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Molecular Characterization of *Pediococcus acidilactici* Pediocin Genes (ped PA-1/AcH) and Plasmid Transfer into *Bacillus subtilis* NRRL B-3749 via Electroporation

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ABSTRACT

Crude pediocin preparations (Class IIa bacteriocin) of selected *Pediococcus acidilactici* strains S3, K2A2-3 and 3G3 inhibited the growth of *Listeria innocua* 026. The *pap* genes (*pap*ABC) of the pediocin operon were amplified from S3, K2A2-3 and 3G3 to further study the mechanism of pediocin production. Pediocin gene sequences were obtained for possible use in downstream and engineering activities. Primer designs for *pap*B and *pap*C gene amplification involved sequence alignments (CLC Sequence Viewer 7.6.1, BLASTn) and computational tools (SnapGene and AmplifX 1.6.1) via homologous sequences derived from the NCBI database. The *pap* gene amplicons were sequenced and protein structures predicted via available online tools such as ExPASY, Phyre2, and COFACTOR. Characterization via generated protein structures revealed the ligand-binding sites which reinforce possible functions of PedA, PedB, and PedC proteins on cell inhibition, immunity, and repair.

Bacillus subtilis NRRL B-3749 competent cells served as the host for the transfer of the plasmid encoding the pediocin operon. The putative transformant B235 was found to be most inhibitory to *Listeria innocua* 026 better than the control strains in plate assays.

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KEYWORDS

pediocin, *pap* genes, plasmid, immunity gene

INTRODUCTION

The search for safe inhibitory compounds for the development of natural biopreservatives may include the use of bacteriocins, such as pediocin produced by the lactic acid bacterium *Pediococcus acidilactici*. The Gram-positive bacteriocins are generally divided into class I (modified peptides, lantibiotics), class II (unmodified peptides, non-lanthionine), and class III (large proteins, heat unstable). Pediocin belongs to Class IIa cationic, nonlantibiotic bacteriocins with known strong antilisterial activity and has been well considered as an antiseptic for a variety of uses (Perez et al. 2015). There is a recurrent necessity to understand the use of bacteriocins in food ecosystems, to study bacterial genomes which may reveal new sources of bacteriocins, and to develop engineered food grade expression systems for improvement of natural products. Nowadays, natural products are perceived to be appealing to health and environmentally-mindful society in ensuring food safety since these are commonly found in familiar consumable food products and usual environments. The agricultural, pharmaceutical, dairy industry and producers of ready-to-eat food products are envisioned to benefit from this research.

Bacteriocins are antimicrobial peptides (AMPs) which are regarded as primary metabolites synthesized in the ribosomes (Papagianni and Anastasiadou 2009). Pediocin then is produced and released by the cell through the operation of *pap* genes *(papA*, *pap* B, *pap*C, and *pap*D) which were all found in the bacterial plasmid (pDNA).

They carry a hydrophilic cationic region with a conserved YGNGVXC motif known as "pediocin box" (Kumar et al. 2011; Zacharoff and Lovitt 2012).

The Class IIa bacteriocins are thermostable wherein Pediocin PA-1/AcH type contains two disulfide bridges in the mature PedA molecule. They are biosynthesized as precursors composed of a leader peptide and a mature bacteriocin. The leader sequence usually has 15 to 30 residues of the double glycine type and is cleaved off at the C-terminal side of the glycine residues. The leader peptide facilitates the signal interaction during translocation and maintains the inactive state of bacteriocin prior to secretion (Zacharof and Lovitt 2012; Fimland et al. 2005).

The N-terminal region of all pediocins contains two cysteines joined by a disulfide bond (Papagianni and Anastasiadou 2009). It contains an additional C-terminal disulfide bridge which plays an important role in stabilizing the helical structure of the C-terminal domain. Most of the time, especially at higher temperatures, these structurally stabilized bacteriocins exhibit higher antimicrobial potencies than those which contain only one disulfide bridge (Marugg et al. 1992; Drider et al. 2006).

The functionality of bacteriocins could be manifested through screenings and bioassays of the collected and prepared crude cell supernatants against an indicator microorganism. The semi-purified pediocin extract of *P. acidilactici* strain K2A2-3 that was previously isolated from carabao intestines, strongly inhibited three *Listeria monocytogenes* strains, *Listeria innocua*, *Bacillus cereus*, *Enterococcus faecium* and *Enterococcus faecalis*, among other species (Villarante et al. 2010; Elegado et al. 2004). Previous studies have also shown that pediocin of *P. acidilactici* K2A2-3 has the ability to inhibit the growth of human colon adenocarcinoma (HT29) and human cervical carcinoma (HeLa) cells by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Villarante et al. 2010). These promising pharmaceutical uses of bacteriocins can be better realized with increased production through recombinant DNA production (Zhong et al. 2014). This in-depth analysis of the pediocin genes (*pap*A, *pap*B, and *pap*C) from selected Philippine local isolates would provide a platform for future transformation and cloning studies. This study generally aimed to sequence, analyze and characterize the pediocin-producing genes of *P. acidilactici* isolated strains using bioinformatics databases and softwares. This also provides initial data on the effect of whole plasmid insertion, which contains the pediocin operon, to a Gram-positive host cell *Bacillus subtilis* via electroporation.

Successful incorporation and expression of these genes via whole plasmid transformation was expected to increase the bacteriocin production per unit substrate input. This system would be more advantageous compared to what the pediococci isolates naturally produce extracellularly, therefore minimizing the cost of downstream processing. *B. subtilis* being found in fermented food products, being considered Generally Recognized As Safe (GRAS), is commonly used as host system in electroporation studies.

An in-depth analysis of the pediocin genes and activities which would demonstrate the transfer of plasmids into a selected competent host cell (*B. subtilis* NRRL B-3749) would provide a platform for future transformation studies.

MATERIALS AND METHODS

The strains used were acquired from the collection of the Food and Feeds Laboratory of the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB), College, Laguna, Philippines. Their accession number, sources, and purpose for this study are listed in Table 1.

Strain Selection and Cultivation

Strains were initially screened using "Spot-On-Lawn" Technique against bacterial indicator *Listeria innocua* 026 (Mayr-Harting et al. 1972) to provide qualitative assessments for bacteriocin-producing strains.

Pediococcus sp. strains were grown in 25 ml de Man Rogosa and Sharpe (MRS) Broth (De Man et al. 1960) for 18-24 hours at 37 ^{O}C without shaking (Papagianni and Anastasiadou 2009). The supernatant of the cells was collected via centrifugation at 10,000 rpm for 7 minutes. Subsequently, the crude supernatant was pH-adjusted (1M NaOH, pH 6-7), filter-sterilized (0.2 µm), and diluted in a two-fold manner (0.85% NaCl as diluent).

