Purification and structure elucidation of paraplantaricin TC318, a new lantibiotic produced by *Lactobacillus paraplantarum* OSY-TC318

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Abstract

A new *Lactobacillus paraplantarum* strain isolated from a Turkish cheese showed potent antimicrobial activity that inhibits *Bacillus cereus*, *Micrococcus luteus*, *Lactobacillus casei* and *Pediococcus pentosaceus*. The producer strain, designated *Lactobacillus paraplantarum* OSY-TC318, was identified using rapid MALDI-TOF mass spectrometry (MS), 16S rDNA sequencing and further confirmed by whole genome sequencing (WGS). The antimicrobial agent was extracted from cells of OSY-TC318 with 70% isopropanol, purified by high-performance liquid chromatography, and structurally analyzed using Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometry. The MS and WGS analyses, taken together, elucidated the structure of the novel lantibiotic consisting of 22 amino acids and four thioether bridges. The molecular mass of the new lantibiotic, designated paraplantaricin TC318, is 2263.900 Da. Paraplantaricin TC318 contains one didehydroalanine (Dha), one didehydrobutyric acid (Dhb), one methyllanthionine (MeLan), two lanthionine (Lan) residues and a C-terminal S-[(Z)-2-aminovinyl]-D-cysteine (AviCys) residue. The proposed primary sequence of paraplantaricin TC318 is: F-K-A-W-Dha-L-A-Abu-F-G-A-G-H-Dhb-G-A-F-N-A-F-A. This lantibiotic, which differs from mutacin 1140 at positions 9, 12, 13 and 20, is a new member of the epidermin group in class I lantibiotic.

Key Words: bacteriocin, lantibiotic, *Lactobacillus paraplantarum*, paraplantaricin TC318

Introduction

Bacteriocins are proteinaceous antimicrobial agents produced by bacteria. Bacteriocins produced by Gram-positive bacteria such as lactic acid bacteria (LAB) can be modified or unmodified after translation. The most studied LAB bacteriocin, nisin produced by *Lactococcus lactis*, was discovered in 1928 (Rogers, 1928). Nisin was approved by FDA in 1988 to be used commercially as a natural food bio-preservative, which increases the interest in other bacteriocins produced from LAB (McAuliffe et al., 2001). LAB have been used in the manufacture of fermented food due to their role in the flavor and texture development besides their ability of inhibiting the growth of pathogenic and spoilage microorganisms (Abee, 1995; Stiles, 1996).

Lantibiotics are ribosomally synthetized bacteriocins that are post-translationally modified to their biologically functional forms (Chatterjee, Paul, Xie, & Van Der Donk, 2005). The name lantibiotics is referring to lanthionine-containing antibiotic peptides and their biosynthesis associated proteins are designated generally as Lan. Lantibiotics are characterized by the presence of unusual amino acids such as lanthionine (Ala-S-Ala), 3-methyllanthionine (Abu-S-Ala), 2,3-didehydroalanine (Dha) which is a dehydrated serine and 2,3-didehydrobuterine (Dhb) which is a dehydrated threonine (van Kraaij et al., 1999; Guder et al., 2000). Lantibiotics have been classified according to their biosynthetic pathways (Willey & van der Donk, 2007). Class I lantibiotics are modified by LanB (dehydratase) and LanC (cyclase) enzymes. Class II are modified by LanM, a bifunctional single enzyme with both dehydratase and cyclase activity. Class III is modified by LanKC single enzyme and class IV is modified by LanL (van der Donk & Nair, 2014). Additionally, lantibiotic operon includes other enzymes encoding genes to carry out the transportation of the propeptide (*lanT*), the proteolytic processing for cleaving the leader sequence (*lanP*), the immunity (*lanI* and *lanFEG*) as well as the structural gene (*lanA*) (Field, Cotter, Hill, & Ross, 2015).

Nisin is an example of class I lantibiotics. Additionally, class I includes the epidermin group which are characterized by the presence of one Dhb, one methyllanthionine, and two lanthionine residues besides the unusual ring structure containing S-[(Z)-2-aminovinyl]-D-cysteine (AviCys) (Chatterjee et al 2005). Gallidermin, mutacin 1140, mutacin B-Ny266, and mutacin I are other natural variants to epidermin which share the four thioether bridges (Figure 2). Epidermin was isolated from *Staphylococcus epidermidis* Tü 3298 (Allgaier, Jung, Werner, Schneider, & Zähner, 1985) and gallidermin was isolated from *Staphylococcus gallinarum* Tü 3928 (Kellner et al., 1988). Mutacin 1140, mutacin B-Ny266 and mutacin I were isolated from *Streptococcus mutans* JH1140, Ny266 and CH43, respectively (Mota-Meira, Lacroix, LaPointe, & Lavoie, 1997; Qi, Chen, & Caufield, 2000; Smith et al., 2000). In this study, we report a new lantibiotic produced by *Lactobacillus paraplantarum* OSY-TC318 isolated from a Turkish cheese. Paraplantaricin TC318 is a natural variant to the epidermin group and is closely related to Mutacin 1140 with four amino acids difference.

