Molecular Characterization of Some Antilisterial Bacteriocin Genes from *Enterococcus faecium* and *Pediococcus pentosaceus*

Nagwa I. El-Arabi¹, Rasha G. Salim², Nivien A. Abosereh², and Abdelhadi A. Abdelhadi¹*

1Department of Genetics, Faculty of Agriculture, Cairo University, Giza 12613, Egypt
2National Research Center (NRC), Dokki, Cairo, Egypt

Received: March 6, 2018 / Revised: July 12, 2018 / Accepted: July 13, 2018

Introduction

Bacteriocins are proteins or protein complexes with antibacterial activity against a number of other species related or not concerning to the bacteriocine producing bacterium. Their effect can be at the DNA level, synthesis of protein. They produced by some lactic acid bacteria (LAB) strains were contribute to control several pathogenic and spoilage microorganisms [1]. These protein complexes have benefit use due to their potential usefulness as natural food preservatives, in addition to promoting good health. Many LAB, including members of these genera *Lactococcus*, *Lactobacillus*, *Enterococcus* and *Pediococcus* are known to secrete bacteriocins [2].

Three types of bacteriocins operons are found in the LAB, one are located on the bacterial chromosome, another on are located on the bacteria plasmid and the other one was carried on transposon. Bacteriocins operons Class I and Class II are described as a small heat stable peptides. The mutacin II and mutacin III genes that produced by *Streptococcus mutans* are considered one of the bacterial chromosome antibiotic genes examples [3]. Many cases of Chromosomal location are found in the class II bacteriocin operons, such as enterocins A and B that produced by *Enterococcus faecium* [4, 5].

In previous study, Abdelhadi *et al.* [6] identified two bacteriocins bacterial strains; *Pediococcus pentosaceus* AH1 and *Enterococcus faecium* AH2. This work aimed to...
isolation, characterization and distribution of sequences similar to enterocin A and pediocin A structural genes among these strains; *Pediococcus pentosaceus* AH1 and *Enterococcus faecium* AH2. Moreover, this study aimed to evaluate of antimicrobial activity of crude bacteriocin on wild type strains and overexpressed *E. faecium* as a model.

**Material and Methods**

### LAB strains

Two LAB were used in the present investigation; *Pediococcus pentosaceus* AH1 (GenBank accession number LC063691) and *Enterococcus faecium* AH2 (GenBank accession number LC063692).

### Primer design

The primers for some genes responsible for bacteriocin production were designed according to data in Gene Bank Database by using Invitrogen primer design tools.

### DNA isolation

Genomic DNA isolation was carried out using Qiagen Kit (Qiagen Sciences, USA) according to the manufacturer’s instruction manual. Plasmid DNA was obtained either by the alkaline lysis method [7] and QIAprep spin miniprep cat. no 27106 (Qiagen Sciences, USA) according to the manufacturer’s instruction manual.

**PCR amplification**

DNA was extract from the two selected LAB. The genes primers and annealing temperature listed in (Table 1). The PCR mixture and conditions were as follows: 5X Go Taq flexi buffer 10 µl, Go Taq flexi DNA polymerase 0.5 µl, 8 µl MgCl₂ 25 mM, 1 µl primers, 1 µl dNTPs 0.2 mM, 2 µl DNA template, 28 µl dH₂O to complete the final volume to 50 µl. The PCR program was 95°C for 5 min, 35 cycles of (95°C for 1 min, annealing as described in Table 1 and 72°C for 2 min), and the final extension at 72°C for 10 min, −4°C.

### Isolation and partial purification of bacteriocins

Liquid LAB cultures grown in M17 or MRS media incubated for 24 h at optimal temperatures were used for bacteriocin isolation. Cell-free supernatants were obtained by centrifugation at 4,600 rpm for 15 min at 4°C, pH 7. Proteins in supernatant were precipitated with 60% (NH₄)₂SO₄ fractionation at 4°C for 24 h. Pellet containing precipitated proteins was obtained by centrifugation at 4,600 rpm for 20 min at 4°C, then resuspended