The assay plates were incubated at 37 ^{O}C for 24 hours. The bacteriocin-producing strains, yielding distinct zones of inhibitions, were selected and further studied. To maintain plasmid production, the selected strains were cultured under the same conditions with an addition of erythromycin (5 µg/ml).

Plasmid Isolation

The plasmids of potential pediocin producers were extracted using the protocol described by Birnboim and Doly (1979) with some minor modifications. The procedure was a modified method from the conventional alkaline lysis protocol. Initially, 1 ml of the cell culture was centrifuged at 10,000 rpm for 5 minutes. The resulting cell pellet was resuspended in 100 µl of Solution I (25% sucrose) followed by vortexing. To the extracts were added 100 µl of Solution I–lysozyme (10 mg/ml) mix, then vortexed. After 5 minutes incubation at room temperature, 400 µl of Solution II (0.2N NaOH, 3% SDS) was added prior to 10-minute incubation on ice. Solution III (K acetate) was added and the set-up was again incubated on ice for 15 minutes. The precipitate

Table 1: Microbial strains with their respective sources and purpose in the study

SPECIES	STRAIN/ ACCESSION NO.	SOURCE	PURPOSE
Pediococcus acidilactici	S3	fermented sausage	Plasmid source, positive control for pediocin gene amplification
	3G3	fermented pork (*burong babi*)	Plasmid source
	K2A2-3	Philippine water buffalo intestines (*Bubalus bubalis carabanensis*)	Plasmid source
	K2A2-5	Philippine water buffalo intestines (*Bubalus bubalis carabanensis*)	LAB strain, screening
	4E5	fermented rice-tilapia mixture (*burong tilapia*)	LAB strain, screening
	AA-5a	fermented rice-fish mixture (*burong isda*)	LAB strain, screening
Pediococcus lolii	4E10	fermented fish (*burong tilapia*)	Negative control for assays and pediocin gene amplification
Listeria innocua	026	chicken balls	Indicator strain

formed which included the cell pellet was discarded right after centrifugation (10,000 rpm, 15 minutes at 4 ^{O}C). The retrieved supernatant was added with 600 µl ice-cold isopropanol followed by centrifugation (12,000 rpm, 5 minutes, 4 ^{O}C) to precipitate the plasmid DNA. The collected pellet via centrifugation was washed with 70% ethanol, dried and was dissolved in 25 µl of ultrapure distilled water before storage (-20 ^{O}C).

Gene Amplification

The pediocin genes *pap* A, B, and C as described by Motlagh et al. (1994), in which published sequences accessible via NCBI, were amplified using reported and designed primers. The reported primers, sequences described by Perez et al. (2012) and Elegado et al. (2004) were used in this study to amplify the *pap*A gene (Forward 5'CTGCGTTGATAGGCCAGGTTTCA3' and Reverse 5'GCTTCTGTAGTAAAAACTGTAGCC3'), while designed primers were used for *pap*B and *pap*C via homologous sequences derived from the NCBI database (Moon et al. 2005).

Primers were viewed and designed using free and downloadable softwares such as CLC Sequence Viewer 7.6.1 (CLCBio,QIAGEN), AmplifX 1.6.1 (CNRS, France), and SnapGene (trial version). All designed primers in this study were predicted to have good attachments without cross dimers and hairpin loops following the prediction made via Amplifix 1.6.1 (CNRS, France).

In consideration of all necessary parameters, the synthesized *pap*B (Life Technologies, Thermo Fisher Scientific USA) and *pap*C (Integrated DNA Technologies, USA) primers were predicted to yield 379 bp (Ta = 55-56 ^{O}C, molGC% = F50 R50) and 625 bp (Ta = 52 ^{O}C, molGC% = F50 R45.5) length amplicon products, respectively.

The *pap* genes were amplified via Polymerase Chain Reaction (PCR) using the primer sequences recorded in Table 2.

The PCR products were verified by 1% agarose gel electrophoresis (0.07 µl/ml GelRed dye) using 1kb+ MW ladder as the standard marker, followed by visualization under UV light.

Analyses of DNA Sequences and Prediction of Protein Structures

Gene sequences of *pap*A from *P. acidilactici* S3, K2A2-5 and 4E6 were obtained from the record of Molecular Genetics Laboratory of BIOTECH-UPLB. Consensus sequences were obtained and gaps filled prior to submission to Phyre2 (Kelly and Sternberg 2015).

The PCR products of *pap*B and *pap*C were sent to First BASE Asia (Selangor, Malaysia) for purification and sequencing. All the gathered sequences (*pap*A, *pap*B, and *pap*C) were viewed, enhanced and converted using FinchTV (Geospiza), MEGA ver 7 and CLC Sequence Viewer ver 7.6.1 (CLCBio, QIAGEN). Protein Databank Formats files (.pdb) were obtained from Phyre2 prior to modelling (RasWin ver 2.7.5.2). These files were then submitted to PredictProtein (https://predictprotein.org/) and COFACTOR server (https://zhanglab.ccmb.med.umich.edu/COFACTOR/; Michigan University) to obtain possible ligand-binding sites in the predicted 3D protein structure.

The *pap*A, *pap*B and *pap*C protein sequence translations have also been recorded as PedA, PedB and PedC. Coding sequences and active sites were determined in the selected reading frames via tools and databases such as CLC Sequence Viewer ver 7.6.1, UniProt, BLASTn, tBLASTx, Phyre2, and ExPASy. ExPASy indicated that Frame (+2), Frame (+1), and Frame (+1) convey the ideal reading frames for PedA, PedB, and PedC, respectively.

Transformation via Electroporation

Direct transformation via electroporation was performed to observe possible effects of the pediocin genes to a host cell. The process of producing electrocompetent cells and the electroporation method for the transformation of *B. subtilis* B-3749 was performed as described by Xue et al. (1999) and the

Table 2: Primer sequences used for pediocin gene amplification in this study

GENE AMPLIFIED	FORWARD SEQUENCE	REVERSE SEQUENCE
pap A (Perez et al., 2012; Elegado et al., 2004)	$^{5'}$CTGCGTTGATAGGCCAGGTTTCA$^{3'}$	$^{5'}$GCTTCTGTAGTAAAAACTGTAGCC$^{3'}$
pap B (This study)	$^{5'}$CATTATGCTGAGCTGGCATC$^{3'}$	$^{5'}$TACTATTGGCTAGGCCACGT$^{3'}$
pap C (This study)	5'ACGTGGCCTAGCCAATAGTA3'	5'GACGATTTCAAAGATCCATCGC3'

Multiporator/Eppendorf Electroporator Transformation Protocol No. 4308 915.504 - 08/2003 with minor modifications. The competent cells were prepared by culturing the host cells in 50 ml TSB (120 rpm, 37 ^{O}C) containing 0.5M sorbitol prior to cooling in ice (10 minutes) and harvest (4 ^{O}C, 5,000 rpm for 5 minutes). Cells were washed with ice-cold electroporation medium (0.5M sorbitol, 0.5M mannitol, 10% glycerol). Plasmids (10 µl) were added and mixed to 60 µl competent cells and the resulting mixture was incubated in ice for 30 minutes in a pre-chilled cuvette. The actual conditions/input of the electroporation device (Gene Pulser II Bio-Rad, USA) were Resistance: 150 ohms, Voltage: 1900V, Time Constant: 5 ms, Pulses: 2. Putative transformed colonies were chosen via replica plating of yielded smaller colonies exhibiting inhibition to indicator strain *L. innocua* 026. Inhibitory properties of putative transformed colonies were also assessed via microbial inhibitory assays.

