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Plantaricin A of *Lactobacillus plantarum* IYP1718 playing a role in controlling Undesirable Organisms in Soil

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Abstract
Lactobacillus plantarum can control undesirable organisms in soil to create a balanced environment that can protect plant life from diseases by producing plantaricin A. L. plantarum from two conditions was isolated. Six isolates from healthy soil and four isolates from soil that have black rot disease in brassica plants. Existence of plnA in each L. plantarum isolate was demonstrated by PCR using gene-specific primers. Three out of six L. plantarum isolates from healthy soil and one out of four L. plantarum isolates from soil have black rot disease in brassica plant showing the presence of plnA. L. plantarum IYP1718 from soil that has black rot disease in brassica plants, was chosen for sequence analysis.

The amplified PCR fragment was confirmed to have 100% similarity with the plnA sequence in GenBank. A phylogenetic tree based on sequence analysis confirmed that plnA has closeness with plantaricin G and similarity with enterococcin from Enterococcus faecalis BFE 1071 based on the pairwise distance. Sequence analysis *showed that there are 4 ORFs, but only 2 ORFs could be identified. The results of this study imply that L. plantarum plays a role in controlling undesirable organisms in soil by producing plnA.*

Keyword: *Plantaricin A, Lactobacillus plantarum*, Soil, Black Rot Disease.

Introduction

Lactic acid bacteria (LAB) in soil have roles such as enhancing decomposition, release of plant nutrition and increasing soil humus formation by altering the organic materials. During decomposition of organic materials in soil, gas and heat are produced resulting in loss of energy to a cultivated crop and causing harm to plants. LAB facilitates decomposition of organic matter, resulting in less energy loss due to excess heat and gas[8]. Further, *Lactobacillus spp.* can help neutralize soil and remove byproducts that can form a harmful environment. The presence of *Lactobacillus* can inhibit the undesirable organisms in soil to form a balanced environment that can support plant life. *Lactobacillus* thus contributes to decomposition and disease suppression[1]. Black rot is one of the diseases affecting Brassica plants and is caused by *Xanthomonas campestris pv. Campestris*[5]. *X. campestris pv. campestris* can spread quickly to other brassica plants when water splashes from one plant to another.

The symptoms of black rot disease are yellow, wedge-shaped patches on the leaf edges. *Lactobacillus* can control the growth of fungi, yeast and aerobic bacteria[1]. One of the *Lactobacillus spp.* that can control undesirable organisms is *Lactobacillus plantarum. L. plantarum* can control the growth of undesirable organisms by express secondary metabolites such as bacteriocin[6].

In this study, *L. plantarum* was isolated from healthy soil and soil that has black rot disease in brassica plants to compare the expression of the bacteriocin plantaricin A. Plantaricin is a peptide that usually has membrane-permeabilizing activity and contains 25 to 60 amino acid residues. *plnA* has antimicrobial activity and depends on a nonchiral interaction with lipids and the target cell membrane[9].

plnA expressed by *L. plantarum* is inserted in the cytoplasmic membrane of the target cell, thereby promoting membrane depolarization and cell death[2]. This research aims to determine the existence of the bacteriocin biosynthetic cluster of *plnA* in *L. plantarum* and shows the related structural cluster gene ORFs. Based on this study, *plnA* is expected to control the undesirable microorganisms in soil.

Material and Methods

Isolation and Identification of *Lactobacillus plantarum*: Soils from different conditions were collected in Gifu, Japan. Collected soil included healthy soil and soil that has black rot disease in brassica plants. Lactic acid bacteria were isolated from each sample serially diluted technique until 10^{-7} in Man, Rogosa and Sharpe (MRS) broth and were plated on MRS agar at 37°C for 24 hours in an aerobic condition[15]. Lactic acid bacteria were grown on MRS broth and MRS agar (Becton, Dickinson and Company, USA) and incubated at 37°C for 24 h. LAB were then selected and kept at -80°C in MRS broth with 20% glycerol.

