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Original Article

DETECTION AND CHARACTERIZATION OF ENTEROCIN ENCODING GENES IN *ENTEROCOCCUS MUNDTII* STRAIN C4L10 FROM THE CECUM OF NON-BROILER CHICKEN

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ABSTRACT

Objective: This study was carried out to isolate and detect the enterocin genes of the bacteriocin producing strain *Enterococcus mundtii* C4L10 isolated from Malaysian non-broiler chicken.

Methods: Amplified bacteriocin encoding genes were extracted from the total genome of *Enterococcus mundtii* strain C4L10 using specific sets of primers.

Results: The translated protein of the resultant enterocin gene sequence showed that the bacteriocin contain 2 sets of Enterocins; Enterocin_P and Enterocin_B. The enterocin gene was found to be located in the chromosome. The bacteriocin of C4L10 has a molecular mass of approximately 10 kDa. At the amino acid level, bacteriocin of strain C4L10 is 85% homologous to enterocin_P which is similar to class IIa bacteriocins produced by *Lactoctobacillus salivarius* strain. The secondary structure prediction showed that the Enterocin_P contain double glycine motifs and YGNGP instead of the normal YGNGV characteristics of class IIa bacteriocin. Conserved domain search of the encoding gene revealed the presence of Glycosyltransferases domain in the Enterocin_B. The antimicrobial spot/region of the C4L10 enerocins showed the presence of two antimicrobial regions in Enterocin_P whereas only one antimicrobial spot was detected in Enterocin_B.

Conclusion: In this study, two enterocin genes; enterocin_P and Enterocin_B were successfully amplified and characterised from *Ent. Mundtii* strain C4L10. The sequences showed strong similarity with reported class IIa bacteriocin, such as having double glycine and YGNGV motifs, and potential regions with antimicrobials activities which are useful for future exploitation in rational design of antimicrobial peptides.

Keyword: Enterocin, Gene, Glycosyltransferase domain, YGNGP, Antimicrobial Protein.

INTRODUCTION

Enterococci belong to a group of lactic acid bacteria (LAB) with both advantages and harmful aspects. They can prolong shelf-life as a result of the production of antimicrobial agents, synthesize flavor compounds, and contribute to health promotion as probiotic cultures. However, they possess different detrimental attributes: possession of a large number of virulence factors, antibiotic resistance genes and also known to act as an indicator of fecal contamination [1].

Many enterococci are known to produce ribosomal synthesized peptide (bacteriocins) used as a defense mechanism against closely related bacteria [2]. Bacteriocins-producing enterococci have varied ecological niche and many may produce class IIa bacteriocins that are known to be heat stable, cationic, hydrophobic, and low molecular weight peptides [3].

Among enterococci strains, there are some that produce l antibiotic (class I), cyclic (class III), and large bacteriocins (class IV) that remain stable at varying pH values with a broad spectrum of antimicrobial activity [4].

Bacteriocins are produced ribosomally and are known to have bactericidal activity against closely related species of the producer cell. Bacteriocins are heterogeneous compounds with varying molecular weights, biochemical properties as well as inhibitory spectra [5]. The mechanisms of action of bacteriocins are diverse, but the bacterial membrane is the target for most bacteriocins [6]. Many bacteriocins have their names known but other important aspects like molecular, biological functions are not known. Furthermore, the knowledge of their 3D structure is not sort for. Therefore, the aim of this our research is to do a complete annotation of the enterocin gene of strain C4L10 extracted from Kuantan non-broiler chicken.

MATERIALS AND METHODS

Structural gene analysis of C4L10 strain

Detection of enterocin genes by PCR

The purified genomic DNA of bacteriocin producing strain *Ent. mundtii* C4L10, was used as a template in PCR amplifications to determine the existence of structural genes encoding its enterocins using specific sets of enterocin primers. Table 1 showed the sequences of the primer pairs used for PCR-amplification of the enterocin genes from Kuantan non-broiler chicken. Primers used were purchased from First Base Laboratories Sdn. Bhd. Malaysia.

