, NMR spectroscopic data were obtained for CrnA1′. The majority of the proton chemical shifts were assigned based on TOCSY and NOESY data sets.

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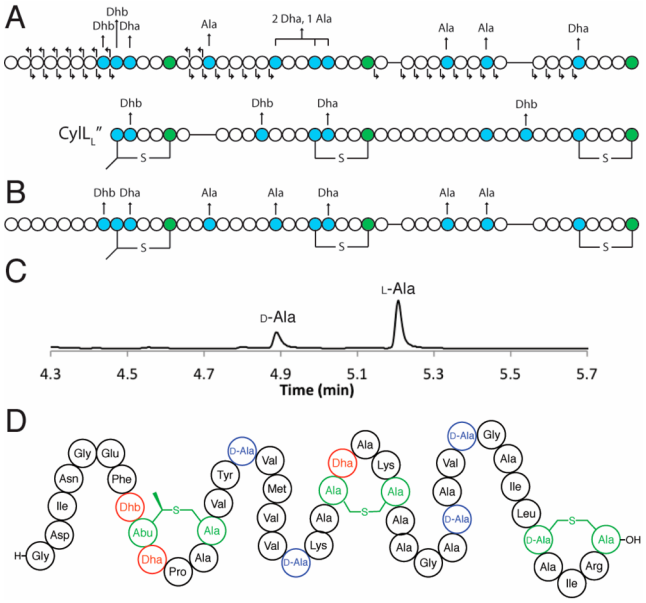

Figure 2. (A) Summary of MS/MS data for CrnA1′ and comparison to CylL$_L$″.[4a] Blue residues are genetically encoded as Ser and Thr, and green residues as Cys. (B) Structure of CrnA1′ based on NMR analysis. (C) GC-MS trace of CrnA1′ Ala residues. (D) Full stereochemical structure of CrnA1′. Lan/MeLan indicated in green, Dha/Dhb in red, and D-Ala in blue.

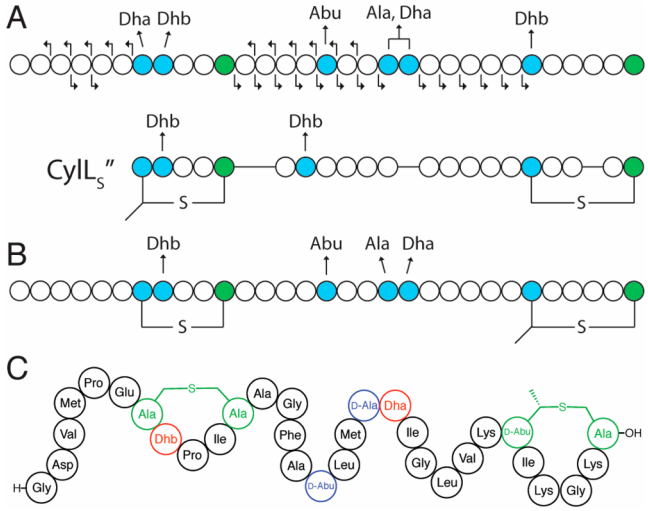

Figure 3. (A) Summary of MS/MS data for CrnA2′ and comparison to CylL$_S$″.[4a] Blue residues are genetically encoded as Ser and Thr, and green residues as Cys. (B) Structure of CrnA2′ based on NMR analysis. (C) Full stereochemical structure of CrnA2′. Lan/MeLan indicated in green, Dha/Dhb in red, and D-Ala/D-Abu in blue.

These data allowed for the determination of the full primary structure, confirming the replacement of four serines with alanines (Figure 2B). Further, the Lan/MeLan connectivities were supported by NOE crosspeaks spanning the thioether linkages (Figure S1).

GC-MS was used to determine the stereochemistry of the Lan and MeLan residues of CrnA1′. Purified peptide was hydrolyzed, and the constituent amino acids were derivatized. Co-injection of the derivatized sample with synthetic standards revealed the exclusive presence of the unusual LL-MeLan stereoisomer (Figure S4). Chiral GC-MS indicated the presence of both DL-Lan and LL-Lan stereoisomers (Figure S5). To determine the position of each Lan stereoisomer, some means of cleaving the peptide between these residues was required. Due to the lack of convenient protease cleavage sites between the Lan rings, CrnA1′ was treated to partial hydrolysis conditions. The fragments were separated by HPLC, and a fraction containing only C-terminal fragments of CrnA1′ was obtained (Figure S2). The peptides in this fraction were fully hydrolyzed and derivatized as before. Co-injection with standards showed that the C-terminal Lan is the DL stereoisomer, indicating that the central ring is LL-Lan (Figure S5).