---

**Table 1. Primers and annealing temperatures used in isolation of some genes responsible for bacteriocin production from selected bacterial isolates.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’--3’)</th>
<th>Annealing temp. (°C)</th>
<th>Size of the amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>entA</strong></td>
<td>F 5’-CACAACTTATCTATGGGGGTACCACTC-3’&lt;br&gt;R 5’-CCCTGGGAATTGCTCACCCTAAAACCC-3’</td>
<td>60</td>
<td>198</td>
</tr>
<tr>
<td><strong>entl</strong></td>
<td>F 5’-ATGAAAAAATGCTAAGCAAATTG-3’&lt;br&gt;R 5’-TTAAAATTGAGATTATCGATAATCTG-3’</td>
<td>50</td>
<td>287</td>
</tr>
<tr>
<td><strong>entF</strong></td>
<td>F 5’-ATGAGTTATCCAAATATATATGTAAGAAT-3’&lt;br&gt;R 5’-CTAAAAACGTGTTTTCTTTCAACTTCTC-3’</td>
<td>53</td>
<td>124</td>
</tr>
<tr>
<td><strong>entR</strong></td>
<td>F 5’-ATGGAAGAAAAAATAAGCATAAAATGCTAA-3’&lt;br&gt;R 5’-TTAGTTACACTTTTTAAAAATAGAAACACA-3’</td>
<td>52</td>
<td>720</td>
</tr>
<tr>
<td><strong>orfA2</strong></td>
<td>F 5’-TTACGCACCGGTCCACAT-3’&lt;br&gt;R 5’-ATGGCATATTAAAAACACAAAACAA-3’</td>
<td>50</td>
<td>213</td>
</tr>
<tr>
<td><strong>orfA3</strong></td>
<td>F 5’-TTATATGTGATAATACATTCTTTCTTTTTTATTT-3’&lt;br&gt;R 5’-TTGATGGAAAATGTGGGCCA-3’</td>
<td>50</td>
<td>192</td>
</tr>
<tr>
<td><strong>PapA</strong></td>
<td>F 5’-ATGAAAAAATGTTGAATATTAGTAAAAAG-3’&lt;br&gt;R 5’-CTACGATTATATGATTATGGACATTTCC-3’</td>
<td>57</td>
<td>140</td>
</tr>
<tr>
<td><strong>PedB</strong></td>
<td>F 5’-ATGAATAAGACTAAGGCACCATATT-3’&lt;br&gt;R 5’-CTATTGGCAGCAGTATTG-3’</td>
<td>57</td>
<td>339</td>
</tr>
</tbody>
</table>

---

http://dx.doi.org/10.4014/mbl.1803.03001
in 10 ml phosphate buffer (pH 6.5). One volume protein mix: fifteen volume methanol-chloroform (1:2, v/v) were mixed and stirred at 4°C for 1 h, followed by centrifugation at 4,600 rpm, at 4°C for 20 min. Supernatant fraction was poured and the pelleted proteins fraction was air-dried. The final pellet was resuspended in 10 ml of ddH₂O stored at −20°C for used for assaying bacteriocins activity.

**Bacteriocin activity quantification**

Procedure-defined units of bacteriocin activities were measured as per Yamamoto et al. [8] using disc diffusion assay and critical dilution method (MIC determination). Briefly, crude bacteriocin aliquots were two-fold serially diluted either in M17 or MRS broth media. Samples of 50 µl crude bacteriocin was applied to 5 mm sensitivity discs placed on the agar medium. Bacteriocin activity was defined as the highest dilution reciprocal (2ⁿ) exhibiting clear inhibition zone of the indicator strains. Accordingly, the arbitrary units (AU) of bacteriocin activity per milliliter (AU ml⁻¹) were defined as 2ⁿ × 1,000/50. The lowest bacteriocin concentration(s) which inhibit visible growth of each indicator strain on agar plate was used to assess MIC.

**Evaluation of antimicrobial activity of bacteriocin on overexpressed E. faecium**

To evaluate the overexpression of entA gene, the PCR product of E. faecium AH2 entA gene was cloned into a linearized pGEMT easy vector (Promega, USA) and chemically transformed into XL1-Blue competent cells. Isolation of plasmid DNA from E. coli was performed using QIAprep® Spin Miniprep Kit (QIAGEN, USA). The DNA fragment was designed with two different restriction sites, SpeI and SphI to enable the cloning of entA gene into the multiple cloning site of pF1A T7 Flexi vector (Promega, USA). The constructed plasmid pF1A T7 Flexi-entA was designed to overexpress the E. faecium AH2 entA gene under T7 promoter according to Abdelaal et al. [9]. The ligation mixture was electroporated into E. faecium AH2 according to the method described previously [10]. Three types of E. faecium AH2 (transformed with pF1A T7 Flexi-entA vector, transformed with pF1A T7 Flexi and wild type) were used for determined the bacteriocine activity and inhibition zone as above.