Materials and Methods

Strain screening

Food samples were collected and screened for antimicrobial-producing bacteria. Thirty-three food products were purchased from local grocery stores in USA and Turkey these included dairy products (kefir, cheddar, blue, Roquefort and fermented Turkish cheeses), herbals (thyme and mint), spices (cumin, black pepper, red pepper and spices mix), meat (Italian salami, fermented sausage, ground meat and Sucuk (Turkish sausage), fermented soybean, kimchi and Turkish beverages (Boza, Salgam and pickle juice). Food samples (10 g each) were homogenized in 0.1% sterile peptone water using a stomacher. Tenfold serial dilutions were made from the homogenate, and a 100-µl aliquot from each dilution was spread plated onto Plate Count Agar, de Man, Rogosa and Sharpe (MRS) agar , Kanamycin Aesculin Azide Agar and Potato Dextrose Agar (Oxoid, Thermo scientific, Waltham, MA,

USA). For the total aerobic mesophilic bacteria, the plates were incubated aerobically at 30°C for 72 h. For LAB, the plates were incubated anaerobically at 30°C for 48 h. For yeasts and molds, the plates were incubated at 26 ºC for 5 days. Hundreds of isolates were screened for their abilities to produce antimicrobial agents using soft-agar overlay technique (Guo et al 2012) with slight modification. Briefly, isolates were inoculated onto MRS Agar and Tryptic Soy Agar (TSA) and plates were incubated at 30ºC for 24 h. The incubated plates were then overlaid with soft agar medium (Luria-Bertani (LB) with 0.75% agar and 0.06% $CaCO_3$ to neutralize the medium) that had been pre-inoculated with *Pediococcus pentosaceus* or *Escherichia coli* K-12. After further incubation at 30°C for 24 h, the overlaid plates were inspected for any zones of inhibition of the indicator strain. Among a few isolates showing antimicrobial activity, one isolate (designated OSY- TC318) produced a clear zone of inhibition against *Pediococcus pentosaceus.* Considering its strong activity against the indicator organism, this isolate was subjected to further analysis.

Bacterial strains and media

Bacterial strains were obtained from the culture collection at the food microbial safety laboratory at The Ohio State University (Columbus, OH, USA). The producer strain, OSY-TC318, which was isolated from a Turkish cheese, was grown on de Man, Rogosa and Sharpe (MRS) agar (Oxoid, Thermo scientific, Waltham, MA, USA). Selected strains (Table 1) were cultivated in MRS broth and tryptic soy broth (Becton Dickinson, Sparks, MD) to test the antimicrobial peptide spectrum.

Producer strain identification using MALDI-TOF mass spectrometry

The antimicrobial peptide producer OSY-TC318 was streaked on a MRS agar plate and incubated at 30°C for 48 hours to obtain well isolated colonies. Before each MALDI-TOF MS run, the Bruker Biotyper (Bruker Daltonics, Billerica, MA) was calibrated using a bacterial test standard *Escherichia coli* (BTS, Bruker Daltonics, Billerica, MA). A working matrix solution was prepared

by adding 250 μL of standard solvent (50% acetonitrile, 47.5% water and 2.5% trifluoroacetic acid) to 2.5 mg of α-cyano-4-hydroxycinnamic acid crystals (HCCA, Bruker Daltonics). This mixture was vortexed until no crystals were present in the bottom of the tube. Using a wooden tooth pick a single colony was immediately spotted on a clean 96-well MBT Biotarget 96 plate (Bruker Daltonics) and allowed to dry. One microliter of the working matrix solution was added onto the spotted well and allowed to dry. The MBT Biotarget 96 was loaded into the Bruker MicroFlex LT mass spectrometer (Bruker Daltonics) instrument. Spectra were collected on the MicroFlex instrument using the standard method as supplied by Bruker. For the spectral analysis and isolate identification, Biotyper software (MTB Compass 4.1; Bruker Daltonics) was used to match the spectrum against the MALDI-TOF MS Bruker library following Bruker's recommended parameters. Scores between 1.70-1.99 were considered appropriate for genus-level identification and those ≥2.0 were considered candidates for species level identification. Three independent experiments were done to confirm the producer identity.

Producer genetic identification using 16S rDNA sequencing

The genomic DNA of the producer isolate OSY-TC318 was extracted using a commercial kit (DNeasy blood and tissue kit; Qiagen, Valencia, CA). Universal primers (16S forward primer 5'CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG3'and 16S reverse primer 5'CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC3') specific for bacterial 16S rRNA gene amplification were used in the polymerase chain reaction, PCR. The 16S rRNA gene was amplified by the use of a DNA polymerase (MyTaq, Bioline, Taunton, MA, USA) under the following thermocycler conditions: an initial incubation at 95°C for 3 min and 35 cycles of denaturation at 95°C for 15 sec, annealing at 52°C for 1 min, and extension at 72°C for 30 sec. The final extension step was conducted at 72°C for 10 min. The amplified 16S rRNA was purified

using a commercial DNA purification kit (QIAquick gel extraction kit; Qiagen, Valencia, CA) following the manufacturer's instructions. The purified PCR product was then sequenced at the Ohio State University Nucleic Acid Shared Resource facility (Columbus, OH, USA). The resultant 16S rRNA was compared to known sequences in in the National Center for Biotechnology Information database (NCBI Genbank) using Basic Local Alignment Search Tool Nucleic Acids (BLAST-N) algorithm.

Whole genome sequencing and bioinformatic analysis of the antimicrobial peptide biosynthetic gene cluster

Genomic DNA from strain OSY-TC318 was isolated using a DNA extraction kit (DNeasy Blood & Tissue kit; Qiagen, Valencia, CA). The whole genome of OSY-TC318 was sequenced using Ion Torrent sequencing technology (Life Technologies, San Francisco, CA). The assembly of short sequencing reads into longer contigs was achieved using a software program (CLC Genomics Workbench, Qiagen).

Antibiotics & Secondary Metabolite Analysis Shell (antiSMASH 4.1.0) (Blin et al., 2018) and the bacteriocin genome-mining tool BAGEL 4.0 (Walsh et al., 2011) were used to identify the paraplantaricin TC318 biosynthetic gene cluster in the draft genome of *Lactobacillus paraplantarum* OSY-TC318. A putative lantibiotic gene cluster was identified in the OSY-TC318 genome. NCBI-BLASTP was used to predict the function of the paraplantaricin TC318 biosynthetic gene cluster (Boratyn et al., 2013). The predicted molecular mass of the resulted peptide was calculated using a program, Protein Prospector v 5.22.0 (Knudsen & Chalkley, 2011).