RESULTS AND DISCUSSION

Strain Selection and Cultivation

The plasmid sources to be used for analysis were selected based on their ability to innately inhibit the indicator strain *Listeria innocua* 026 without modifications or use of inducers. Thus, *P. acidilactici strains* S3, K2A2-3, and 3G3 have been selected and were further studied.

Plasmid Isolation

Inverted images of the gel were presented to facilitate viewing of the faint bands of the plasmids (Figure 1). Strains such as S3, K2A2-3, and 3G3 were the plasmid producers used for sequence analysis. On the other hand, only low copy plasmids were obtained from K2A2-5 and AA-5a while the *P. lolii* 4E10 strain does not produce plasmids. Plasmids were not found in *Bacillus subtilis* NRRL B-3749.

Amplification and Sequence Analysis of Pediocin Genes

The *pap* genes PCR products of *P. acidilactici* strains were about 400-500 bp (published 450 bp), 300-400 bp (predicted 379 bp), and 500-650 bp (predicted 625 bp) for *pap*A, *pap*B, and *pap*C, respectively. Distinct single bands were obtained from the amplifications of *pap*A and *pap*B, while the *pap*C primers provided double bands at the 500-650 bp and 400-500 bp marks. Amplification images of *pap*B (A) and *pap*C (B) using the primer sequences designed for this study are shown as Figure 2.

Homology analysis of the *pap*A consensus nucleotide sequence also revealed a 99% homology to pSMB74, K10 and other *P. acidilactici* strains in the NCBI database. BLASTn analysis of *pap*B consensus sequences of S3, K2A2-3, and 3G3 was found to have 99% homology to *P. acidilactici* K10 (Accession no. AY705375.1) and *P. acidilactici* pSMB74 (Accession no. UO2482.2).

The sequences used for modelling the *pap*A gene were published in the paper "Pediocin structural genes of bacteriocinogenic pediococci isolated from indigenous Philippine and Vietnamese foods" (Perez et al. 2012).

The detailed sequence of *pap*B S3, K2A2-3 and 3G3 strains was presented below having variations only at the first 9 bases: S3 = GTAAAGG(-)(-); K2A2-3 = TAGGAGG(-)(-); 3G3 = GAAAGGGGG and minor variation at the 341st single base residue with S3 having C as compared to G for both K2A2-3 and 3G3.

>consensus S3K2A2-33G3 *pap*B
GAAAGGAGGATTTTGATAGACTAAGTCGGACATATTA
AACAACAAGCTTTGGACTTATTTACTAGGCTACAGTT
TTTACTACAGAAGCACGATACTATCGAACCTTACCAG
TACGTTTTAGATATTCTGGAGACTGGTATCAGTAAAA
CTAAACATAACCAGCAAACGCCTGAACGACAAGCTC
GTGTAGTCTACAACAAGATTGCCAGCCAAGCGTTAGT
AGATAAGTTACATTTTACTGCCGAAGAAAACAAAGTT
CTAGCAGCCATCAATGAATTGGCGCATTCTCAAAAAG
GGTGGGGCGAGTTTAACATGCTAGATACTACCAATAC
GTGGCCTAGCCAATAGTAA

On the other hand, the FASTA format of the whole *P. acidilactici* S3 *pap*C sequence is:

>*pap*C S3
GGTATAGCATTGTAGTTGTCTAGAATTTTGGTCAATAT
CTTTTTAGCATTAGGCGTCTTTCTTGCTTTTGCAGGAG
TTGCTACCATATCGGTGAGTGCTGACAGTTCCGCTAC
TATAGAATCAAATACTAGCTCGAAAATCATCGATGGT
GCAACTTATGAAGAAAACATCAGGGGCGTTATTCCTA
TTACGCTAACTCAATATTTGCATAAAGCTCAAACTGG
AGAAAAAATTTATTGTCTTTGTCGGGTTCAAGGAGTGT
GTGCATTGTCGTAAATTTTCTCCAGTCATGAAACAGT
ACTTACAACAAAGTCAGCATCCCATTTATTACTTAGA
CTATGGGAACAACGGGTCTTTCAGCATGGCTTCTCAA
AAACAAATAACTGATTTCTATTCAACTTTTGCAACCC
CCATGAGTTTTATGGGAACGCCAACTGTTGCCTTGCT
CGATAATGGTAAGGTGGTATCAATGACCGCTGGTGAT
GATACCACTTTATCTGATTTACAACAGATTACTGCTG
ATTACAATAATCAGTAGTCACCTGGTTAATATGGTTTT
GTAACCAATGTAAAAGGCGATGGATCTGAAAAATCG
TTCAAAA

There were no observed major variations found within the selected *pap*B and *pap*C *P. acidilactici* sequences from the local strains in comparison to the sequences in the NCBI database. However, minor variations have been observed: 1.) The *pap*B sequences of S3, K2A2-3 and 3G3 were all found to have gaps in a common ATG codon and A residue near the start of the gene (Figure 2, A); 2.) An A residue instead of the common C (Figure 2, B); 2.) In the whole *pap*C S3 sequence, a TCAAA variation