Genomic Identification of Microorganism

DNA Isolation: Lactic acid bacteria were grown in MRS Broth at 37˚C for 18 hours. Cells were harvested and LAB genomic DNA was extracted using the Extrap Soil DNA Kit Plus Ver.2. (Nintetsu Sumikin Kankyo Kabushiki Gaisya, Japan) according to the manufacturer's protocol.

Electrophoresis was performed on 1% agarose gel in tris acetic acid EDTA (TAE 1X) buffer and photographed under UV light.

16S rRNA Sequencing: The 16S rRNA gene fragment of ~1.5 kb was amplified using a pair of universal primers 27 F: (5′- GAGTTTGATCCTGGCTAG-3′) and 1525 R: (5′-AGAAAGGAGGTGATCCAGCC-3′)[15]. Polymerase chain reaction (PCR) was carried out in a fast reaction tube (Applied Biosystems, USA) in a total volume of 25 µl containing 12.5 µl 2 × Green Master Mix PCR (Promega, USA), 1.25 µL of each primer 27F and 1492R (concentration 0.05 pmol/µL), 9 µL nuclease free deionized water and 1 µL template DNA and was run under the following temperature program: initial denaturation of DNA for 5 min at 95°C, 25 cycles of 1 min at 94°C, 1 min at 56°C and 1.5 min at 72°C and final extension for 7 min at 72°C. Then, 5 µl aliquots of the PCR product were analyzed by electrophoresis using 1% (w/v) agarose gel in TAE 1X buffer at 100 V for 30 min.

The gel was then placed in an Electronic U.V. Transilluminator to detect the presence a 1500 bp band. The size of the DNA fragments was estimated using a FastGene 100 bp DNA Ladder (Nippon Genetics, Germany). Fast Gene™ Gel/PCR Extraction kit (Nippon Genetics, Germany) was used for purification before sending the extracted DNA for sequencing according to the manufacturer's instructions.

An average of 500 bp nucleotides for each sequence from each side was read and compared against the NCBI database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Six isolates had been chosen from healthy soil and four isolates were chosen from soil that has black rot disease in brassica plants. Isolates with 98% or higher similarity in sequences were identified as the same species.

Phylogenetic Tree Construction: The DNA sequences obtained were then assembled into contiguous sequences (contigs) using DNAStar; about over a hundred genomic contigs were edited using BioEdit and were aligned using the Bioedit-ClustalW Multiple Alignment. A phylogenetic tree was drawn using Mega7 Construct/ Test Neighbor-Join Tree with 1000 bootstrap replicates.

Plasmid Extraction from *L. plantarum* isolated from Soil: Plasmids were extracted with a protocol based on *Lactobacillus spp.* oriented high-quality methods[12] with some modification. *L. plantarum* was cultured in 2 ml for 18 h. The culture was centrifuged at 15000 rpm for 5 min. The supernatant was removed and the pellet was resuspended in 25% sucrose containing 30 mg/mL lysozyme, with a final volume of 200 µL. Next, 200 µL of 0.5M EDTA was added to the solution and incubated at 37°C for 15 min. The solution was mixed with 400 µL of alkaline SDS solution (3% SDS on 0.2N NaOH) incubated for 7 min at room temperature and then added with 300 µL of ice-cold 3M sodium acetate (pH 4.8).

The solution was mixed immediately and centrifuged at 14000 rpm for 15 min at 4°C. Supernatant was transferred to a new tube and 650 µL of isopropanol (R/T) were added, mixed immediately centrifuged at the max speed for 15 min at 4°C. All the liquid was removed and the pellet was resuspended in 320 µL of sdH_2O, 200 µL of 0.5M EDTA, 200 µL of 7.5M ammonium acetate and 350 µL phenol: chloroform (1:1).

The solution was mixed immediately and centrifuged at the max speed for 5 min at room temperature. The upper phase was transferred to a new tube along with 1 ml ethanol. The solution was mixed immediately and centrifuged at the max speed for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended in 40 µL of TE buffer.