Antimicrobial activity determination of OSY-TC318 crude extract

Our preliminary data showed that OSY-TC318 produced antimicrobial agent when grown on MRS agar plates but not in MRS broth. Therefore, OSY-TC318 was inoculated on 300 MRS plates by

spread plating 100 µl of overnight culture. The inoculated plates were incubated at 30°C for 48 hours. The cell pellets containing antimicrobial agents were harvested and active antimicrobials were extracted using 70% isopropanol for 3 hours. After the evaporation of the solvent, the concentrated suspension (crude extract) was further analyzed. Antimicrobial activity was determined using spot-on-lawn method (He et al., 2007). *Bacillus cereus*, *Micrococcus luteus*, *Lactobacillus casei*, *Pediococcus pentosaceus*, *Staphylococcus aureus* and *Enterococcus faecalis* were used as indicators. Aliquots (10 µL) of overnight indicator bacterium were transferred into 10 mL of sterile soft molten MRS agar, fortified with 0.75% agar (Fisher Scientific, St. Louis, MO, USA). The mixture was poured onto a basal MRS agar plate. After incubation at 30°C overnight, clear inhibitory zones were observed.

HPLC purification of the antimicrobial agent produced by *Lactobacillus paraplantarum* OSY-TC318

Active antimicrobial agent was purified from the crude extract using a high-performance liquid chromatography (HPLC) system (Hewlett Packard 1050, Agilent Technologies, Palo Alto, CA). The stationary phase was a reverse-phase C_{18} column (Discovery BIO C_{18} column, 25 cm × 4.6 mm I.D., 5 µm, Supelco, Bellefonte, PA, USA). The mobile phase consisted of (A) acetonitrile with 0.1% trifluoroacetic acid (TFA), and (B) HPLC-grade water with 0.1% TFA. For each run, aliquots (100 µl) of crude extract were loaded and separated on the column by a linear gradient of solvent A from 0 to 100% over 30 min followed by 100% solvent A for 5 min at a flow rate of 1.0 ml/min. Elution was monitored using a UV-detector at a wave length of 220 nm. Fractions were collected at a rate of one fraction per minute using Waters Fraction Collector II (Waters Cooperation, Milford, MA). Fractions with the same retention time from multiple runs were combined and dried using Speed-Vac concentrator (Savant SPD131DDA, Thermo scientific,

USA). The resulting dry fractions were dissolved in 70% acetonitrile and the antimicrobial activity of each fraction was tested. *Pediococcus pentosaceus* was used as an indicator for TC318 HPLC purified fractions. When spot on the surface of soft-agar plate (0.75% agar), the solvent (70% acentonitrile), which evaporate quickly, didn't show antimicrobial activity after overnight incubation. Fractions containing the semi-purified antimicrobial agent from multiple runs were further purified by re-injection in to the HPLC using the same conditions described above. The fraction with the strongest antimicrobial activity and the highest purity was stored at -80°C for further analyses.

Structure determination of the antimicrobial peptide using Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometry analysis

Active HPLC fractions were subjected to MALDI and electron spray ionization (ESI) for structure determination using Fourier Transform Ion Cyclon Resonance (FT-ICR) instrument. The MALDI analysis was used to determine the accurate mass of the singly charged ions. The ESI analysis was used to determine the accurate mass of the double charged ions. A Bruker 15T SolariXR FT-ICR instrument was used with a 4M transient that provided the required resolution for accurate mass measurement (< 1 ppm error)**.** Additional QCID MS/MS analysis was performed to fragment the singly charged peptide and obtain its amino acid sequence. For MALDI, samples were mixed with the HCCA MALDI matrix solution in a ratio of 1:5 (vol/vol). One microliter of the mixture was spotted on the MALDI plate. The mass spectrometer was calibrated using peptide calibration standard II (Bruker Daltonics). Ions were detected in the range of *m/z* 500-4000. A Yag/Nd laser (351 nm) was used for ionization with a varying laser power of 20-25% to ionize/desorb the peptide ions. Quadrupole MS/MS fragmentation of the singly charged 2265. 90304 *m/z* $[M+H]^+$ precursor ion was performed with collision-induced dissociations (CID) at 80 eV collision energy (with a

collision gas of Argon) and 40% laser power. For ESI, the original HPLC fractions were diluted tenfold first and these diluted solutions were sprayed with standard ESI conditions (ESI spray voltage: 4500V, capillary voltage: 250V, capillary temperature: 180°C, flow rate: 2.5 μL/min).

Results

Strain identification

The producer strain OSY-TC318 is a Gram-positive bacterium. The whole cell MALDI-TOF Mass Spectrometry identified the isolate as *Lactobacillus paraplantarum.* The strain identity was also conformed using 16S rRNA sequencing with 95% similarity with *L. paraplantarum* JCM12533. Whole genome comparison also revealed that OSY-TC318 is closest to *L. paraplantarum* DSM 10667 (data not shown). Therefore, the new isolate was designated as *Lactobacillus paraplantarum* OSY-TC318. The OSY-TC318 crude extract showed antimicrobial activity against *Bacillus cereus, Micrococcus luteus, Lactobacillus casei* and *Pediococcus pentosaceus* (Table 1).