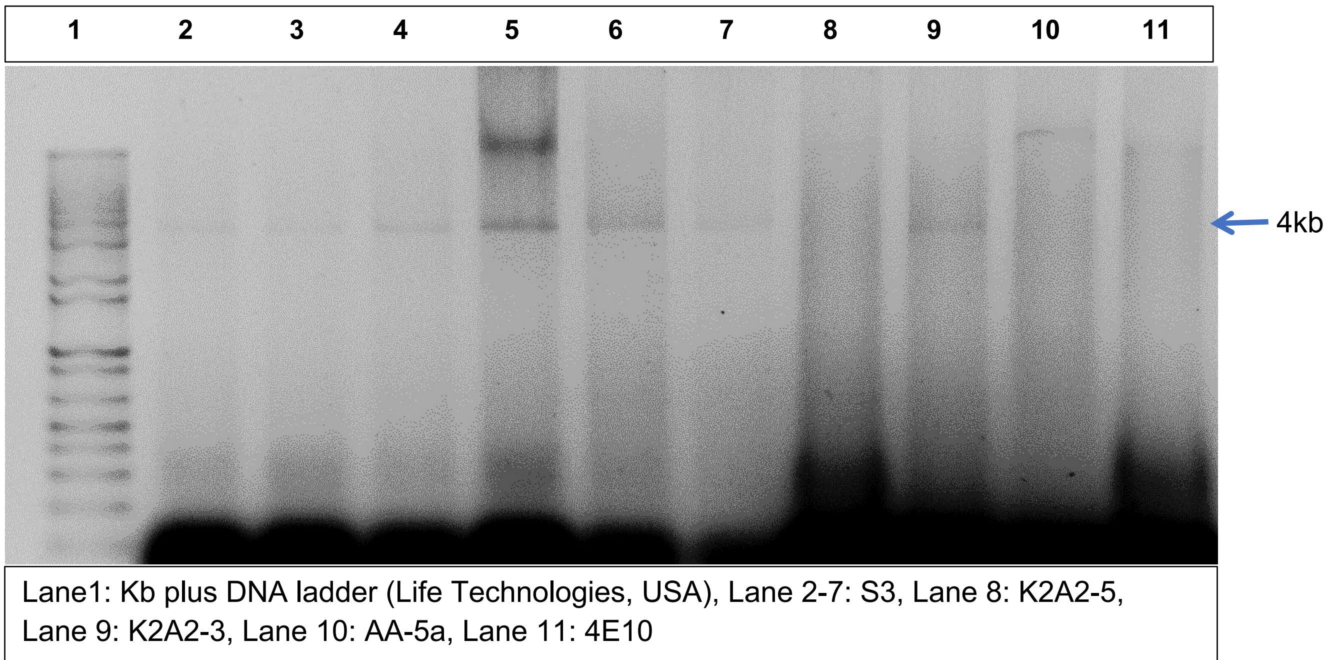

Figure 1: Inverted images of plasmid DNA isolated from LAB strains. Lane 2-7: S3 and Lane 9: K2A2-3 plasmids as faint visible bands; Blue arrow indicate the 4kb size of plasmid

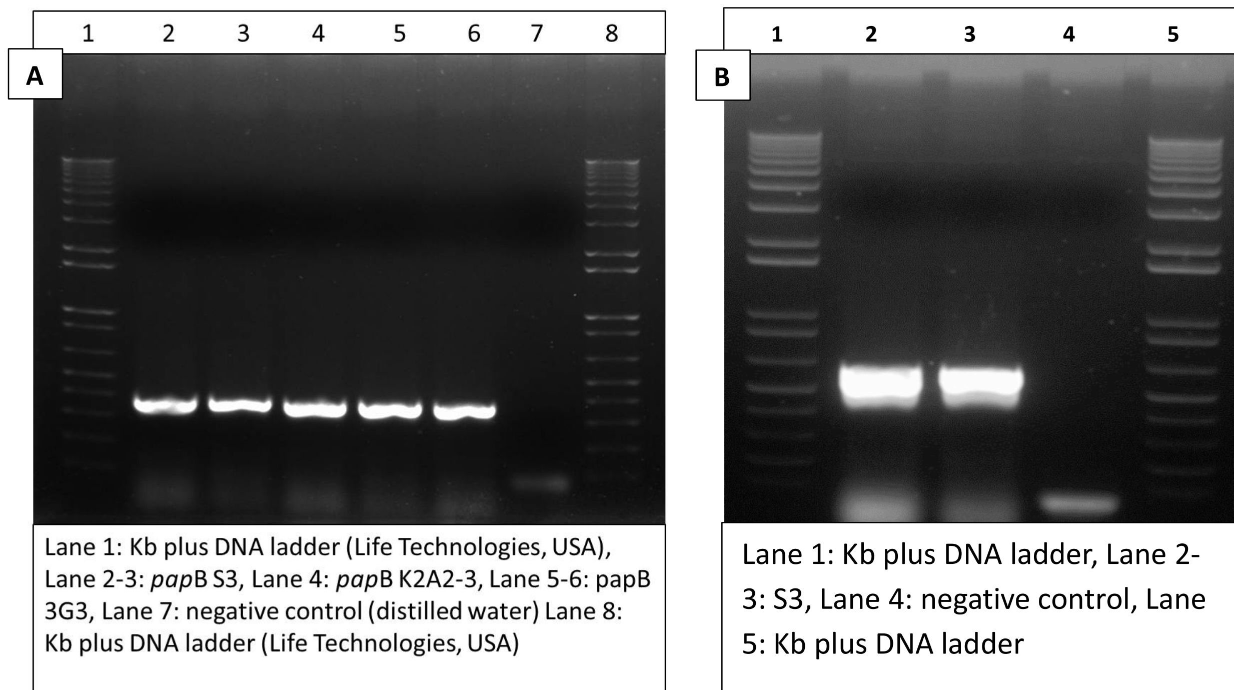

Figure 2: Image of pediocin genes *pap*B (A) and *pap*C (B) amplification using designed primers for this study. (A) papB, predicted 329bp; and (B) papC, predicted as 625bp lengths whereas primer dimers were seen as light bands below 100bp mark.

near the end was found as compared to the common CTTTTT of other *P. acidilactici* strains. The C residue of the TCAAA variation is similar to the ACTGC sequence of the *E. coli* microcin genetic system (AJ009631.3) at the same position.

Analyses of Translated Sequences and Prediction of Protein Structures

Nucleotide analysis and three-dimensional structures for each translated protein sequence were generated.

a. PedA

The following amino acid sequence for PedA (Frame 2+) was the predicted open reading frames suitable to form functional protein (ExPASy):

>PedA consensus sequence (S3, K2A25, and 4E6)

MKKIEKLTEKEMANIIGGKYYGNGVTCGKHSCSVDWG KATTCIINNGAMAWATGGHQGNHKC

Based on the generated protein structures, the presence of the highly conserved amino-terminal sequence YGNGV (Tyr21-Gly22-Asn23-Gly24-Val25) box with CXXXXCXV motif at the N-terminal half (strand, Cys27-Gly28-Lys29-His30-Ser31-Cys32-Ser32-Val34) indicated that the nucleotide sequence is pediocin PA-1. In this generated structure, the disulfide bonds most likely to interact could be C-27 and C32, similarly with C-42 and C-62, that would be forming somewhat a hook structure to be inserted to the cell wall and cause lysis to other competitive