Next, it was of interest to determine if the Ala residues derived from Ser were D-amino acids. GC-MS analysis of derivatized CrnA1′ revealed the presence of both D- and L-Ala stereoisomers (Figures 2C, S3). Furthermore, the relative peak areas suggested a ratio of 9 L-Ala to 4 D-Ala. This is consistent with the exclusive formation of the D-Ala stereoisomer from serine residues. Based on these data, the full structure of CrnA1′ was proposed (Figure 2D).

As with CrnA1′, CrnA2′ was analyzed by tandem mass spectrometry. MS/MS data suggested a similar Lan/MeLan ring topology to CylL$_S$″ (Figure 3A). The data indicated the presence of an alanine in a position genetically encoded as a serine. Furthermore, the data suggested the presence of an α-aminobutyrate (Abu) residue in place of a threonine, which is to our knowledge an unprecedented amino acid in a prokaryotic ribosomally synthesized protein.

To clarify the structural ambiguities and to confirm the presence of Abu, CrnA2′ was isolated from a 20 L culture of *C. maltaromaticum* C2 and analyzed by NMR spectroscopy. The majority of the chemical shifts were assigned using TOCSY and NOESY data sets. These data revealed the extent of post-translational modification, indicating an N-terminal Lan ring and a C-terminal MeLan ring (Figures 3B, S1). Furthermore, these data supported the presence of Abu in position 16.

To examine the amino acid stereochemistries, CrnA2′ was hydrolyzed and derivatized as described above. Co-injection with Lan standards indicated that CrnA2′ contains only the unusual LL-Lan stereoisomer (Figure S8), while coinjection with MeLan standards revealed only the more common DL-MeLan stereoisomer (Figure S7). Analysis of the Ala residues demonstrated the presence of D-Ala, consistent with the expected conversion of a single serine into an alanine (Figure S6). Furthermore, coinjection experiments confirmed the presence of D-Abu (Figure S6). Based on these data, the full structure of CrnA2′ was proposed (Figure 3C).

The positions and sizes of the Lan/MeLan rings are similar between carnolysin and cytolysin.[4a] Furthermore, the placement of the unusual Lan and MeLan LL- stereoisomers are the same, as is the presence of Dha/Dhb residue within these rings. The positions of other Dha/Dhb residues differ between these structures. The most notable difference is the presence of D-Ala and D-Abu in carnolysin, residues not found in cytolysin. To our knowledge, carnolysin is only the third lantibiotic to contain D-Ala (along with lacticin 3147 and lactocin S),[7,8] and the first ribosomally synthesized peptide to contain D-Abu. The D-Ala residues of lacticin 3147 are introduced by the reduction of select Dha residues by LtnJ.[7b] Replacement of a serine residue (ultimately converted into D-Ala) with threonine was found to result in the formation of Dhb, but not D-Abu.[7b] Reductase CrnJ, which does not have a homologue in cytolysin biosynthesis, is the most likely candidate for the analogous transformation.[5] CrnJ

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may be more promiscuous than LtnJ, capable of reducing both Dha and Dhb.

To confirm the role of CrnJ in D-Ala and D-Abu formation, a heterologous expression system was used.[4a] A plasmid was created expressing lantibiotic synthetase CrnM with His_6-CrnA1 (pRSF-Duet-crnM-crnA1), or with His_6-CrnA2 (pRSF-Duet-crnM-crnA2). A second plasmid was prepared encoding CrnJ (pET-Duet-crnJ). For protein expression, *E. coli* BL21(DE3) was cotransformed with pET-Duet-crnJ and either pRSF-Duet-crnM-crnA1 or pRSF-Duet-crnM-crnA2. Following culture growth and induction of protein expression, the resulting peptides were purified by affinity chromatography and HPLC.

From the culture expressing His_6-CrnA1, an HPLC fraction containing the post-translationally modified peptide was identified (Figure S11). The mass of this peptide was consistent with 10 dehydrations, four reductions, and the loss of fMet-1. MS/MS analysis supported the identity of this isolate, as well as the positions of several modifications. Similarly, the culture expressing His_6-CrnA2 was found to produce the fully post-translationally modified peptide with six dehydrations and two reductions (Figure S11).