Bioinformatics analysis of lantibiotic gene cluster and structure prediction

The putative biosynthetic gene cluster of the antimicrobial peptide was identified in contig 14 of the assembled draft genome of OSY-TC318 using BAGEL 4.0 and antiSMASH 4.1.0. Both tools showed a typical type I lantibiotic gene cluster and the predicted amino acid sequence of the core antimicrobial peptide. Additionally, antiSMASH analysis showed that the TC318 gene cluster is closest to mutacin 1140 and mutacin B-Ny266 gene clusters and the program also predicted the molecular weight of lantibiotic TC318 as 2265.6 Da. The identified biosynthetic cluster comprised 11 open reading frames (ORFs). The proposed function of each ORF, length, closest homolog, percent identity and the percent query cover are shown in Table 2. The 11 open reading frames had homology to the structural gene and genes encoding modification, transport, protease,

transposases and regulation enzymes. Paraplantaricin TC318 biosynthetic gene cluster includes the structural gene (*lanA*), modification enzyme genes (*lanB*, *lanC*, and *lanD*), one protease enzyme gene (*lanP*), three secretion ABC-type transporter genes (*lanT*), two regulatory genes (*lanK* and *lanR*), three transposases genes (*tra*) and one genes with unknown function (Figure 1). The predicted sequence of lantibiotic TC318 consisted of 22 residues that differ from mutacin 1140 sequence by four amino acids (Figure 2). Based on the genomic analysis, the chemical formula of the TC318 neutral form is ($C_{106}H_{133}N_{27}O_{22}S_4$) that corresponds to a theoretical molecular mass of *m/z* 2264.907409$^+$ calculated using Protein Prospector v 5.22.0.

The TC318 proposed biosynthesis includes a structural gene expression to make the pre-propeptide. The pre-propeptide consisted of a propeptide at the C-terminus and a leader peptide at the N-terminus. The leader peptide is the signal responsible of the processing and the cleavage of the propeptide by peptidase. The specific post-translational modifications of TC318 are introduced by enzymes encoded in the biosynthetic gene cluster. Based on the sequencing of this biosynthetic gene cluster, it can be inferred that these enzymes catalyze the dehydration (Lan B) and cyclization (LanC) of the serine and threonine residues in the propeptide that can condense with the neighboring cysteine residue (Chatterjee et al., 2005) leading to the formation of lanthionine and methyllanthionine (thioether) bridges, respectively. The enzyme encoded by (Lan D) may catalyzes the decarboxylation of the C-terminal cysteine residue, forming the C-terminal S-[(Z)-2-aminovinyl]-D-cysteine (AviCys) residue (Cotter et al, 2005). After the dehydration and the cyclization, the leader peptide is removed by the protease as the last step in the biosynthesis (Sahl & Bierbaum, 1998).

Purification of lantibiotic TC318 and structure verification using Fourier transform ion cyclotron resonance mass spectrometry

To verify the predicted lantibiotic from strain TC318, a reverse phase HPLC was used to purify active antimicrobial agent from the crude extract. A representative chromatograph and its active fraction is presented in Figure 3. Active factions were eluted and collected from multiple runs and re-purified using the same conditions. The re-purified TC318 was used for the mass spectrometry analysis. HPLC-purified TC318 was subjected to MALDI and ESI analysis using FT-ICR instrument for accurate mass determination. The MALDI analysis showed the peaks of the singly charged ions ($[M+H]^+$, $[M+O]^+$ and $[M+Na]^+$) for intact TC318 with the observed molecular masses of *m/z* 2264.9071, 2280.90219 and 2286. 88937, respectively (Figure 4). The accurate monoisotopic mass of TC318 singly charged ion, $[M+H]^+$, *m/z* 2264.90718 was observed using FT-ICR/MALDI that matched the calculated mass with 0.1 ppm error as shown in (Figure 5). The accurate molecular mass of the TC318 double charged ion, $[M+2H]^{2+}$, *m/z* 1132.95792 was observed using FT-ICR/ESI that matched the theoretical calculated mass m/z 1132.957343 with 0.5 ppm error as shown in (Figure 5).

Additional QCID MS/MS analysis was performed to fragment the singly charged peptide and obtain its amino acid sequence. The peptide was fragmented using QCID MS/MS analysis by isolating the $[M+H]^+$ peak to obtain the sequential information of this peptide. The resulted fragments were compared with the proposed structure based on the genomic analysis and Mutacin 1140 published structure. As shown in table S1 and Figures S1, S2, S3, S4 and S5, a series of MS/MS product ions were observed which matched the predicted structure. However, the ring structure made it difficult for complete fragmentation. Therefore, the structure of the new paraplantaricin TC318 lantibiotic (Figure 6) was proposed based on the bioinformatics analysis and mass spectrometry analysis.

Discussion

TC318 is characterized by a poor production in the liquid media. This was observed in Mutacin 1140 due in part to inhibition of structural gene transcription by the mature lantibiotic (Hillman et al., 1998). They hypothesized that growing the producer on solid medium allows the diffusion of the lantibiotic away from the inoculated cells and increases the synthesis time before reaching an inhibitory concentration. For the production of TC318, we used the MRS agar plates to grow and harvest the cells by scraping for the lantibiotic extraction using 70% isopropanol.

Buchman et al. (1988) suggested that the ability of lantibiotics production is dispersed among Gram-positive bacteria by the transfer of lantibiotic encoding mobile elements such as plasmids or transposons. Genes for lantibiotic biosynthesis can be found on chromosomes (e.g., subtilin, epilancin K7, salivaricin A, and variacin), on plasmids (e.g., epidermin, Pep5, cytolysin, lacticin 481, lactocin S, and streptococcin A-FF22) or on conjugative transposable elements (e.g., nisin) (Chatterjee et al., 2005; Chen, Qi, Novak, & Caufield, 1999). The presence of transposases genes *tra1*, *tra2* and *tra3* adjacent to the biosynthetic gene cluster of TC318 suggested that the ability of this lantibiotic production in *Lactobacillus paraplantarum* TC318 could be obtained from an ancestral transposon (Chen et al., 1999). The leader peptide sequence predicted from the structural gene *lanA* showed limited homology to the leader sequences of other reported lantibiotics (Figure 2). However, the propeptide sequence showed that mutacin 1140 is the most related to TC318 as they share 18 of the 22 amino acids that forming the mature lantibiotic. To the best of our knowledge, this is the first report of a mutacin variant produced by a lactic acid bacterium. The mass of the pure lantibiotic was determined through two approaches using FTICR instrument. The FTICR/MALDI analysis of the singly charged ions ($[M+H]^+$ showed an observed molecular masses of m/z 2264.9071 with 0.1 ppm error. The FTICR/ESI analysis of the TC318 double charged ion $[M+2H]^{2+}$ showed an observed molecular mass of *m/z* 1132.95792 with 0.5 ppm error. Based on the two observed masses, the TC318 monoisotopic neutral mass was calculated to be 2263.900 with 0.1 ppm error. The observed mass matched the calculated mass predicted from the propeptide amino acid sequence and the chemical formula of the mature peptide ($C_{106}H_{133}N_{27}O_{22}S_4$).