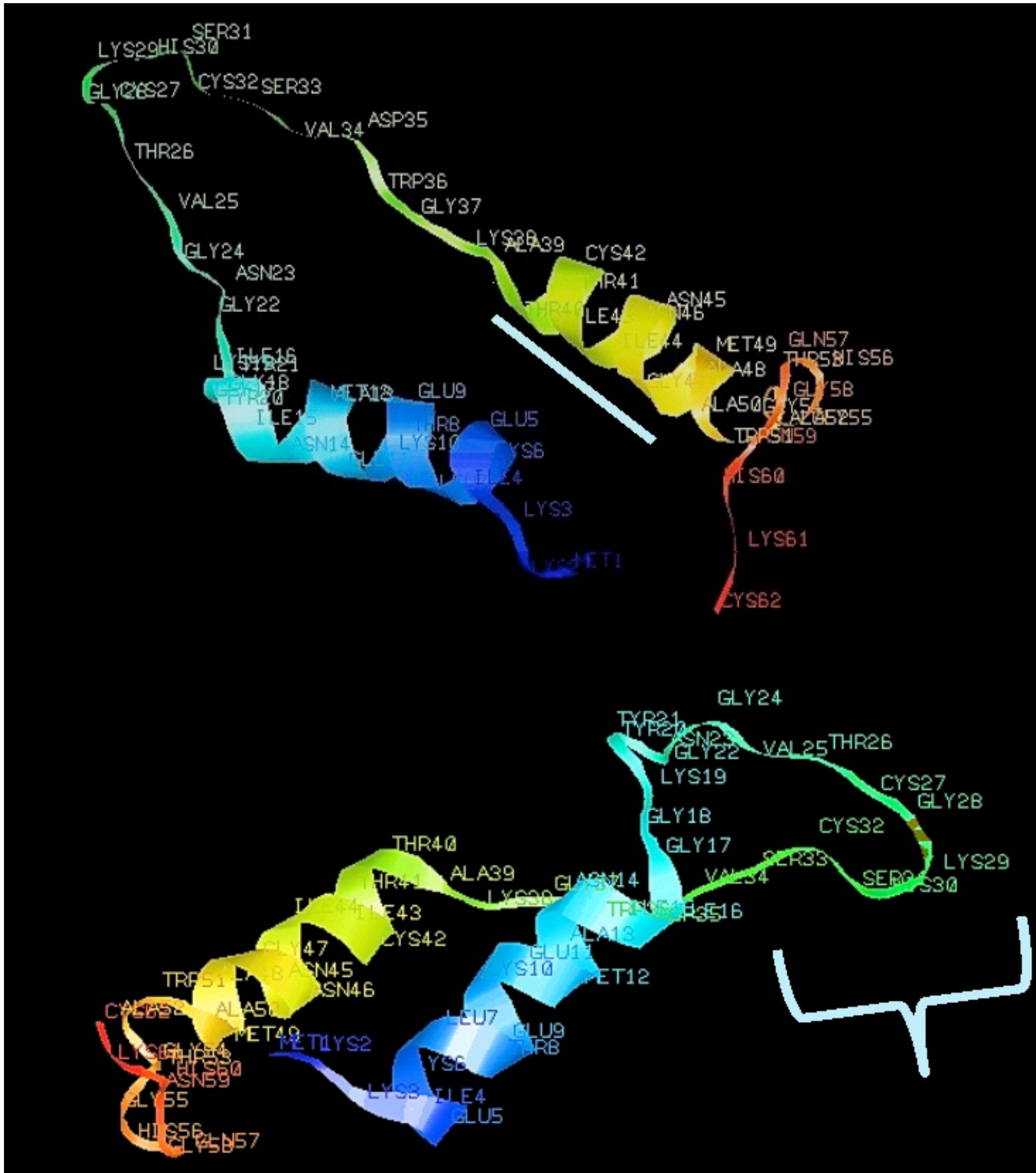

Figure 3: Generated protein structure of PedA (Ribbon model by RasWin ver 2.7.5.2). N-terminal (Blue) and C-terminal hydrophobic regions (Yellow) consists of helical structures; White and orange arrow points to the YGNGV conserved sequence and 4 Cys residues that forms the disulfide bonds, respectively, while bracket shows the CXXXXCXV motif.

cells nearby (Drider et al. 2006). The predicted protein PedA to be a bacteriocin molecule with disulfide bonds and is not localized in the cell. Its highest amino acid composition is Gly, followed by Lys which is both hydrophobic (PredictProtein).

b. PedB

The following amino acid sequence for PedB (Frame 1+) was the predicted open reading frames suitable to form functional protein (ExPASy):

> PedB consensus sequence (S3, K2A23, and 3G3)

HIKQQALDLFTRLQFLLQKHDTIEPYQYVLDILETGISKT
KHNQQTPERQARVVYNKIASQALVDKLHFTAEENKVL
AAINELAHS

Analysis of the protein consensus sequence (tBLASTx) revealed 100% homology to pediocin PA-1 immunity proteins (Accession number WP002834573.1 and ACQ77170.1) and pediocin *P. acidilactici* (Accession number AAT72009.2).

The structural basis of the predicted PedB protein (Figure 4) was compared with the proposed high resolution crystal structure and function of Kim et al. (2007) wherein the α-helices are arranged