Identification of the Plantaricin Gene from *L. plantarum* using Specific Primers: Identification of plantaricin was performed using PCR. Specific primers of the bacteriocin used in this study are shown in table 1. The amplicons were purified using a purification kit (Nippon Genetics, Germany) and DNA sequence was determined using a Multi – capillary DNA Sequencer "ABI Prism 3100/3130 Genetic Analyzer" (Gifu University, Japan).

Table 1
Primers used throughout this study and their amplification details

Name	Sequence (5′ → 3′)	Annealing
plnA-F	TAGAAATAATTCCTCCGTACTTC	55[3]
plnA-R	ATTAGCGATGTAGTGTCATCCA	
plnEF-F	TATGAATTGAAAGGGTCCGT	54[3]
plnEF-R	GTTCCAAATAACATCATACAAGG	
plnW_F	CACGTCACAGCTAATCTGG	61.5[10]
plnW_R	CTAATTGCTGAATGGTTGGT	
plnS_F	GCCTTACCAGCGTAATGCCC	56[14]
plnS_R	CTGGTGATGCAATCGTTAGTTT	
plnNJK_F	CTAATAGCTGTTATTTTTAACC	55[7]
plnNJK_R	TTATAATCCCTTGAACCACC	
pln423A_F	GTCGCCCGGGAAATACTATGGTAATGGGG	58[13]
pln423A_R	GCGTCCCGGGTTAATTAGCACTTTCCATG	

DNA Sequencing: Plasmid DNA sequencing was performed using the Multi - capillary DNA Sequencer "ABI Prism 3100/3130 Genetic Analyzer" (Gifu University, Japan). Sequences were translated to amino acids using the CLC Sequence Viewer program. Computer alignment and BLAST (basic local alignment search tool) analysis of the sequence were performed using BioEdit for Windows.

Results

Isolation and Identification of *L. plantarum* from Soil: Six LAB isolates from healthy soil and four LAB isolates from soil that have black rot disease in brassica plants were isolated. Their morphology on the MRS agar plate was presented as milky-white in color, circular and convex. The 16SrRNA locus for LAB was amplified using universal primers to confirm the species. The amplicon products of 1500 bp size were used for species identification. All the sequences were found to be *L. plantarum.* The potential *L. plantarum* from soil that has black rot disease in brassica plant was chosen for the next experiment. *L. plantarum* isolated from soil that has black rot disease in brassica plant was designated as *L. plantarum* IYP1718. The sequence of *L. plantarum* IYP1718 was deposited in NCBI Data GenBank under the accession number MK743941.

A phylogenetic tree was drawn to determine the closeness of the relationship of the species based on their genetic similarities and differences (Fig. 1). A phylogenetic tree of *L. plantarum* based on 16S rRNA sequence analyses was constructed using Mega7 Construct/Test Neighbor-Joining Tree which showed the phylogenetic placement of representative strains. The result obtained from the 16S rRNA sequences revealed 100% similarity with the 16S rRNA sequence of *L. plantarum.*

Identification of Plantaricin Gene from *L. plantarum* using Specific Primers: Six specific primers of plantaricin were used for amplification, but only *plnA* was found to exist in *L. plantarum*; the other plantaricins could not be detected by PCR. Cells were harvested at the beginning of the stationary phase to reach the maximum production of *plnA* (data not shown). The genes encoding bacteriocins are located on operon clusters which may be placed on plasmids. Plasmid isolation showed that all *L. plantarum* isolates had plasmids. All plasmid of *L. plantarum* were approximately ~10 kbp. The results showed that a fragment DNA containing the *plnA* gene of approximately ~550 bp was amplified from the plasmid of *L. plantarum.*

The presence of the *plnA* gene encoded on the plasmid was thus confirmed (Fig. 2) based on the observation of the PCR product using specific primers for *plnA.* Among the six isolates of *L. plantarum* from healthy soil, three produced *plnA*, whereas one of four *L. plantarum* isolates from soil that has black rot disease in brassica plant produced *plnA* (Fig. 2). *L. plantarum* COY2906 isolated from Virgin Coconut Oil that had confirmed presence of *plnA*, was used as a positive control.