PCR reaction

The amplification of bacteriocin genes from strain C4L10 was carried out in an Eppendorf AG-22331 Hamburg Mastercycler Gradient Germany, with the following conditions: Initial denaturation temperature was at 95°C for 5 min, denaturation temperature was at 95°C (30 sec); Annealing temperature 50°C for 30 sec. Extension temperature was at 72°C for 2 min; 35 cycles. Final extension temperature was at 72°C 5 min, and final hold of 4°C. The PCR products were analyzed on 1.0 % agarose gel with 1X TAE buffer at constant voltage of 150V. Fermentas GeneRuler 1kb DNA Ladder was used to compare the PCR amplicons. Four pair of primers used for amplification of bacteriocin genes are listed in table 1.

Conserved domain search

Conserved domains (CD) in proteins which play crucial role in protein interactions, DNA binding, enzyme activity, and other important cellular processes, were searched through the Enterocin of C4L10 strain using sequence similarity search tool with close homologous family members available in various protein databases. CDD-BLAST [7] bioinformatics web tool [8-10], was used to detect the presence of hypothetical proteins, which shows the ability to search for defined conserved domains in the sequences and assist in classifying the proteins in appropriate family.

Protein 3D structure prediction

The three dimensional structure of Enterocin of strain C4L10 was generated by homology modeling I-TASSER [11].

Determination of antimicrobial proteins and regions from their amino acid sequence

This calculates bactericidal propensity index for each amino acid, using report from a high –throughput experimental data as

reference, thereby identifying the potentially active stretches within the protein sequence. The methods corroborates against positive and negative datasets with 5% accuracy and 90% sensitivity, providing with information on active sites [12].

Table 1: The sequences of the four primer pairs used for PCR-amplification of the enterocin genes

Primer	Sequence	Reference
Entcin_A-F	5'-AAA TAT TAT GGA AAT GGA GTG TAT-3'	[13]
Entcin_A-R	5'-GCA CTT CCC TGG AAT TGC TC-3'	[13]
Entcin_B-F	5'-GAA AAT GAT CAC AGA ATG CCT A-3'	[13]
Entcin_B-R	5.-GTT GCA TTT AGA GTA TAC ATT TG-3'	[13]
Entcin_P-F	5'-TAT GGT AAT GGT GTT TAT TGT AAT-3'	[13]
Entcin_P-R	5'-ATG TCC CAT ACC TGC CAA AC-3'	[13]
Entcin_L50-F	5'-STG GGA GCA ATC GCA AAA TTA G-3'	[13]
Entcint_L50-R	5'-ATT GCC CAT CCT TCT CCA AT-3'	[13]

Table 2: PCR amplifications of 16S rRNA from strain C4L10 using enterocin gene specific primer to determine the existence of structural genes encoding four enterocins

Strains	Enterocin genes			
	Enterocin_A	Enterocin_B	Enterocin_P	Enterocin_L50
C4L10	-	+	+	-

(+) positive, (-) negative amplification

RESULTS AND DISCUSSION

From table 2, it could be seen that strain C4L10 produced amplification product with Enterocin_B and Enterocin_P. *Ent. mundtii* C4L10 strain carried the enterocin B and P structural genes. The detection of more than one bacteriocin encoding gene was also observed by PCR in the Enterococcus strains isolated from fermented foods [14-16]. Several findings have shown that multiple bacteriocin production by enterococci is a frequent phenomenon [17, 18]. Furthermore, the production of multiple bacteriocins by single strains and repeated isolation of the same enterocin by different groups may reflect the efficient gene transfer and the diversity of enterococci in nature [18].

Strompfová and Lauková [4] reported the presence of only enterocin A and P structural genes among 5 bacteriocin producing *E. faecium* strains isolated from the caecum, ileum and crop of chickens. Among rabbit enterococci, enterocin A, P, L50B genes were determined but not enterocin B [19]. On the other hand, enterocin B gene was found in enterococci isolated from horses [19].

Table 3 is a representation of BLAST search from Bactibase database result of the translated gene encoding the bacteriocins for strain C4L10. The gene translation was done using the software Geneious 6.1.2 [20] created by Biomatters available from http://www.geneious.com/(table 3).