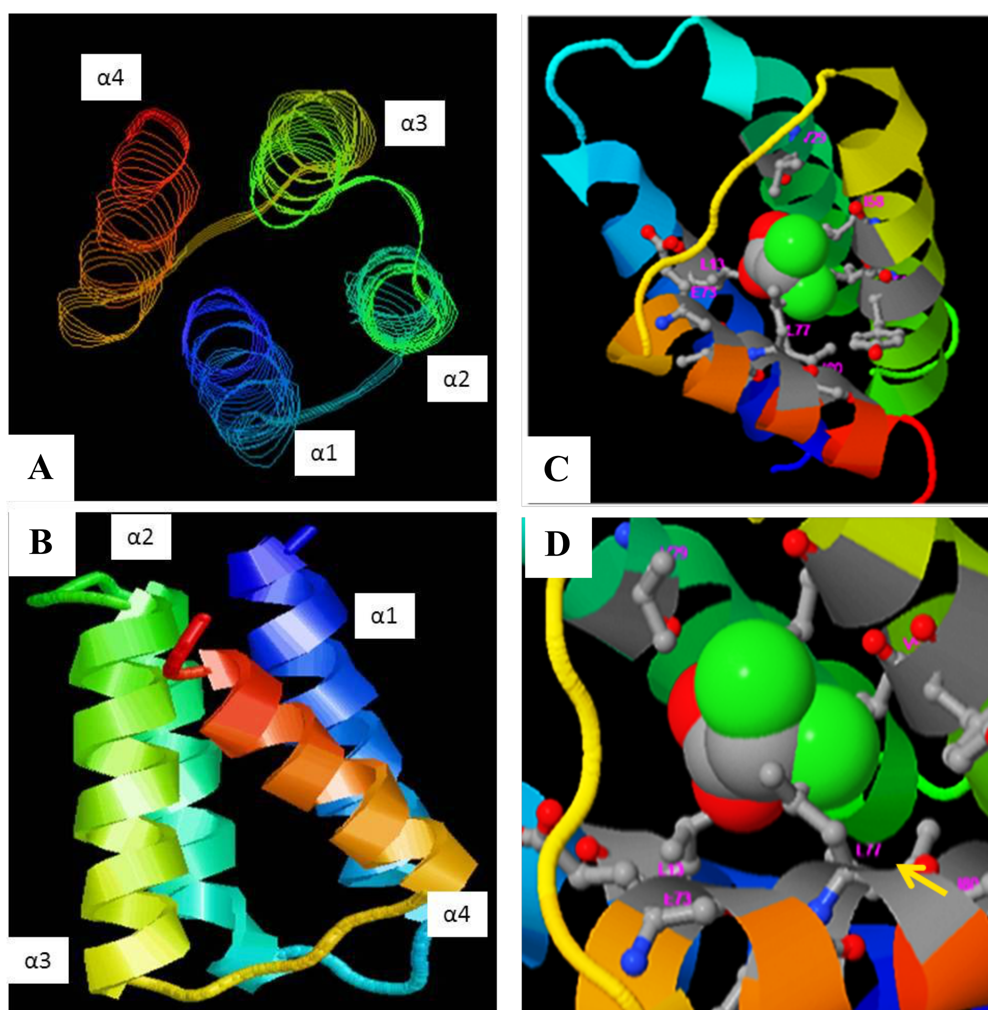

Figure 4 Predicted protein structures of PedB from consensus sequence. (A and B) Generated Strand and Cartoon Models by RasWin ver 2.7.5.2 showing the positions of the 4 α-helices. (C and D) COFACTOR generated models showing possible ligand-binding sites **L13**, V29, V54, Y55, **I58**, E73, V76, **L77** (yellow arrow), I80 of chlorine and oxygen molecules (green and red).

in a way that α1 and α3 run in the same direction and α2 and α4 in the opposite direction, forming an antiparallel four-helix bundle compact globular domain.

Two hydrophobic residues from the connecting loop (Leu and Phe) also penetrates into the interhelical space between α3 and α4, resulting in a stable triangle-like conformation (Kim et al. 2007). The predicted protein structure is composed of four (4) helices α1, α2, α3, and α4, wherein α3 and α4 comprise the C-terminal half. It was predicted that the possible ligand-binding sites are amino acid residues L13, V29, V54, Y55, I58, E73, V76, L77, and I80; where I58 and L77 may also serve as attachment-site for chlorine molecules, and L13 for oxygen.

Compounds within the cells and on the surface of cell membranes which contain oxidizable material react with chlorine dioxide, causing disruption of cell. Chlorine dioxide reacts directly with disulfide bonds in proteins and other nucleotides in the cell. Bacteria appear to employ overlapping stress response systems to counteract the toxic effects of reactive chlorine species such as defences against oxidative stress and protein unfolding (Gray et al. 2013). Researchers are searching for ways on how oxidative stress are being handled by microorganisms and discovered that certain proteins such as RidA in *E. coli* protects bacteria from chlorine (Muller et al. 2014). As regarded, since the immunity protein PedB has attachment sites for chlorine molecules, it may also be established in future studies whether PedB could act as an agent for protection against protein unfolding and lysis due to sublethal concentrations of chlorine.

The immunity protein PedB protects the pediocin-producing cells from being lysed by their cognate bacteriocins. It was reported to interact with the same mannose permease indirectly via recognition of receptor-bacteriocin complex, binding with it and blocking the pore of the cell membrane. It acts in association with the internal side of the cell membrane. The C-terminal half of PedB contains a region involved in the specific recognition of the bacteriocin to which it confers immunity (Liu, Academic Dissertation in Microbiology).

The predicted protein PedB was identified to be an immunity protein localized in the cytosol (PredictProtein). Its highest amino acid composition is Leu (hydrophobic), followed by Gln (polar, hydrophilic).

c. PedC

The following amino acid sequence for PedC (Frame 1+) was the predicted open reading frames suitable to form functional protein (ExPASy):

>PedC (S3)
TYEENIRGVIPITLTQYLHKAQTGEKFIVFVGFKECVHCR
KFSPVMKQYLQQSQHPIYY
LDYGNNGSFSMASQKQITDFYSTFATPMSFMGTPTVAL
LDNGKVVSMTAGDDTTLSDLQQITA

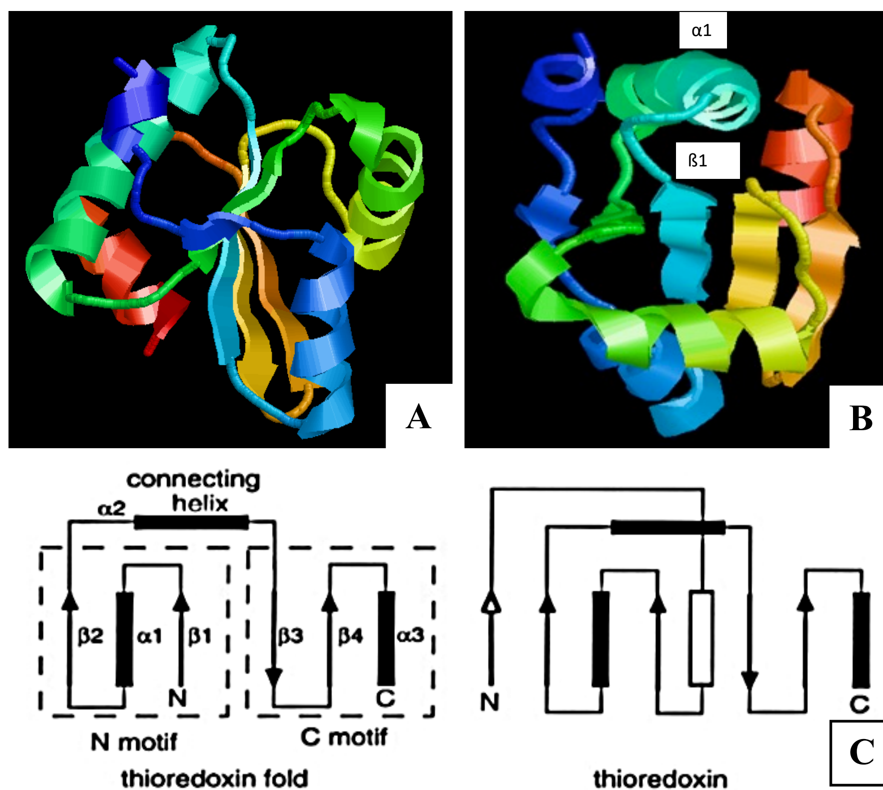

Figure 5: PedC S3 predicted structure as compared to the thioredoxin-fold. (A) Protein structure generated from S3 sequence; (B) Top view of protein PedC S3 showing possible position of active site between α1 and ß1; (C) Thioredoxin fold and thioredoxin arrangement (Martin 1995).