Sequence Analysis: The potential *L. plantarum* IYP1718 from soil that has black rot disease in brassica plants, was chosen for sequence analysis.

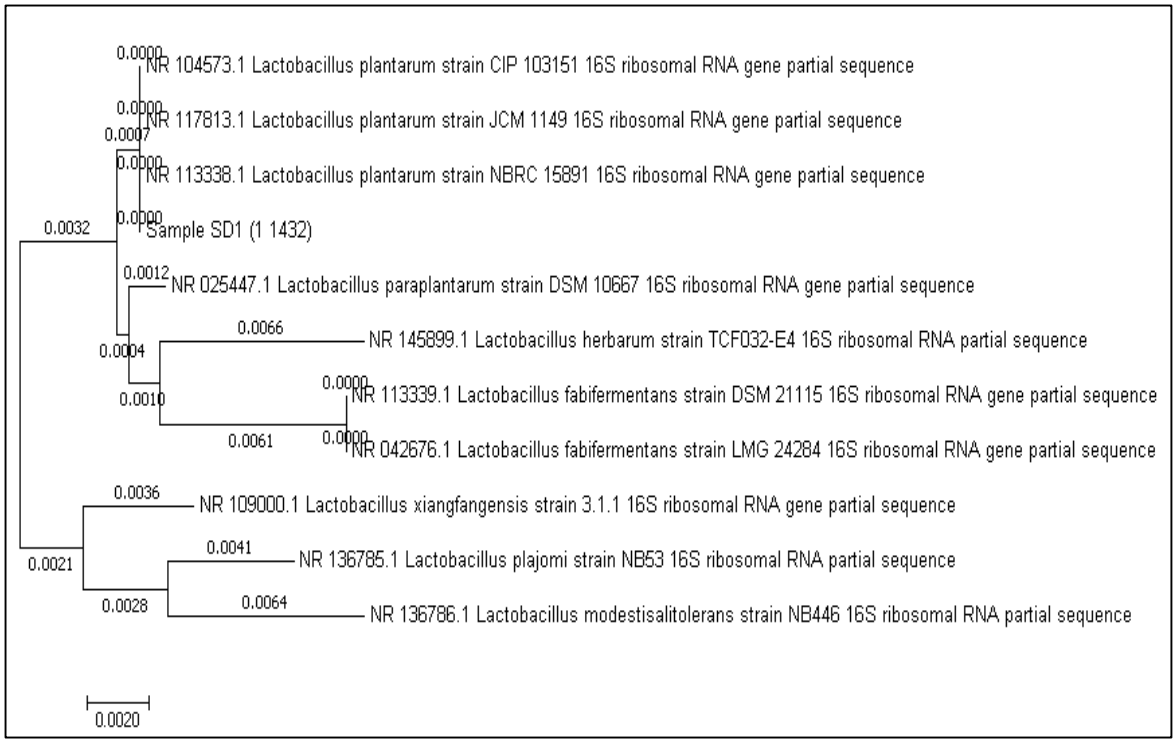

Fig. 1: Phylogenetic tree of *L. plantarum* based on the 16S rRNA sequence analyses using Mega7 Construct/Test Neighbor-Join Tree, showing the phylogenetic placement of representative strains; Sample SD1 is *L. plantarum* IYP1718

The identity of the amplified *plnA* gene was confirmed by DNA sequence analysis whereby high DNA sequence identity confirmed 100% correspondence to *plnA* from GenBank originating from *L. plantarum* with accession number AFJ79564.1. This sequence of ~528 bp was translated using CLC Sequence Viewer. The DNA sequence of *plnA* was analyzed to determine the start – end position of transcription and translation. Computer analysis of the ORF indicated *plnA* translation start at the first AUG codon. The DNA sequence from the sample using a specific primer showed that the start position of transcription and translation is 60 and that the end position of transcription and translation is 206.