The Bactibase protein database homology search of the deduced peptides showed that strain C4L10 produced 2 types of Enterocin, P and B, both of which have strong homology to Bacteriocin L-1077 produced by *Lactobacillus salivarius* [21] and Sakacin G produced by *Lactobacillus sakei* 83% and 46% identity, respectively [22]. The two enterocins carry leader peptides or signal peptides (fig. 2) and belong to class IIa bacteriocins family.

The encoding gene for Bacteriocin L-1077 is unclassified while "skgA2" is for Sakacin G. Since, strain C4L10 enterocin P recorded a higher homology of (83% identity) with bacteriocin L-1077, it can be said to be the enterocin of strain C4L10 (table 2). The 104 residues of enterocin P were determined, and the theoretical molecular weight was calculated to be 11.1 kDa. This is almost identical to the observed size on SDS-PAGE of approximately 10kDa [23]. It also contains isoelectric point of 11.9 and Extinction Coefficient of 1, 740M^{-1} cm^{-1}. Furthermore, the bacteriocin contains the consensus amino acid sequence Tyr-Gly-Asn-Gly-Pro (YGNGP) instead of the Tyr-Gly-Asn-Gly-Val, which is a common motif within the pediocin-like bacteriocins [24].

In addition, it may also be said to belong to the subgroup of class IIa bacteriocins (which are characterized as small heat-stable bacteriocins, non-lantibiotic with strong anti listerial activity [25]. The screening indicated the presence of combinations of more than one enterocin gene in one strain in our study. The same result seen in our study was observed by De Vuyst, Moreno and Revets [17], who observed the presence of two, three or four different enterocin genes in one strain. However, it was shown that not all enterocin genes must be expressed at the same time. In addition, multiple bacteriocin production in single strains has been demonstrated [24, 26]. Moreover in our study, enterocin P and B were isolated and all of them belong to the class IIa bacteriocins Among the possible combinations of two enterocin genes, the combination of enterocin B and P was the most frequently found. However, L De Vuyst, MRF Moreno and H Revets [17] observed that A and P are the most frequently detected enterocins in a single strain. V Strompfová, A Lauková, M Marciňáková and Z Vasilková [27] in their study, observed the presence of all four enterocin genes in 34 strains which indicated the high genetic potential of some strains to produce various bacteriocins.

As shown in fig. 1, the structural gene for enterocin_P strain C4L10 revealed an open reading frame (ORF) with two disulphide bridges (positions 269-72 and 260-62) with potential Shine Dalgarno ribosome binding site sequence (AAGG, position 77-80). The enterocin_P of strain C4L10 contain double-glycine leader peptides position 26 to 27 aa as shown in fig. 1. This is in line with the report that the majority of the class IIa bacteriocins possess a double-glycine sequence situated at the N terminus, which helps in the recognition of signal needed for peptide procession and secretion The detection of double-glycine at the N-terminus in our study is in line with the work of De Kwaadsteniet et al., [44] who showed that most class IIa bacteriocins posses double-glycine sequence located at the N terminus, whose main function is to serve as signal for the recognition of peptide procession and secretion.[28]. The translocation of the bacteriocin across the cell membrane is aided by ATP-binding cassette (ABC) transporters [29]. A few class IIa bacteriocins make use of a signal peptide instead of a double-glycine leader sequence for these processes [30, 31]. The leader peptide is usually positively charged and has a hydrophobic core and cleavage region. There is the procession of the peptide during translocation across the membrane by a signal peptidase [32]. Bacteriocin L-1077 sequences was obtained from Bactibase database. The alignment shows identical sites of 13 (18.1%) (Pairwise % Identity: 18.1%), mean molecular weight: 4.003kDa, mean Isoelectric point: 10.33 and mean Extinction Coefficient: 2, 980M^{-1} cm^{-1}(fig. 2).

As shown in fig. 3, the predicted secondary structure of C4L10 Enterocin_P has 4 α-Helix, 10 β-strands 7 Coil and 11 turn, making a total of 32 structures. Together with the presence of the double glycines and YGNGP motifs, this cationic N-terminal beta-sheet domains could have involved in the binding of class IIa bacteriocin to the target cell membrane. Our enterocin P possessed a novel N-terminal sequence made up of a change of Val to Prol at position 20. In the same line, bacteriocins like bacteriocin 31 [30], sakacin 5X [33], and plantaricin C19, [34], also have altered N-terminal sequence.