Protein analysis of the S3 sequence from tBLASTx has revealed conserved domain hits for thioredoxin-like superfamily of PedC_BrcD. It also has 98% homology to both thiol reductase thioredoxin of *P. acidilactici* (Accession no. WP00598571.1) and a hypothetical protein O209_14225 from *Lactobacillus plantarum* WHE92 (Accession no. EYR70533.1). A 99.9% homology was also obtained from the Protein d1zmaa1 from the Phyre2 database indicating that the sequence is categorized to have a thioredoxin fold (Superfamily: thioredoxin-like; Family: thioltransferase).

In the PedC predicted structure and arrangements (Figure 5) of S3, the thioredoxin arrangement, a blue ß-sheet can be observed prior to a blue α-helix at the N-terminal end. PedC was also predicted (PredictProtein.org, Phyre2) to have transmembrane helices which was believed to have association in the cell membrane and forms the export machinery together with *pap*D, and are required for the membrane translocation and the removal of the leader peptide (Dimov et al. 2014).

PedC S3 was categorized as an oxidoreductase which acts on sulfur group of donors (EC1.8), considering it as an enzyme. It was said that Thioredoxin (Trx) reduces or correct improper disulfide bonds, protects against many different types of damaging stresses, and remove potentially damaging free-radicals. Trx relieves incorrect disulfide bonds in a 'ping-pong' manner of transferring electrons peroxides (Pan and Bardwell 2006).

Transformation via Electroporation

The colonies of *B. subtilis* B-3479 emerged after electroporation were counted having a colony forming unit per ml (cfu/ml) computed to be 1.37×10^{9}. The selection of putative transformants was based on possible inhibitions against an indicator strain *L. innocua* 026 in the replica plates. The chosen *B. subtilis* colony "B235" is a smaller colony which has grown in the agar assay and was notable for the characteristic inhibition against the indicator and competing cells (Iqbal et al. 2014; Cursino et al. 2006).

Transformation efficiency based on occurrence in replicate plates is 1:5. Gene amplification was done for cell lysates and possible isolated plasmids of putative transformants, although PCR products were not obtained from using crude cell lysates. Gene amplifications from isolated plasmids of *pap*A and *pap*B genes served as gene markers for the putative transformant B235 although faint bands manifested in the gel may be due to having low copy number of plasmids (Williams et al. 2006).

Using these two markers in amplification, the presence of *pap*A and *pap*B amplicons in B235 may indicate a successful transformation activity. However, successful incorporation of plasmid may not indicate successful survival of strain or plasmid replication for long time use and incompatibility may result in loss of incorporated plasmid. Thus, it is suggested that this preliminary presumption needs further in-depth study and protocol optimization. In addition, bacteria tend to maintain fewer copies of plasmids if they contain large inserts or genes that create a toxic product (Williams et al. 2006). Thus, the growth curve and biomass production of B235 should also be further studied.

Crude bacteriocin preparation from cultured B235 (putative transformant) directly obtained from the master plate was collected and tested via 'Spot-On-Lawn' assays, as compared to B-3749 (control/ host) and plasmid source S3 (Figure 6). One-way analysis of variance of inhibition zones means of B235, S3, and NRRL B-3749 against *L. innocua* 026 was observed to be $13.83mm_{(a)} \pm 0.75 > 13.17mm_{(b)} \pm 0.75 > 7.33mm_{(c)} \pm 5.72$, respectively. As noted, NRRL B-3749 (control) had a standard deviation of ± 5.72 which indicates weak or unstable inhibitory effect to the indicator strain, in comparison to the plasmid recipient S3 and putative transformant B235 both having less than 1 (0.75). Significant difference in the mean of inhibition

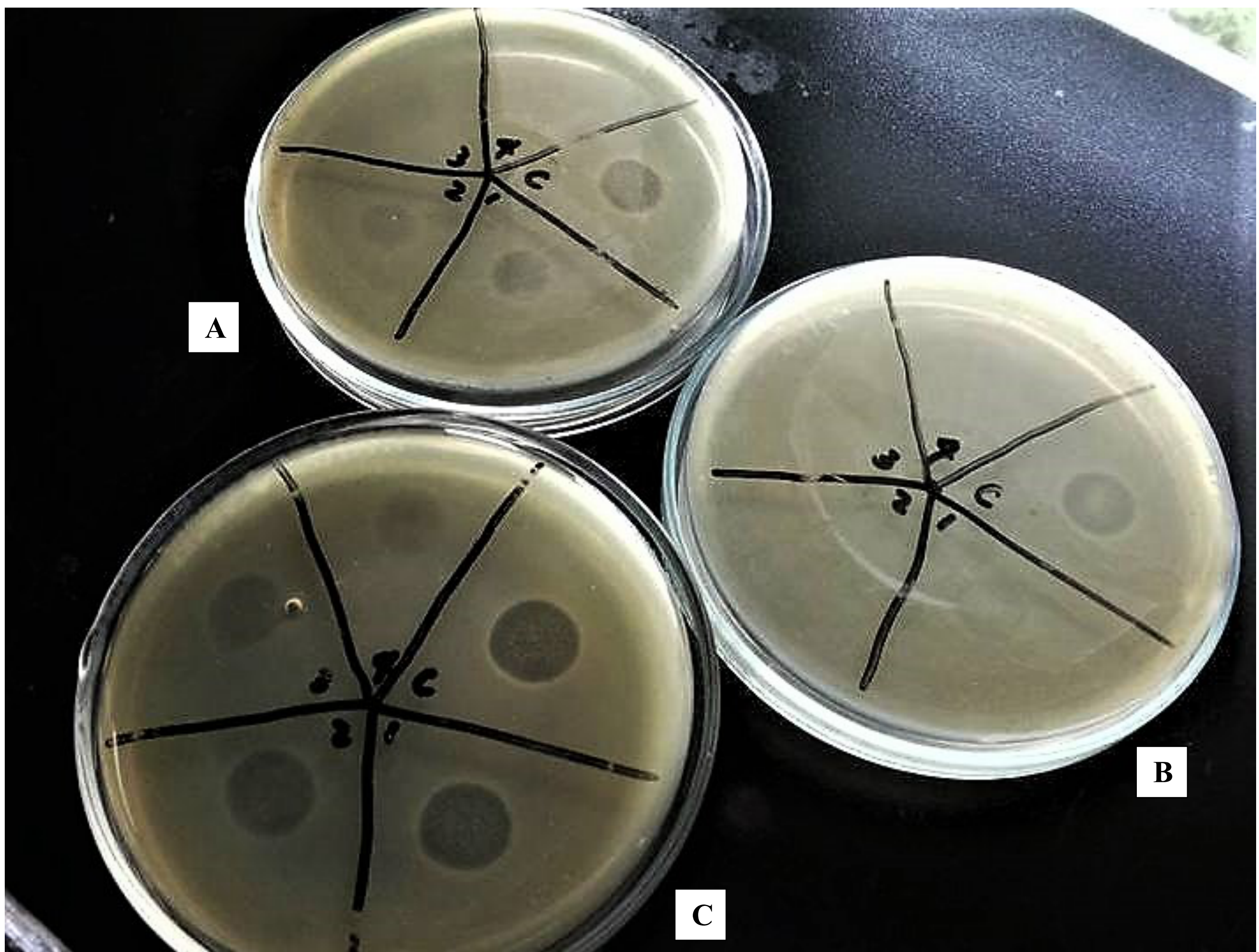

Figure 6: Spot-on-Lawn Assay showing inhibition zones of (A) *P. acidilactici* S3, (B) *Bacillus subtilis* B-3749, and (C) putative transformant B235 in successive 1:1 dilution. B235 expressed inhibition until the 4th dilution.