Bioedit ClustalW Multiple Alignment was used for sequence alignment. *plnA* from GenBank was compared with the *plnA* from *L. plantarum* IYP1718. No differences were observed between *plnA* GenBank and *plnA* from *L. plantarum* IYP1718 and both sequences were identical with 100% similarity homology between *plnA* from GenBank and *plnA* from *L. plantarum* IYP1718. Structurally, *plnA* has an Open Reading Frame (ORF) of 147 bp that encoded 48 amino acid residues (Fig. 4). Comparison of the amino acid sequence to known proteins in the database revealed significant homology with class II bacteriocins.

It was found that *plnA* from *L. plantarum* IYP1718 and GenBank showed 18% similarity with Plantaricin G based on pairwise distance (Fig. 3) (data not shown).

Even though *L. plantarum* IYP1718 DNA sequence analysis showed high DNA sequence identity of 100% corresponding to *plnA* from GenBank, the plantaricin gene was observed elsewhere. The cluster structure of the plantaricin encoding region was not shown around the *plnA* gene region. The DNA fragment containing the *plnA* operon as the probe revealed extensive homology, has several restriction enzyme sites: *ApoI, BbsI* and *BsaBI* (Fig. 4). The first ORF encodes a protein consisting of 48 amino acid residues, followed by the TAA stop codon.

Computer analysis of the ORF indicated that the *plnA* translation starts at the first AUG codon. ORF1 was identified as *plnA* that consists of 146 bp, ORF 2 was identified as Structure of Importin Beta Bound to the Ibb domain of Importin Alpha that consists of 71 bp. However, the function of ORF2 has not been related to any bacteriocin production. Computer analysis showed ORF3 and ORF4 but these could not be identified in NCBI GenBank and encoded for a peptide with unknown function. All ORFs larger than 20 bp were compared against the protein database using the BLAST server as shown in table 2.

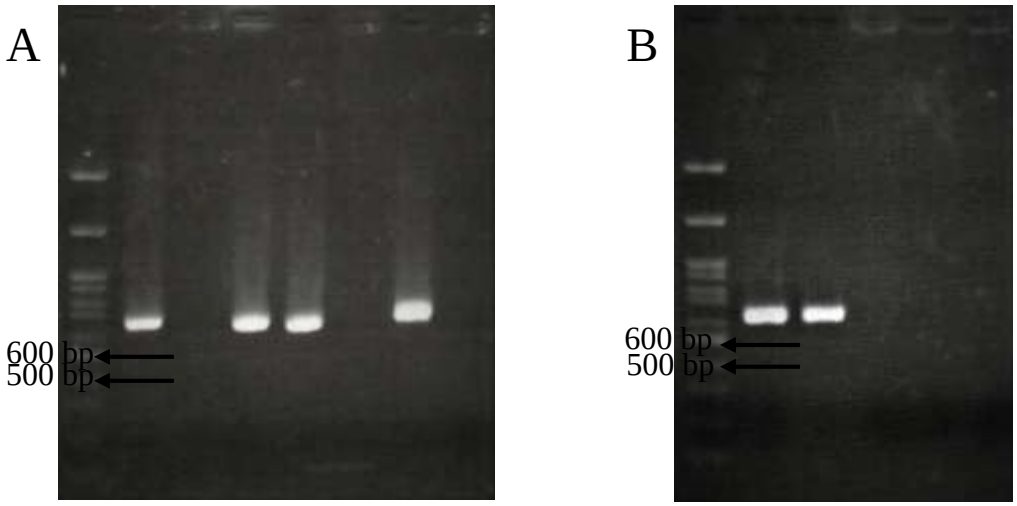

Fig. 2: A. Detection of *plnA* sequence in *L. plantarum* plasmid isolated from healthy soil B. Detection of *plnA* sequence in *L. plantarum* plasmid isolated from soil with black rot disease in brassica plants. M: Molecular Weight Marker 100bp DNA Ladder. 1. Positive control, Amplification of *plnA* from *L. plantarum* COY2906 isolated from Virgin Coconut Oil; 2-10. Amplification of *plnA* from *L. plantarum*

Table 2
Characteristics of the predicted ORFs encoded from *L. plantarum* IYP1718

