Short-Communication

Cloning of pediocin PA-1 and its immunity genes from *Pediococcus acidilactici* K7 using pAMJ shuttle vector into *Lactococcus lactis* MG1363

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Running title: ELECTROPORATION OF *LACTOCOCCUS LACTIS*

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The matured pediocin PA-1 encoding gene, *ped*A and its immunity counterpart, *ped*B from *Pediococcus acidilactici* K7 were cloned by PCR technique and ligated into the *E. coli*-lactic shuttle, protein expression vector pAMJ2008. The recombinant pAMJ*AB* was constructed and sequenced to confirm the intactness of reading frame of the deduced pediocin PA-1 fusion protein. *Lactococcus lactis* MG1363 was electroporated with the plasmids and the transformation efficiency of ~ 2-3 X 10^3 μg^{-1} was observed. This was further confirmed by plasmid DNA isolation and analysis.

Heterologous expression of proteins in a food-grade organism such as lactic acid bacteria (LAB) is found to have immense commercial applications. Food-grade systems not only offer high quantity but also an economic production of the target protein[1,2]. LAB, due to their GRAS status are being employed of late for the heterologous expression of various enzymes, hormones, bioactive compounds, antigens and membrane proteins. The large number of host-vector systems that have been developed are found to be valuable tools due to their food-grade controlled expression of foreign genes[3]. Among them the P170[4], nisin inducible[5] and the sugar inducible controlled expression systems are known to be commercially available[6].

P170 expression system constitutes an important module of gene expression in *Lactococcus lactis* due to several reasons. Host bacterium *L. lactis* requires simple fermentation technology where in the amount of native protein produced in the culture filtrate (CF) is low. Besides, P170 offers other advantages such as limited proteolytic activity, N-terminal signal peptide for secretion and a promoter regulated by pH and growth phase and elimination of the need for addition of an exogenous inducer[1].

Pediocin PA-1 is 4.6 kDa cationic membrane acting protein produced by *Pediococcus acidilactici*, that is being used for biopreservation and pharmaceutical applications[7]. However, the production in native condition is limited. In the recent past, a large number of LAB-based

heterologous systems for pediocin PA-1 expression have been developed[8]. However, majority of them suffer due to low production levels or poor secretion apparatus of the host bacterium[9]. Expression of pediocin PA-1 in *E. coli* made some progress, however, due to the disulfide nature of the protein, the over-expressed protein gets accumulated in inclusion bodies (IBs) of *E. coli* that needs additional steps of isolation, purification and refolding[10]. The prime objective of this study was to clone the pediocin PA-1 gene into the *L. lactis* expression system. The recombinant was electroporated into the host *L. lactis* MG1363 for expression.

A laboratory isolate of *Ped. acidilactici* K7 producing pediocin PA-1 was used as a source for the pediocin PA-1 biosynthesis genes[11]. This culture was grown in de Man, Rogosa and Sharp (MRS) broth (Himedia, Mumbai) at 37^0C in a static condition. The total DNA of *Ped. acidilactici* K7 was isolated by the method of Mora et al.[12] and used as a template for PCR. The oligonucleotide primers (Sigma Aldrich) used for PCR cloning of *ped*AB genes were LlpF: 5' CCAATATCATTGG**AGATCT**ATACTACG 3' as forward and BexR: 5'CAATATC**CTGC AG**ATCAGTACTATTGG 3' as reverse primers. The restriction sites for *Bgl*II and *Pst*I, marked in bold were introduced in the forward and reverse primers, respectively. PCR was carried out in a Perkin Elmer (USA) thermocycler using Deep Vent DNA polymerase (MBI, Fermentas, Luthiana), following standard procedure described by Sambrook and Russell[13]. The PCR product obtained (~528 bp) was double digested with *Bgl*II and *Pst*I, purified by using Qiagen column and ligated to the double digested vector pAMJ2008 using T4 DNA ligase (MBI) to obtain the recombinant pAMJ*AB*. Transformants were obtained using *E. coli* DH5α and analyzed by plasmid DNA isolation (alkaline-lysis method) and restriction digestion. Partial sequencing of the recombinant plasmid was carried out using the primer P170 fwd 5'CTGCCTCCTCTCCCTA GTGC 3' at the University of Delhi, South Campus, New Delhi. For the construction of recombinant molecule and fusion protein translation, Clone Manager Ver. 4.0 was used.

The vector pAMJ2008 and the recombinant pAMJ*AB* were electroporated into the competent cells of *Lactococcus lactis* MG1363 as per the protocol described in the P170 expression system[14] manual (Biotechnologie Institute, Denmark) using the Electroporation equipment of Biorad (Gene pulser X cell ^{TM}). The conditions set for electroporation pulse were capacitance, 25 μF; voltage, 2 KV and resistance 200 Ω using a 0.2 cm cuvette. The transformed colonies of MG1363 were selected based on their growth on M_{17} agar medium (Hi Media) containing 0.5% glucose and erythromycin (Hi Media), 1 μg/ml. Plasmids from *L. lactis* MG1363 were isolated by the method of O'Sullivan and Klaenhammer[15] followed by gel analysis.