Table 3: Bacteriocin activity of crude bacteriocin preparation samples (S3, B-3749, B235) against *Listeria* sp. strains

INDICATOR	BACTERIOCIN ACTIVITY PER SAMPLE (AU/ml)		
	S3	B-3749	B235
L. innocua 026	267	133	1067
L. innocua FB101	111	0	133
L. *innocua* MB118	0	0	200
L. monocytogenes NB40	133	0	0
L. monocytogenes NB6	533	0	178

zones across the three samples of solvents (S3 ≠ B-3749 ≠ 235; p-value =0.01 at α = 0.05) have been observed.

The bacteriocin activity in arbitrary units (AU/ml) against listerial indicator strains was computed according to Yamamoto et al. (2003) and is shown in Table 3.

Putative transformant B235 numerically had the highest bacteriocin activity against *L. innocua* 026, *L. innocua* FB101 and *L. innocua* MB118 (1067 AU/ml, 133.333 AU/ml and 200 AU/ml, respectively) as compared to *P. acidilactici* S3 (267 AU/ml, 111 AU/ml, n/a) and *B. subtilis* B-3749 (133 AU/ml, n/a). However, *P. acidilactici* S3 was observed as most effective against *L. monocytogenes* NB40 and NB6 strains (133 AU/ml and 533 AU/ml, respectively).

It should be noted that the putative transformant B235, being *B. subtilis* in nature, may not only produce pediocin but other inhibitory substances as well (e.g. subtilosin). Subtilosin is a macrocyclic bacteriocin which may also have inhibitory effects to listerial species (Stein et al. 2004).

In this study, B-3749 in its natural state generally did not exhibit any inhibition against most of the listerial strains tested except for slight and inconsistent inhibitions on indicator strain *L. innocua* 026 (133 AU/ml). Thus it may be said that the

transformation activity could have had some effects on its metabolite production due to a computed increase in bacteriocin activity (Table 3).

In an observation that *L. monocytogenes* strains were more resistant to the inhibitory substances produced by *B. subtilis* B-3749, and in turn more specifically susceptible to pediocin, it would be rational to consider that *P. acidilactici* S3 pediocin sample would have higher inhibitory effects than B235's mixture of inhibitory compounds.

It was also observed that B235 only had faint amplification bands for both *pap*A and *pap*B markers which may be due to low copy of plasmids. Thus in an assumption that *L. monocytogenes* would be selectively susceptible to pediocin, *P. acidilactici* was observed to have higher inhibitory value (133, 533 AU/ml) than Colony 235 (0, 178 AU/ml) against *L. monocytogenes* strains NB40 and NB6, respectively.

The varying observations may also imply existing differences between listerial strains. The membrane proteins IIC and IID man-PTS of *Listeria* sp. together form a membrane-located complex that may serve as specific receptors for both Class IIa (pediocin) and other Class IIc bacteriocins (lactococcin A).

Little was known on how *Listeria* sp. may acquire resistance to bacteriocins, however it was reported that strains may produce mechanisms of resistance such as downregulation or reduced expression of man-PTS gene expression for naturally-resistant and mutant strains. It was also noted that although Class II bacteriocins are known to have strong antilisterial activity, natural isolates of *L. monocytogenes* were reported to differ greatly in their sensitivities to these bacteriocins (Kjos et al. 2009).

SUMMARY AND CONCLUSION

Local *Pediococcus acidilactici* strains were positively screened for the presence of plasmids bearing the pediocin-producing genes (*pap* genes). Published primer sequences (Motlagh et.al. 1994) were used to amplify the *pap*A gene (PedA) that was present in all the bacteriocin-producing isolates (S3, K2A2-3, and 3G3) but not in the non-bacteriocin producing control isolate *P. lolii* 4E10. On the other hand, the primer sequences that were designed for both *pap*B (PedB) and papC (PedC) were used to amplify and sequence the genes.

Protein structures were presented substantiating their type and functions in the cell. The hydrophobic bacteriocin PedA predicted from the consensus sequences of S3, 4E6
and K2A2-5 is similar to PA-1/AcH type having 4 Cys residues. It is complete with the hydrophobic and conserve regions having C27 at the CXXXXCXV loop motif as the donor of hydrogen molecule prior to formation of disulfide bridges.

On the other hand, PedB is the immunity protein composed of 4 helices having a triangular conformation between the α3 and α4 carboxyl end with ligand binding sites for chlorine.

The thioredoxin-like accessory protein PedC in *P. acidilactici* K10 (AY 707375.1), has a conserved CXXC motif. This same motif and observations were found in local strain PedC S3 as C86-V87-H88-C89. In eukaryotes, the formation of disulfide bonds occurs easily at the oxidized environments such as in the endoplasmic reticulum or cell membranes, not in the reducing environment of cytosol. Thus it may be further studied, based on the tool-generated structure the functional Trx-like protein, encoded in PedC sequence may have functions in repair mechanism, protein folding, and removal of hydrogen peroxide residues in the cell.

In this study, previously tested B-3749 in its natural state has been known **not** to exhibit inhibitions against most of the listerial strains except for slight and inconsistent inhibitions on *L. innocua* 026. Thus, the transformation activity could have had some effects on its metabolite production due to primary observable increase in inhibitory ability. Subsequent culturing and revival of B235 also yielded observable changes in the inhibitory effects of B235.

The inhibitory effect of the putative transformant against the indicator strain *L. innocua* 026, however visually decreased in the 2nd passage was still observed after the 7th passage. The decrease in inhibition via subculturing may be attributed to the principle in which multiplying cells tend to increase their biomass in a growth medium without a stressor. In the process, removal of plasmids which may slow down their metabolic rates may occur since their total cell energy was set in proliferation rather than in defense. The occurrence of "toxic products" as an effect of plasmid insertion may also be considered since this could also be a reason for removal.

A basic understanding on how the pediocin operon works could boost the bacteriocin research in our country. Research on the cloning, transformation and expression of these pediocin genes extracellularly in GRAS competent hosts would be a great step in increasing production and downstream processing of bacteriocin, as well as promoting pediocin as a safe and competitive antimicrobial product.

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CONFLICT OF INTEREST

There are no known conflicts of interest in this study.

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