The MS fragmentation of the purified TC318 strongly supported the predicted structure using bioinformatic analysis. The TC318 amino acid sequence partially matches the sequence of Mutacin

1140 with four amino acid difference. As shown in (Figure 6), TC318 consists of 22 amino acids (Phe-Lys-Dha-Trp-Dha-Leu-Cys-Dhb-Phe-Gly-Cys-Gly-His-Dhb-Gly-Dha-Phe-Asn-Dha-Phe-Cys-Cys). The proposed structure of TC318 contains four thioether rings and is similar to other type A lantibiotics such as Mutacin 1140 (Smith et al., 2003), Mutacin B-Ny266 (Mota-Meira et al., 1997) , Mutacin I (Qi et al., 2000), gallidermin and epidermin (Götz, Perconti, Popella, Werner, & Schlag, 2014). Thioether rings contain a single sulfur atom and link two amino acids through their β-carbons. The fragmentation was achieved between the rings. However, the chemical formula, the calculated mass and the observed mass of the fragments confirmed the entire structure of the rings. In future study, nuclear magnetic resonance spectroscopy analysis could fully reveal the chemical structure of the new lantibiotic compound.

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Table 1. Antimicrobial activity of Lactobacillus paraplantarum OSY-TC318 crude extract

Strain	Media	Diameter of inhibition zone (mm)
Lactobacillus casei ATCC 7469	MRS	15
Pediococcus pentosaceus	MRS	10
Micrococcus luteus Maga	TSB	10
B. cereus ATCC 12826	TSB	10
B. cereus ATCC 49064	TSB	3
B. cereus ATCC 33019	TSB	2
Staphylococcus aureus ATCC 25923	TSB	N/A
S. aureus ATCC 12600	TSB	N/A
S. aureus ATCC 29213	TSB	N/A
Staphylococcus epidermidis 838	TSB	N/A
Streptococcus agalilactiae JOY	TSB	N/A
Enterococcus faecalis ATCC 29212	TSB	N/A
E. faecalis OSU 48	TSB	N/A
Pediococcus acidilactici PO2	MRS	N/A
P. cerevisiae	MRS	N/A
L. acidophilus ATCC 19992	MRS	N/A
L. bulgaricus OSU 135	MRS	N/A

Table 2 Open reading frames (ORFs) analysis of the putative TC318 biosynthetic gene cluster

ORF no.	Proposed function	Length (aa)	GenBank accession no, Closest homolog (In NCBI database on 7/11/2018)	Percent Identity (Percent Query Cover)
1	No function determined	101	WP_063487862.1, hypothetical protein [*Lactobacillus plantarum*]	93% (91%)
2	LanK (Sensor histidine kinase)	327	WP_062688923.1, sensor histidine kinase [*Lactobacillus plantarum*]	99% (100%)
3	LanR (Transcriptional regulatory protein)	225	WP_062688921.1, DNA-binding response regulator [*Lactobacillus plantarum*]	100 %(100%)
4	LanT1 (ABC transporter ATP-binding protein)	547	WP_062688919.1, ABC transporter ATP-binding protein [*Lactobacillus plantarum*]	100 %(100%)
5	LanP (Leader peptide processing protease)	461	WP_032531395.1, peptidase S8 lantibiotic specific protease[*Streptococcus mutans*]	44 %(97%)
6	LanD (Decarboxylation enzyme)	186	WP_002304546.1, Lantibiotic biosynthesis protein [*Streptococcus mutans*]	49 %(94%)
7	LanC (Lanthionine synthetase C family protein)	437	WP_002289980.1, lantibiotic epidermin biosynthesis protein EpiC [*Streptococcus mutans*]	99 %(100%) 37% (90%)
8	LanB (Lantibiotic dehydratase domain protein)	1005	AAG48566.1, *MutB [Streptococcus mutans*]	32 %(98%)
9	LanA (Prepropeptide of TC318)	62	WP_002268802.1, gallidermin/nisin family lantibiotic [*Streptococcus mutans*]	57% (87%)
10	No function determined	114	WP_062688908.1, hypothetical protein [*Lactobacillus plantarum*]	100 %(100%)
11	LanT2 (ABC transporter, ATP-binding protein)	649	WP_062688906.1, ABC transporter permease [*Lactobacillus plantarum*]	99 %(100%)
12	LanT3 (ABC transporter, ATP-binding protein)	246	WP_062688904.1, ABC transporter ATP-binding protein [*Lactobacillus plantarum*]	100 %(100%)
13	Tra1 (Transposase)	95	ERO39513.1, putative transposase [*Lactobacillus plantarum* WJL]	74% (100%)
14	Tra2 (Transposase)	76	WP_076644604.1, transposase [*Lactobacillus plantarum*]	93% (100%)
15	Ttra3 (Transposase)	139	WP_016527020.1, transposase [*Lactobacillus plantarum*]	90% (97%)