To confirm that the heterologously produced peptides were consistent with the natural isolates, proteolytic cleavage was required. The protease domain of ABC transporter CrnT was likely responsible for this conversion.[9] Plasmid pET-28-crnT-150 was constructed and transformed into *E. coli*.[9] Following expression and purification (Figure S12), protease CrnT-150 was incubated with His_6-CrnA1* and His_6-CrnA2*. MS analysis of the digests revealed ions consistent with CrnA1′ and CrnA2′ (Figures 4, S13, and S14). MS/MS data for these peptides were

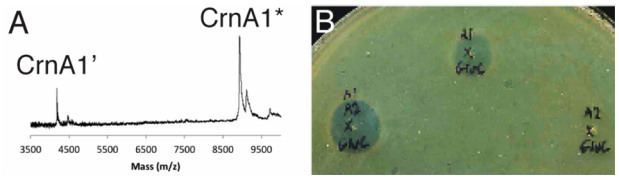

Figure 4. (A) CrnT-150 digest of His_6-CrnA1*. (B) Activity test, from left to right, of (1) CrnA1′ and CrnA2′ with GluC, (2) CrnA1′ with GluC, and (3) CrnA2′ with GluC. Activity testing was performed using carnolysins isolated from *C. maltaromaticum* C2.

consistent with the natural isolates. Furthermore, LC-MS coinjection of the CrnA1′ from *E. coli* with the natural isolate resulted in only a single peak of the expected mass (data not shown). The reaction of CrnA2′ with the reducing agent required for CrnT-150 activity[9a] precluded similar coinjection experiments (Figure S14). However, based on these data, the post-translational modifications installed by CrnM and CrnJ in *E. coli* are the same as those in *C. maltaromaticum* C2. Expression of CrnM with CrnA1 or CrnA2 in the absence of CrnJ did not yield any ions consistent with reduced carnolysin, as judged by mass spectrometry (data not shown). CrnJ is therefore responsible for the formation of D-Ala and D-Abu in CrnA1′ and CrnA2′.

CrnA1′ and CrnA2′ were not antimicrobially active at the levels tested.[5] An additional proteolytic cleavage was predicted to be required to obtain active peptides, analogous to cytolysin.[3] Based on the different N-terminal sequences of CrnA1′ and CrnA2′, it is unclear whether cleavage of both peptides occurs. Putative protease CrnP, a homologue of CylA,[3] is encoded within the carnolysin gene cluster. Based on the similarity of the CrnA2′ N-terminus to those of $CylL_L'$ and $CylL_S'$, CrnP may be expected to selectively cleave CrnA2′. However, efforts to express CrnP (removal of signal sequence,[10] varied position of His_6-tag) yielded insoluble protein. Cleavage assays following attempts to refold CrnP did not demonstrate any observable activity (data not shown).

The predicted cleavage site of CrnA2′ follows Glu-6, a cleavage site for endoproteinase GluC. However, GluC cleavage was slow (Figure S9), likely impeded by neighboring Pro and Lan residues. The digest was mixed with CrnA1′ and tested for activity against *Lactococcus lactis*, but no inhibition was observed. Only following GluC digestion of both CrnA1′ and CrnA2′ was strong antimicrobial activity observed (Figures 4, S10). This may result from cleavage C-terminal to Glu-6 of CrnA1′, yielding a peptide more similar to $CylL_L''$ (Figure S9). Unexpectedly, GluC-treated CrnA1′ alone was weakly active against *L. lactis*, although not to the same extent as when it was combined with GluC-treated CrnA2′ (Figure 4).

The carnolysin GluC digest was tested for its spectrum of activity (Table S1). Like most lantibiotics, these peptides were only active against Gram-positive indicator strains.[1] The scope of Gram-positive bacteria inhibited by carnolysin was fairly broad, with strong inhibition observed of strains including *L. lactis*, *Enterococcus faecium*, and *C. maltaromaticum*. Contrasting cytolysin, no hemolysis was observed following testing of the carnolysin digest on sheeps blood agar. Therefore, this analogous structure may provide insight into the hemolytic activity exhibited by cytolysin. It is unclear whether this is a function of amino acid identity, differing post-translational modifications, or the extent of proteolytic processing.

Overall, we describe the full connectivity and stereochemistry of carnolysin, a novel two-component lantibiotic. Carnolysin is the first lantibiotic to contain the unusual LL-Lan and LL-MeLan stereoisomers in combination with D-amino acids. Furthermore, this is to our knowledge the first example of a ribosomally synthesized peptide containing D-Abu. Through heterologous expression, reductase CrnJ was shown to be involved in the formation of D-Ala and D-Abu. Following proteolytic cleavage, antimicrobial activity of carnolysin was achieved. Furthermore, no hemolytic activity was observed at the levels tested.

The ability of CrnJ to reduce Dha and Dhb residues may allow for enzymatic introduction of D-amino acids into other peptides. Appending the carnolysin leader sequence to other peptides could allow for the installation of D-Ala and D-Abu. The promiscuity of CrnJ for dehydro residues will be explored, to see if other transformations (e.g., conversion of dehydrovaline to D-Val) are catalyzed.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Purification and expression protocols, NMR chemical shifts and spectra of important NOEs, GC-MS traces, MALDI-TOF MS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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