The YGNGV consensus motif plays a role in the recognition step involved in the mechanism by which class IIa bacteriocins act [35, 36], though, it is not certain whether YGNGV consensus motif play any prominent part in pediocin PA-1 initial binding step [37]. However, Quadri et al., 1997 observed a reduction in the activity of Carnobacteriocin B2 when there was a replacement within the Y 3 GNGV motif (Tyr 3 to Phe).

Moreover, Miller et al., 1998 observed a dramatic reduction in Pediocin AcH activity due to a mutation within the YGN 5 GV motif (Asn 5 to Lys). In another study conducted by Yamazaki et al., (2005), showed that their piscicocin CS526, with a Lys instead of a Val (YGNGL sequence), had anti-Listeria activity characteristic of class IIa bacteriocins. In conclusion, the presence of Val within the YGNGV motif could not be a prerequisite for the antimicrobial activity of class IIa bacteriocins.

C4L10 Enterocin_P has 4 putative antigenic regions, with the first having length: 8, Interval: 89->96, residues: PSSLLTRS and Score: 1.087. Second predicted antigenic site has length: 15, Interval: 4->18, residues: PLLGSKKPIIKITKC and Score: 1.112. The third has Length: 40, Interval: 38->77, residues: NSTLIAGSIICEGAVSLTLIA and score: 1.171. The fourth has length: 6, Interval: 31->36, residues: KTCIKL and score: 1.073 (fig. 4).

As shown in fig. 5, the predicted secondary structure of C4L10 Enterocin_B have 9 α-Helix, 14 β-strands 18 Coil and 26 turn, making a total of 67 structures. It could be seen that in fig. 6 Enterocin_B of strain C4L10 contains 9 antigenic regions and 2 signal cleave regions. The presence of the double glycine (GG) and the YGNGP in Enterocin_P with antibacterial activity differentiate it from Enterocin_B that possess the glucosyltranferaces enzyme that is known to participate in antiproliferative activity on cell lines we observed (in press).

Prediction of the antimicrobial proteins and antimicrobial regions from their amino acid sequence

Using Antimicrobial Sequence Scanning System AMPA online tool [12] putative antimicrobial regions are predicted in the protein sequences. We were able to predict the active spot in the enterocin sequence of C4L10 examined. A protein is classified as antimicrobial if it has at least one antimicrobial region. The enterocin of C4L10 protein has 2 bactericidal stretches having a mean antimicrobial value of 0.233 the first Antimicrobial stretch found.

Table 3: Results of the Bactibase sequence analyses of the bacteriocin gene that produces significant alignment with strain C4L10 enterocin genes

Strain	Enterocin gene	Produces significant alignment (Bactibase Database)	Score bits	E-Value	Identities %	Positive %	BB producing organism (Bactibase Database)	Gene	ORF	Leader peptides	Class
C4L10	C4P	Bacteriocin L-1077	15.8	7.5	83	83	*Lact. salivarius*	UI	+	+	IIa
	C4B	Sakacin G	18.5	1.8	46	50	*Lactobacillus sakei*	skgA2	+	+	IIa

In C4L10 occur between residues 4 to 18 with Propensity value of 0.219 (6 %), and the second Antimicrobial stretch occur between residues 25 to 39 with a Propensity value of 0.218 (5 %) (fig. 7). Only one antimicrobial stretch was found in C4L10_B and this occurs between residues 149 and 165.

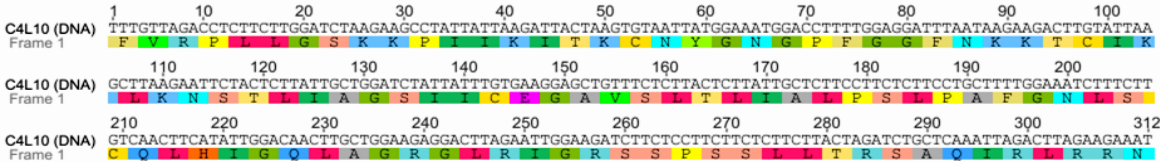

Fig. 1: Nucleotide sequence of the region encoding enterocin P of *Ent. mundtii* C4L10 and the deduced amino acid sequence