Figure legends

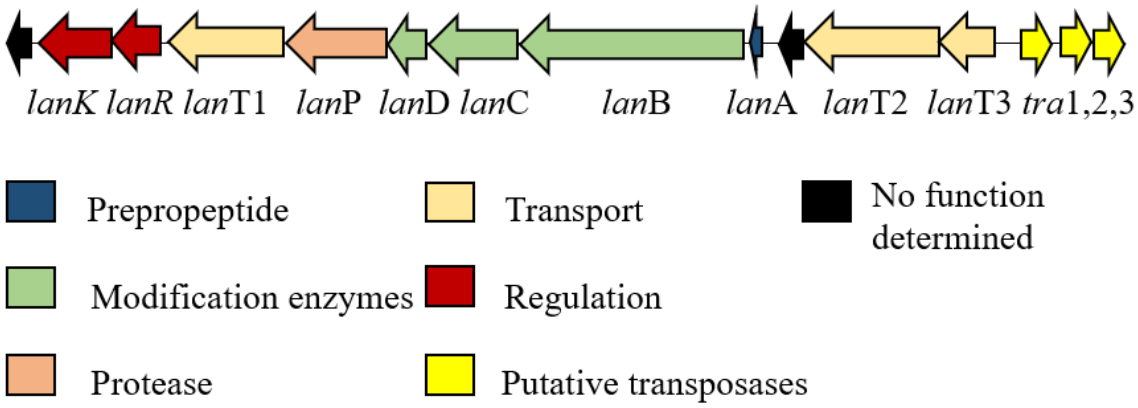

Figure 1. Genetic organization of TC318 biosynthetic gene cluster

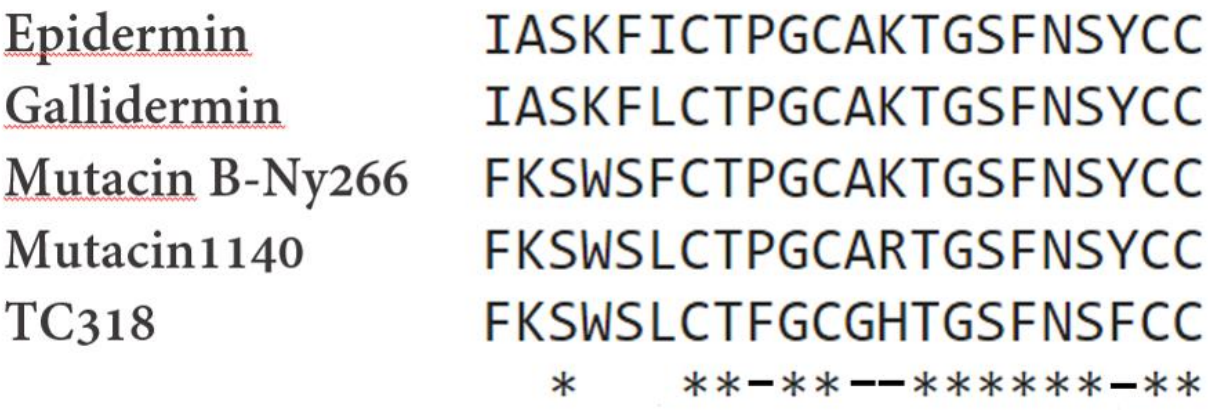

Figure 2. Primary sequence alignment of TC318 with those of mutacin 1140, mutacin B-Ny266, gallidermin and epidermin using ClustalW tool. The conserved residues are indicated with stars. Mutacin 1140 is the most related to TC318. The residues that are different between TC318 and mutacin 1140 are underlined.

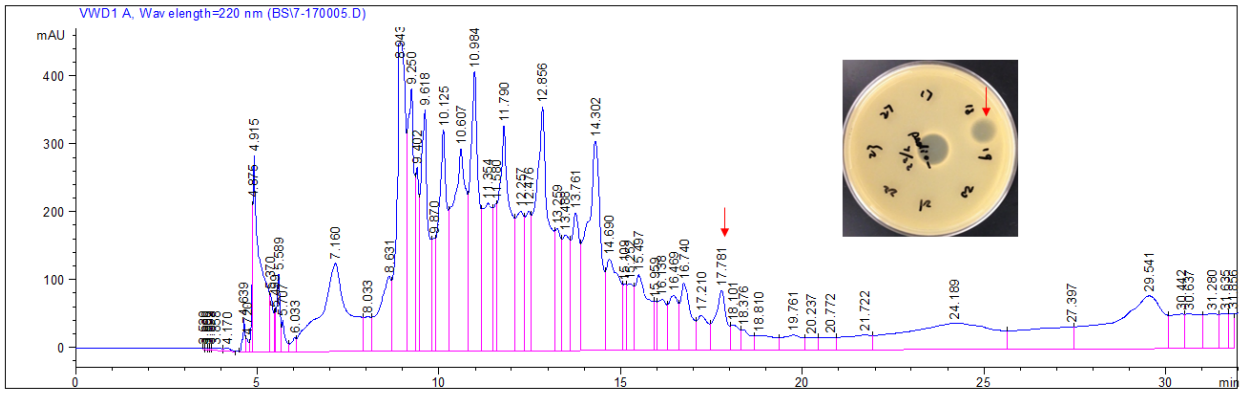

Figure 3. Separation of crude extract using HPLC. Red arrow indicates the single peak showing antimicrobial activity against an indicator strain *Pediococcus pentosaceus*