**Results and Discussion**

**Bacteriocin genes localization**

Before genes amplification, plasmids were isolated from target strains to detect localization of bacteriocin genes on plasmid or in chromosome, both Qiagen plasmid mini prep and manual preparation were used for plasmid isolation. The result showed that there was no plasmid in the bacterial strain (data not shown). This result indicted the present of bacteriocin genes on bacteria chromosomal. This results were similar to Franz et al. [11] and Du Toit et al. [12]. Their result indicated that the bacteriocin genes are located on the chromosome DNA and no plasmid could be extract from Enterococcus faecium.

According to Moreno et al. [13], entA, entB and entP genes were located on Enterococcus faecium and E. faecalis strains genome. Bacteriocin gene was isolated by Kang and Lee [14] from E. faecium GM-1 chromosomal DNA with high similarity with enterocin P by using PCR and direct sequencing methods. The results of Abriouel et al. [15] were in contrast with our result, they found structural genes for enterocin P (entP) in enterococci isolated from food sources using plasmid hybridization. Also Achemchem et al. [16] isolated structural genes for F-58 A and B from a 22-kb plasmid using the polymerase chain reaction.
Primers design

Multi coding sequence of bacteriocin genes were used for primers design of the bacteriocin producing genes in *E. faecium* AH2 strain and *Pediococcus pentosaceus* AH1 and aligned for detecting conserved sequences of the bacteriocin gene. Two highly conserved sequences were selected for primers design, forward (F) and reverse (R) primers, to amplify the open reading frame.

Enzymatic amplification of genetic material using PCR technique was used in this study to amplify the corresponding sequence of the bacteriocin genes. Several trials of optimization of the temperature profile of the enzymatic amplification of DNA using PCR. The experiment gave the best possible amplification pattern. PCR amplifications of the bacteriocin genes were carried out using F and R primers to amplify the expected fragments of the genes.

Detection of *papA* and *pedB* genes

*PapA* and *pedB* genes were involved in pediocin A production from *Pediococcus pentosaceus* AH1 strain. F-PapA and R-PapA primers were used to amplify the coding sequence of *PapA* gene with expected size about ≈140 bp. The amplification of *pedB* gene was carried out.
using F- pedB and R- pedB primers to amplify the complete coding sequence with about ≈339 bp fragment size (Fig. 1). Many studies have been focused on isolation and genetic manipulations these genes in different strains suitable for industrial applications via genetic engineering [17, 18].

Many application have been found for pediocin as an antimicrobial peptide in biotechnology. The overproduction of pediocin is has been a long-standing challenge due to the complexity of the underlying pathway and the difficulty in genetic modification of lactic acid bacteria. Another described of pediocin from P. acidilactici H is Pediocin AcH. Other Pediococcus spp linked to pap ABCD operon including immunity function (papB), pediocin AcH structural gene (papA) and ABC transport proteins (pap C and pap D) that have a very significant role in processing and translocation of active pediocin AcH. The random mutagenesis was used by Miller et al. [19] to provide that the pediocin AcH four cysteine residues are essential for its activity, and they have an important function in secondary structure stabilization of this peptide. In other organisms, another genes are found associated with bacteriocin operon that encoded for his-kinase and C39-protease. These enzymes are involved in secretion and production of active bacteriocins by indirect way.

Nowadays many studies have been focused on the pediocin family which produced by LAB specially Pediococcus sp. It is another commercially available for food applications. Different strains of P. pentosaceus able to produce the pediocin, those strains are genetically identified, it’s have some similarity with other bacteriocins and mode of action. Pediococcus spp that have pediocin A activity could be useful in controlling Listeria and Staphylococcus contamination in fermented meats. Also this peptide may have potential as biopreservatives in a different types of food [20].

Detection of enterocin A production genes

The entAIFKRTD operon is responsible for encoded the enterocin A protein. The genesent A encode the enterocin A prepeptide, entI encode the immunity protein, and theentF encode the induction factor prepeptide. In this work, Six genes; entA, entI, entF, entR, orfA2
Fig. 4. The phylogenetic tree for the pedB gene.

Fig. 5. The phylogenetic tree for the entA gene for pre-peptide of enterocin A.
and orfA3, involved in enterocin A production were isolated from *Enterococcus faecium* AH2 strain. The coding sequence of these genes were amplified and synthesized about ≈198, 287, 124, 720, 213 and 192 bp fragment size (Fig. 2).