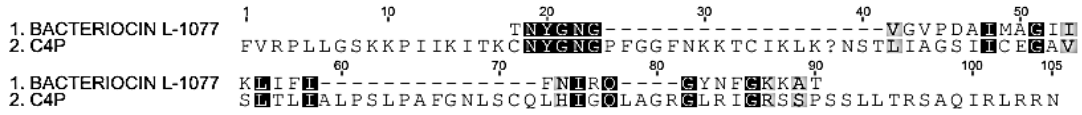

Fig. 2: Alignment of precursor peptides of enterocin P, and bacteriocins of the Bacteriocin L-1077 family. Identical residues in enterocin and Bacteriocin L-1077 are shown in bold

Fig. 3: Predicted secondary structure and cleavage site in Enterocin_P of strain C4L10 (Geneious 6.1.2)

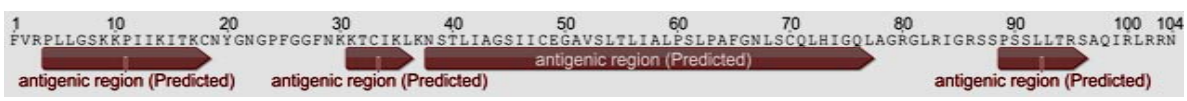

Fig. 4: Predicted antigenic region of strain C4L10 Enterocin_P (Geneious 6.1.2)

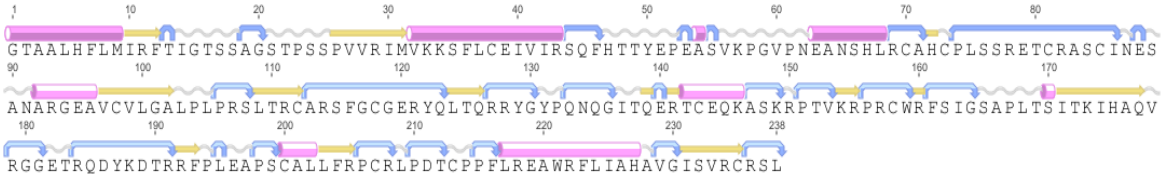

Fig. 5: Predicted secondary structure in Enterocin_B of strain C4L10 (Geneious 6.1.2)

Fig. 6: Predicted antigenic region and cleavage site in Enterocin_B of strain C4L10 (Geneious 6.1.2)

Fig. 7: Prediction of the antimicrobial proteins and antimicrobial regions in C4L10 enterocin_P amino acid sequence

From fig. 7 it could be seen that the YNYGNGP sequence is located between the first and second antimicrobial segments, while the double glycine (GG) is located within the second antimicrobial segments. The combination of these sequences can lead to the production of therapeutical agent to combat certain disease.

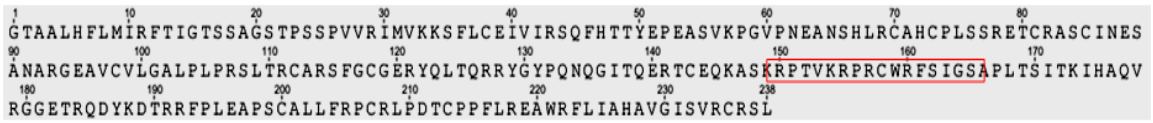

Fig. 8: Prediction of the antimicrobial proteins and antimicrobial regions in C4L10 enterocin_B amino acid sequence showing the location of the antimicrobial spot sequence (KRPTVKRPRCWRFSIGS) within the C4L10 Enterocin_B sequence

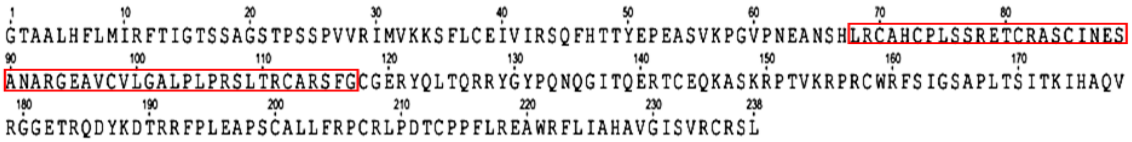

Fig. 9: Shows the conserved domain sequence of Glycosyltransferases in the C4L10 Enterocin_B in the red box