ORF	Location (bp)	Size (bp)	Gene	Protein accession no.	% Identity
ORF1	60-206	146	*plnA, L. plantarum*	AFJ79564.1	100%
ORF2	230-311	71	Structure of Importin Beta Bound to the Ibb Domain of Importin Alpha	1QGK_A	42%
ORF3	342-428	86	undetected		
ORF4	444-506	62	undetected		

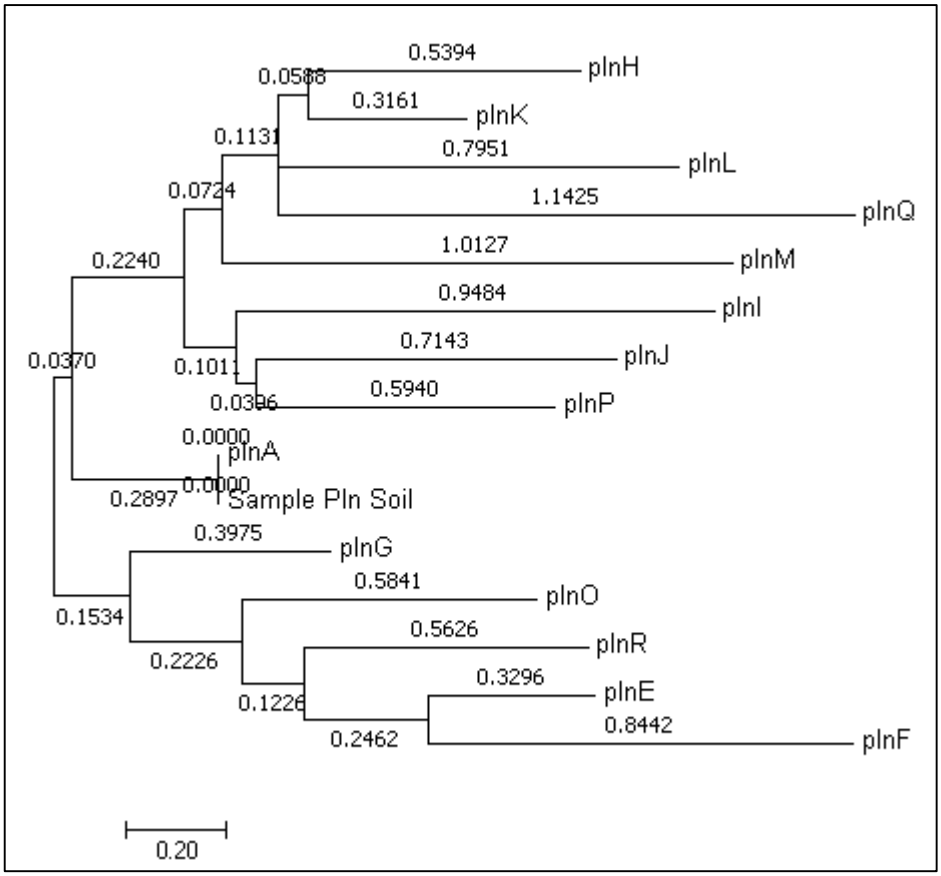

Fig. 3: Phylogenetic tree based on the sequence analyses of *plnA* between *L. plantarum* IYP1718 and plantaricin from *L. plantarum* GenBank. The phylogenetic tree shows the closeness of the relationship of plantaricin based on the genetic similarities.

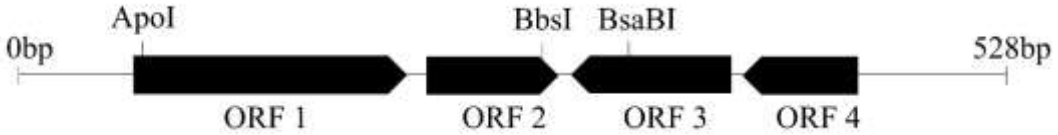

Fig. 4: Schematic representation of the *plnA* gene cluster. ORF 1 indicates the structural gene *plnA*.