Preceding investigation reported that more than one bacteriocin encoding gene in the *Enterococcus* strains were isolated from fermented foods using the polymerase chain reaction [21, 22]. The presence of structural gene for enterocins has been studied [23]. Among 20 isolates identified by polymerase chain reaction, 65% of strains were identified as *Ent. Faecium* and 35% as *Ent. faecalis*. Class II a bacteriocin such as Enterocin A which produced by *Enterococcus faecium* and pediocin A which produced by *pediococcus spp*. have attracted more attentions so it has strong antilisterial activities which were grown under 4°C and couldn’t combat by traditional methods such as antibiotics. Enterocin A is a small peptied, heat-stable, with antimicrobial properties especially against *listeria sp*. The chromosome contain about 10,879-bp which have about twenty open reading frames (ORFs), seven of them may have an important role in the biosynthesis of enterocin [4].

The enterocin genes occurrence was studied [24], the factor of virulence, and antibiotic resistance in the bacteriocin-producer *Enterococcus faecium* EYT17, EYT31, and EYT39 strains. PCR studies showed that the enterocin A and B structural genes (*entA* and *entB*) was found in all *E. faecium* strains, while the enterocin P structural gene (*entP*) was carried in *E. faecium* EYT17 and EYT31 strains. This study indicated that the multiple enterocin-producer *E. faecium* EYT17, EYT31, and EYT39 strains are safe and these strains may be used for food preservation.

**Sequence analysis using bioinformatics tools**

*PapA* and *pedB* genes were sequenced and then the sequence analysis was performed by blasting with available sequences in the national center for biotechnology information (NCBI). The nucleotide and amino acid

![Fig. 6. The phylogenetic tree for the entI gene.](image-url)
Fig. 7. The phylogenetic tree for the entF gene.

Fig. 8. The phylogenetic tree for the entR gene.
Fig. 9. The phylogenetic tree for the orfA2 gene.

Fig. 10. The phylogenetic tree for the orfA3 gene.
sequences of **PapA** and **pedB** genes using the Expasy software and the genetic distance of these genes according to blast results were done (Figs. 3 and 4).

The isolated genes, **entA**, **entI**, **entF**, **entR**, **orfA2** and **orfA3**, were sequenced blasted with available sequences in NCBI. The genetic distance of these genes according to blast results were shown in (Figs. 5-10). The nucleotide protein sequences of genes using Expasy software were carried out.

**Gene Bank database**

All genes sequences were deposited in the gene Bank under accession numbers. The gene names, accession numbers, molecular weight of amplified fragment, protein names, number of amino acids, and molecular weight of proteins were listed in Table 2.

**Antimicrobial activity of crude bacteriocin**

Antimicrobial activity of the crude bacteriocin fraction aliquots against ten bacterial isolates were tested (Fig. 11). Bacteriocins showed variable degree of activity on **E. faecium** AH2 and **P. pentosaceus** AH1 strains. The **P. pentosaceus** AH1 strain displayed the highest activity of 2610 AU ml⁻¹ with large and evident inhibition zone of 26 mm. Whereas, the **E. faecium** AH2 exhibited the lowest bacteriocin activity (690 AU ml⁻¹) with inhibition zone of 19 mm.

Bacteriocins synthesized by lactic acid bacteria are efficient growth inhibitors of Gram-positive bacteria over Gram-negative bacteria since the outer membranes of the latter are barrier for bacteriocins to breach into inner cell membranes [25]. The obtained results are in agreement with Trias et al. [26], who showed that most