Conserved domain prediction

Protein domains are associated with particular aspects of molecular function such as catalysis or binding, moreover, they represent discrete units of three-dimensional (3D) structure. Detecting domains in protein sequences may provide the first clues as to their molecular and cellular function. The search was done using the online tool at http: //www. ncbi. nlm. nih. gov/Structure/cdd/wrpsb. cgi with an E-value threshold of 0.01 and Maximum number of hits: 500. The conserved domain search showed that the Enterocin_B contain the Glycosyltransferase sequences (LRCAHCLSSRETCRASCINESANARG EAVCLGALPLPRSL TRCARSFG) in its domain (fig. 9) which belongs to the superfamily cl10013. The glycosyl transferases are universal catalytic enzymes that help in glycosylations. It is also known to inhibit tumor cell proliferation through assembly of glycoconjugates found found ubiquitous in nature (fig. 9).

As shown on fig. 10, an example of 3-dimensional structure generated by homology modelling tool (I TASSER) [38].

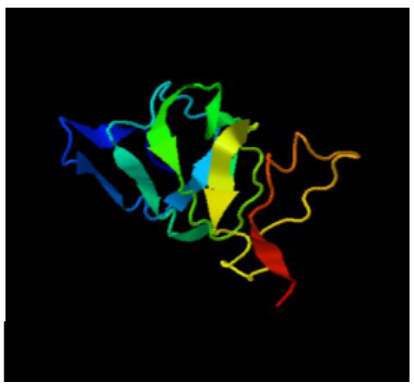

Fig. 10: Three dimensional structure of Enterocin_P of strain C4L10 generated by homology modeling I-TASSER Zhang, I. Y (2008) N terminal translocation domain (yellow color), central receptor (blue color) and C terminal catalytic domain (Red color).

Chromosomal Origin of Enterocin_P and Enterocin_B from Strain C4L10

In this work, we observed that Enterocin_P and Enterocin_B could only be detected from genomic DNA of C4L10 strain but not from its plasmid.. In a similar development, [13, 39] were not able to detect enterocin gene in the plasmid of *Enterococcus faecium* proving the presence of the gene responsible for bacteriocin activities to be embedded on the chromosome DNA. Furthermore, Moreno, Callewaert, Devreese, V Beeumen and de Vuyst [40] recorded the isolation of Enterocin_A, enterocin_B and enterocin_P genes from the genome of the already isolated strains of *Enterococcus faecium* and *Enterococcus faecalis*. In addition, Kang and Lee, Hsu, Mantovani and Russell [41] were able to isolate the bacteriocin gene of a similar nature to enterocin_P by PCR and direct sequencing from the total genomic DNA of *Ent. faecium* GM-1. On the contrary, Abriouel, et al., [42] found the structural genes for enterocin P (enterocin_P) through hybridization studies to be located on the plasmid. Still on the contrary, Achemchem [43] through their studies found that the structural genes for F-58 A and B were embedded in a 22-kb plasmid harbored by that strain through the amplification of PCR fragments.

CONCLUSION

Previously isolated *Ent. Muntdii* strain C4L10 from Malaysian non-broiler chicken was found to be class IIa bacteriocins producer. This bacteriocin was also able to elicit inhibitory activities towards human cancer cell lines. Two types of bacteriocin genes; namely Enterocin_P and Enterocin_P were detected from the chromosomes of C4L10 strain. Enterocin P contains double glycine and YGNGP motifs characteristic of class IIa bacteriocin, although the last position Val was replaced with Pro. Meanwhile, Enterocin_B contains part glycosyltransferase motifs, thought to plays roles in the synthesis of glycoconjugates of which could have indirectly involved in anti-proliferative activities we observed on human tumour cell lines. Several antimicrobial segments were detected in these bacteriocins. Further exploration on these elements in the bacteriocin sequences could open up more understanding on enterocin diversity and future exploitation of these peptides as a useful antimicrobial agent.

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CONFLICT OF INTERESTS

Declared None

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