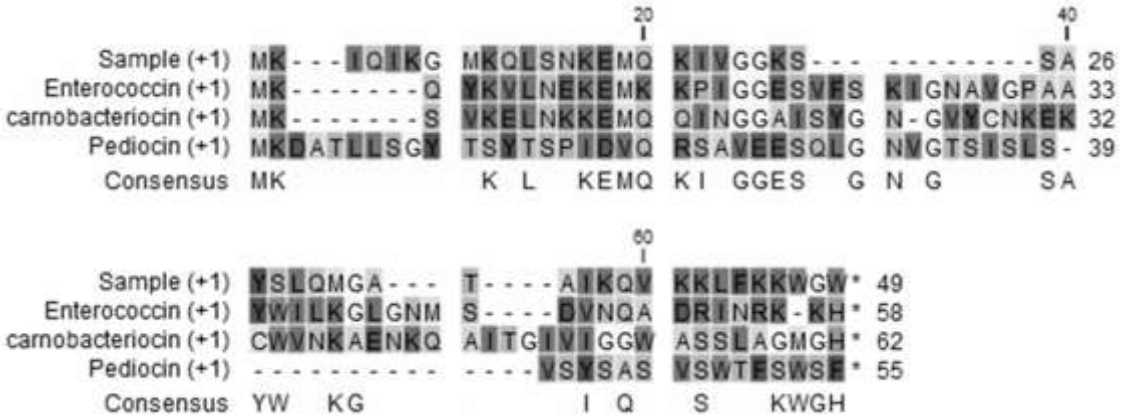

Fig. 5: Multiple-sequence alignment of known and putative bacteriocin precursors using Bioedit ClustalW Multiple Alignment. The known sequences are *Enterococcin, Carnobacteriocin BM1* and Pediocin

Multiple-sequence alignment was performed based on bacteriocin class IIa to show closeness relative to other bacteriocins. Pairwise distance confirmed that the amino acid sequence of *plnA* showed 22% similarity with Enterococcin from *Enterococcus faecalis BFE 1071* based on the pairwise distance (data not shown). It remains to be determined whether *plnA* has the promoter motif of the plantaricin operon with a similarity of 22% with

enterococcin.

Discussion

In this study, *L. plantarum* was isolated from healthy soil and soil with black rot disease in brassica plants. Six species of *L. plantarum* isolated from healthy soil showed three *L. plantarum* isolates with *plnA* whereas one of four *L. plantarum* isolates from soil with black rot disease in brassica plants had *plnA*. *plnA* from *L. plantarum* of healthy soil plays a role to control the undesirable organisms in soil in order to form a balanced environment that can support plant life. Under healthy conditions, *L. plantarum* produced more plantaricin compared to that in *L. plantarum* isolated from soil with black rot disease in brassica plant.

L. plantarum that did not have *plnA* indicated an incompatible plasmid. The plasmids that could not receive this gene are considered to be deficient in some genes and specific function that allow compatible plasmids in the cell to accept plantaricin genes. Moreover, if the plasmid is incompatible, it is released from the cell. The manner of transfer depends on their mechanism of replication in a single cell. This mechanism helps control the undesirable organisms in soil to form a balanced environment that can support plant life.

L. plantarum IYP1718 isolated from soil with black rot disease in brassica was selected for the next experiment because of its ability to survive in soil that has black rot disease. It was confirmed that the *plnA* biosynthetic cluster was located on a plasmid of *L. plantarum*. *L. plantarum* IYP1718 survived in an environment that has an undesirable organism by producing *plnA*.

This is obtained through *L. plantarum* from healthy soil and transfer of genetic material from one bacterial cell to *L. plantarum* IYP1718, either through direct contact or a bridge between the two cells, but some plasmids contain genes called transfer genes that facilitate the beginning of conjugation.

During their life cycle, bacteria must adapt to several environments by coping with environments containing few nutrients and with other bacteria that produce the required metabolic products. *plnA* is a secondary metabolite that also functions as self-defense from undesirable organisms. *plnA* can dissipate the proton motive force by disrupting the trans-membrane potential of the sensitive cell. Two bacteriocin peptides appear to form relatively specific pores, thus dissipating the trans-membrane potential[4,17].