---

**Table 2. Gene names, protein names, molecular weights and GenBank accession numbers.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Source</th>
<th>Molecular weight of amplified fragment (bp)</th>
<th>coding sequence (CDS)</th>
<th>GenBank Accession number</th>
<th>Protein</th>
<th>Number of amino acids</th>
<th>Molecular weight of protein (Da)</th>
<th>Theoretical pl (isoelectric point)</th>
<th>High amino acid composition</th>
<th>GenBank Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>entA</strong></td>
<td><strong>Enterococcus faecium AH2</strong></td>
<td>198</td>
<td>complete ccds</td>
<td>LC064146</td>
<td>pre-peptide of enterocin A</td>
<td>65</td>
<td>6887.10</td>
<td>9.36</td>
<td>Gly (16.9%)</td>
<td>BAR94721</td>
</tr>
<tr>
<td><strong>entI</strong></td>
<td><strong>Enterococcus faecium AH2</strong></td>
<td>287</td>
<td>partial ccds</td>
<td>LC064147</td>
<td>immunity protein for enterocin A, partial</td>
<td>94</td>
<td>11177.58</td>
<td>5.37</td>
<td>Leu (12.8%)</td>
<td>BAR94722</td>
</tr>
<tr>
<td><strong>entF</strong></td>
<td><strong>Enterococcus faecium AH2</strong></td>
<td>124</td>
<td>partial ccds</td>
<td>LC064148</td>
<td>pre-peptide of inducing peptide, partial</td>
<td>40</td>
<td>4309.08</td>
<td>9.26</td>
<td>Lys (17.5%)</td>
<td>BAR94723</td>
</tr>
<tr>
<td><strong>entR</strong></td>
<td><strong>Enterococcus faecium AH2</strong></td>
<td>720</td>
<td>partial ccds</td>
<td>LC064149</td>
<td>response regulator homologue, partial</td>
<td>239</td>
<td>28211.66</td>
<td>8.36</td>
<td>Leu (10.5%)</td>
<td>BAR94724</td>
</tr>
<tr>
<td><strong>orfA2</strong></td>
<td><strong>Enterococcus faecium AH2</strong></td>
<td>213</td>
<td>complete ccds</td>
<td>LC064150</td>
<td>hypothetical protein</td>
<td>70</td>
<td>7376.43</td>
<td>8.10</td>
<td>Gly (17.1%)</td>
<td>BAR94725</td>
</tr>
<tr>
<td><strong>orfA3</strong></td>
<td><strong>Enterococcus faecium AH2</strong></td>
<td>192</td>
<td>complete ccds</td>
<td>LC064151</td>
<td>hypothetical protein</td>
<td>63</td>
<td>7791.18</td>
<td>10.23</td>
<td>Arg (15.9%)</td>
<td>BAR94726</td>
</tr>
<tr>
<td><strong>PapA</strong></td>
<td><strong>Pediococcus pentosaceus AH1</strong></td>
<td>140</td>
<td>partial ccds</td>
<td>LC101300</td>
<td>bacteriocin-II, partial</td>
<td>46</td>
<td>4823.53</td>
<td>8.58</td>
<td>Gly (19.6%)</td>
<td>BAT70754</td>
</tr>
<tr>
<td><strong>PedB</strong></td>
<td><strong>Pediococcus pentosaceus AH1</strong></td>
<td>339</td>
<td>complete ccds</td>
<td>LC101789</td>
<td>immunity protein</td>
<td>112</td>
<td>12993.71</td>
<td>6.97</td>
<td>Leu (10.7%)</td>
<td>BAT72570</td>
</tr>
</tbody>
</table>

---

http://dx.doi.org/10.4014/mbl.1803.03001

**Fig. 11. The bacteriocin activity of Enterococcus faecium AH2 and Pediococcus pentosaceus AH1 strains.** *mean of Inhibition zone diameter ± standard error.*
Antilisterial Bacteriocins from Lactic Acid Bacteria

September 2018 | Vol. 46 | No. 3

LAB originating from vegetables and fruits exhibited rational activity against foodborne pathogens, such as *E. coli*, *Listeria monocytogenes* and *Salmonella typhimurium*. Also, Malik et al. [27] reported that some LAB that yield bacteriocins play important role as antibacterial agents and able to inhibit human pathogenic bacteria such as *E. coli* and *Salmonella* sp.

The overexpressed *E. faecium* AH2 with pF1A T7 Flexi-entA vector showed the highest bacteriocin activity (1260 AU ml⁻¹) that represent about 1.6 fold with inhibition zone of 24 mm than both *E. faecium* AH2 with pF1A T7 Flexi vector, and the wild type (Fig. 12). The results obtained by Borrero et al. [28] suggest that the production, secretion, and antimicrobial activity of the EntA produced by the recombinant LAB strains depend on the signal peptide, the expression vector, and the host strain. Previous studies have shown the heterologous production of EntA by cloning and expression of the entAITD cassette under control of a constitutive promoter in *L. lactis* [29]. The heterologous expression of EntA fused to a cellulose-binding domain in *E. coli* resulted in a fused protein with antilisterial activity [30].

We concluded that bacteriocins are bioactive peptides produced by LAB, which prevents food spoilage and pathogens. The isolation and characterization of lactic acid bacteria (LAB) that having the ability to produce bacteriocin were the major tasks of many studies.

Wherefore, the identification and characterization of the genes responsible for bacteriocin production and activity had the great importance.

**Acknowledgements**

We wish to thank Prof. Dr. Naglaa A. Abdallah for offering lab facilities and her suggestions to the experimental design.

**Conflict of Interest**

The authors have no financial conflicts of interest to declare.

**References**


**Fig. 12. Comparison between antimicrobial activity of overexpressed *Enterococcus faecium* AH2 and wild type strains.**

*mean of Inhibition zone diameter ± standard error.*


http://dx.doi.org/10.4014/mbl.1803.03001