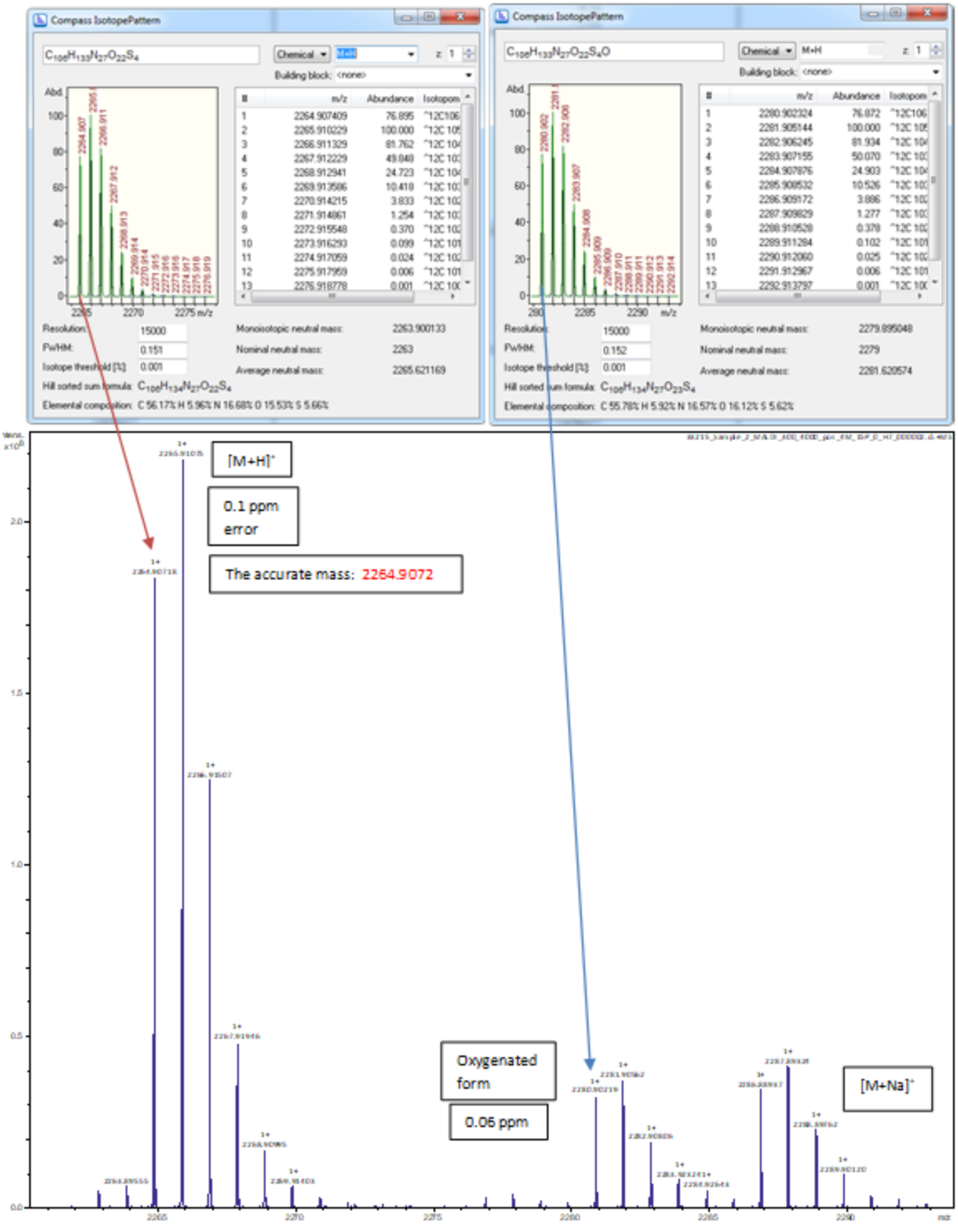

Figure 4. FTICR/MALDI analysis of the singly charged ions ([M+H] +, [M+O] + and [M+Na] +) for intact TC318 with the observed molecular masses of m/z 2264.9071, 2280.90219 and 2286. 88937 respectively. TC318 accurate mass is 2263.900 0.1 ppm based on monoisotopic neutral mass and the TC318 chemical formula is $C_{106}H_{133}N_{27}O_{22}S_4$