All plasmids of *L. plantarum* were harvested in the early stationary phase to reach the maximum production. Similar to *L. plantarum* J-51, the plantaricin was detected at the end of the exponential phase and during the early stationary growth phase[11]. The 528 bp amplicon was obtained by PCR analysis of *L. plantarum* IYP1718 using a specific primer pair for the *plnA* gene. Computer analysis showed that *plnA* of the sample has an Open Reading Frame (ORF) of 147 bp that encodes 48 amino acid residues. The DNA fragment was sequenced and confirmed to have 100% identity with *plnA* from NCBI gene bank as expected from strains of the same species.

However, some reports have shown a mutation: Gly7 mutated to Ser7 in pln A of *L. plantarum J-51*. The mutation (Ser7) was located at a double-glycine leader peptide and the putative active peptides of strain J-51 remain identical to those *plnA* peptides of *L. plantarum* C11[11]. In addition, the *plnA* encoded 47-48 amino acid residues.

plnA with 37-residues and a C-terminal moiety corresponding to the amino acid of plantaricin 423 with 19 or 18 residues have N-terminal extensions with a glycine-glycine (GG) cleavage site[13]. It is suggested that *plnA* from *L. plantarum* IYP1718 and GenBank showed 18% similarity with *plnG* based on pairwise distance. The 18% similarity with *plnG* indicates the closeness relative plantaricin between *plnA* and *plnG*. These peptides of class II primary and three-dimensional bacteriocin consist of two functional domains: a well-conserved hydrophilic N-terminal β-sheet domain and diverse hydrophobic or amphiphilic C-terminal α-helical domain [13].

Schematic representation showed 4 ORFs; ORF1 and ORF2 are *plnA* from *L. plantarum* and structure of Importin Beta Bound to the Ibb domain of Importin Alpha respectively. ORF3 and ORF4 have undetected functions. In addition, some reports have shown that *Lactobacillus brevis* encodes brevicin 925A. Plasmid pLB925A04 carried many ORFs other than the bacteriocin-biosynthesizing gene cluster and most of the ORFs cannot be annotated[18]. However, an ORF could describe the unknown function encoded in the *plnA* locus.

Multiple-sequence alignment was done to show the closeness relative to other bacteriocins. The amino acid sequence of *plnA* showed 22% similarity with enterococcin from *Enterococcus faecalis BFE 1071* and 18% similarity with *plnG* based on pairwise distances. *L. plantarum* I-UL4 showed that the promoter motif of pln operon was also found in other bacteriocin systems such as the gene cluster of sakacin A, sakacin P, carnobacteriocin A, carnobacteriocin B2 and enterococcin A indicating a similar regulatory mechanism for bacteriocin production[16]. That report also showed similarity with this report.

This sequence has not been fully characterized and many genes essential for bacteriocin export including the mode of action that regulates the production and synergistic actions of the bacteriocin are topics of ongoing research. Class II bacteriocin production is organized within operon clusters and consists of a structural gene encoding the prepeptide, immunity gene, an ABC transporter gene and a gene encoding an accessory protein; in some cases, presence of a regulatory gene has been reported.

Conclusion

L. plantarum was isolated from healthy soil and soil with black rot disease in brassica plants. Six species of *L. plantarum* isolated from healthy soil showed three *L. plantarum* isolates with *plnA*, whereas one of four *L. plantarum* isolates from soil with black rot disease in brassica plants had *plnA*.

Sequenced DNA fragment confirmed to have 100% identity with *plnA* from NCBI gene bank. The amino acid sequence of *plnA* showed 22% similarity with enterococcin from *Enterococcus faecalis BFE 1071* and 18% similarity with *plnG* based on pairwise distances. More studies to determine the full operon and whether other genes encoding other bacteriocins are present in the *L. plantarum* IYP1718 plasmid and chromosome are required. The initiation sequence reported here will be useful for functional analysis of ORFs located in the plasmid of the *plnA* biosynthetic gene cluster in *L. plantarum*.

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