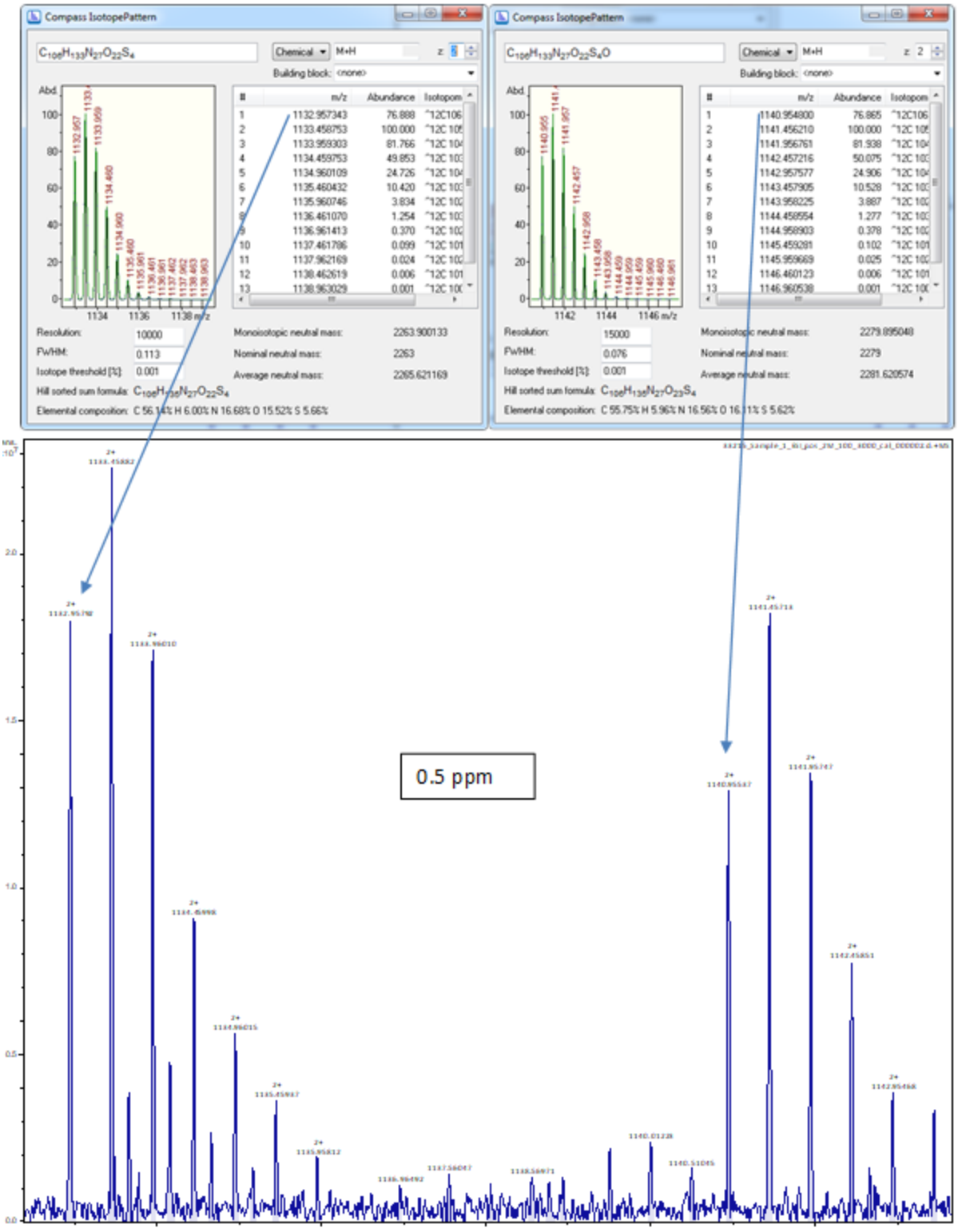

Figure 5. FTICR/ESI analysis of the TC318 double charged ion $[M+2H]^{2+}$ with an observed molecular mass *m/z* 1132.95792.

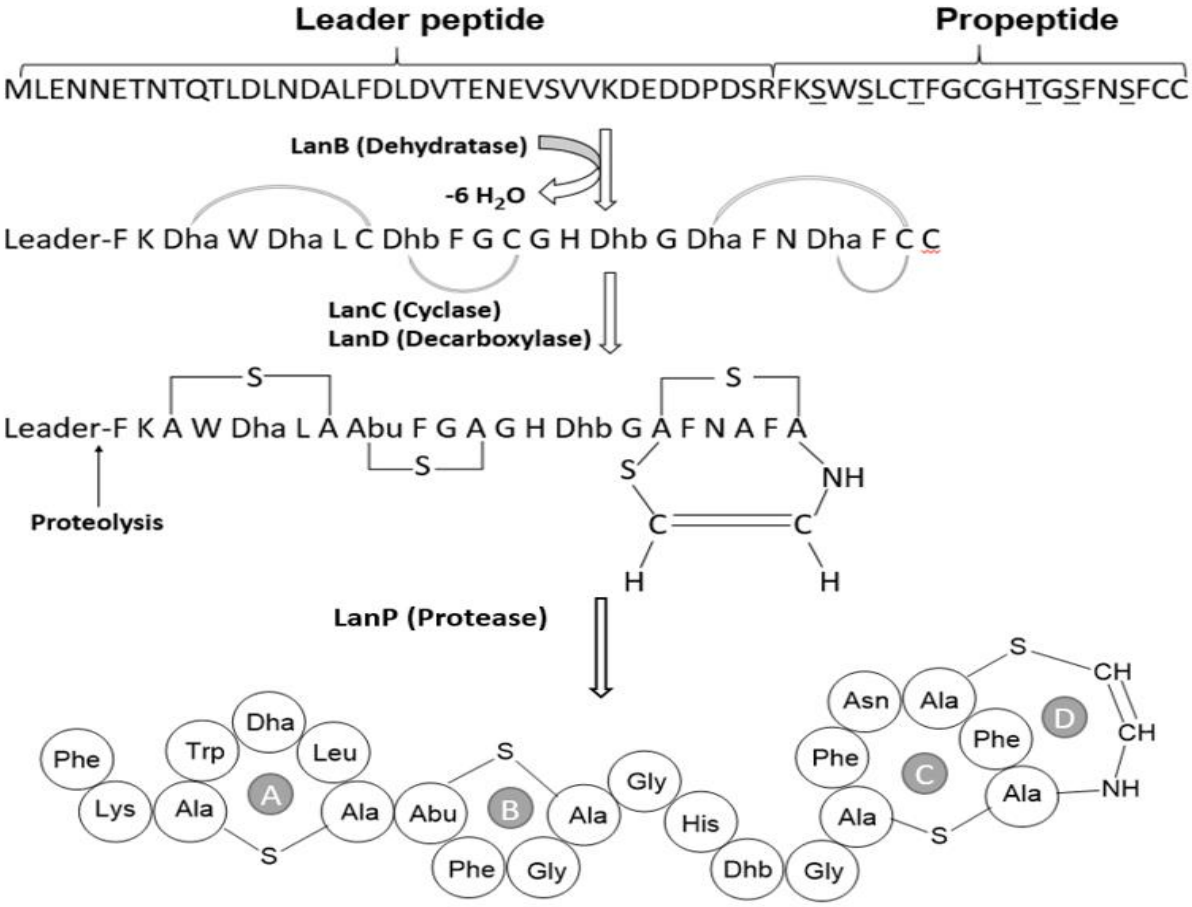

Figure 6. Proposed post-translational modifications and structure of paraplantaricin TC318. TC318 contains 4 rings (A–D). Dha, dehydroalanine; Dhb, dehdrobutyrine.