In order to express matured pediocin PA-1 (without signal peptide) and its immunity counterpart, *ped*AB genes were cloned by PCR and ligated to the shuttle vector pAMJ2008 into its *Bgl*II/*Pst*I sites. The deduced translated fusion protein obtained upon DNA sequencing is shown in Fig. 1. The result shows the intactness of the reading frame with the DAQA amino acids at the N-terminal of the target protein that act as a recognition site for cleavage by signal peptidase. The translated fusion protein has the following features. The expected size of PedA fusion protein should be 8.5 kDa and the matured pediocin PA-1 of 5 kDa is expected to be secreted into the culture filtrate.

The partial DNA sequence of *ped*B gene was also obtained that had been truncated at 3'end due to incomplete sequencing. Sequencing results in combination with BLAST analysis further confirm cent percent homology of *ped*AB genes with that reported in literature, Acc nos. M83924[16] and our previously reported sequences[11]. In this study, *ped*B gene was cloned to co-express in combination with *ped*A gene since *L. lactis* MG1363 is sensitive to the pediocin PA-1

(data not shown). In heterologous expression of pediocin in LAB, co-expression of immunity protein is required since majority of the lactic hosts are sensitive to the pediocin PA-1[17].

The *E. coli* shuttle vector pAMJ2008 and the recombinant were electroporated into the host *L. lactis* MG1363 and the efficiency of transformation was calculated based on colonies obtained in M17 agar plate containing erythromycin as a selection marker. The results indicated high efficiency transformation. The transformation efficiency was 2 X 10^3 recombinant μg^{-1} of DNA when compared to the vector (3 X 10^3 transformants μg^{-1}). The field strength and time constant for electroporation was also within the range of expected results (data not shown). Transformation results were further confirmed by plasmid DNA isolation and analysis (Fig.2). The two independent clones were subjected to gel analysis and were compared with the plasmid isolated from *E. coli*.

Results described in this study show the successful construction of the recombinant plasmid pAMJ*AB* molecule and upon its restriction analysis and sequence determination, electroporation into *L. lactis* MG1363. Since *L. lactis* is one of the GRAS status bacteria, approaches towards the production of pharmaceutical compounds and bioactive peptides are attractive. Further, P170 expression system is an environmentally regulated gene expression system being used recently for the production of several antigens and bioactive peptides. In this study, we made an attempt to clone pediocin PA-1 gene for its hyper-expression. For bacteriocins that are being produced at very low amount by the native microorganism and require complex cultivation medium for the growth, recombinant DNA approach is being suggested. Since pediocin PA-1 is an anti-listerial low molecular weight peptide to be used for food conservation and pharmaceutical purposes, heterologous production using P170 system is an attractive concept. Our studies on optimization of the conditions for expression of biologically active peptide is underway.

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References

1. Bredmose L, Madsen S M, Vrang A, Ravn P, Johnsen M G, Glenting J, Arnau J & Israelsen H, Development of a heterologous gene expression system for use in *Lactococcus lactis, Rec Prot Prod Prok Eukar, Cell* (2001) 269-272.

2. Nga B N, Genome analysis of lactic acid bacteria in food fermentations and biotechnological applications, *Curr Opin Microbiol*, 8 (2005) 307-312.

3. Nouaille S, Ribeiro L A, Miyoshi A, Pontes D, Le Loir Y, Oliveira C, Langella P & Azevedo V, Heterologous protein production and delivery systems for *Lactococcus lactis*, *Genet Mol Res*, 2 (2003) 102-111.

4. Ravn, P., Arnau, J., Madsen, S. M., Vrang, A. & Israelsen, H. Optimization of signal peptide SP310 for heterologous protein production in *Lactococcus lactis, Microbiol*, 149 (2003) 2193-2201.

5. Zhou X X, Li W. F, Ma G X & Pan Y J, The nisin-controlled gene expression system: construction, application and improvements, *Biotechnol Adv*, 24 (2006) 285-295.

6. de Vos W M, Gene expression systems for lactic acid bacteria, *Curr Opin Microbiol,* 2 (1999) 289-295.

7. Rodriguez J M, Martinez M I, & Kok J, Pediocin PA-1, a wide spectrum bacteriocin from lactic acid bacteria, *Crit Rev Food Sci Nutrition*, 42 (2002) 91- 121.

8. Rodriguez J M, Martinez M I, Horn N & Dodd H M, Heterologous production of bacteriocins by lactic acid bacteria, *Int J Food microbiol,* 80 (2003) 101-116.

9. Moon G-S, Pyun, Y-R, Park, M S, Ji G. E. & Kim, W J, Secretion and recombinant pediocin PA-1 by *Bifidobacterium longum*, using the signal sequence for Bifidobacterial α-amylase, *Appl Environ Microbiol*, 71 (2005) 5630-5632.

10. Halami P M & Chandrashekar A, Heterologous expression, purification and refolding of an anti-listerial peptide produced by *Pediococcus acidilactici* K7, *Electr J Biotechnol,* 10 (2007) 1-7.

11. Halami P M, Ramesh A, Chandrashekar A, Fermenting cucumber, a potential source for the isolation of pediocin-like bacteriocin producers, *World J Microbiol Biotechnol*. 21 (2005) 1351-1358.

12. Mora, D, Fortina, M. G. Parini, C., Ricci, G., Daffonchio, D.,. Manachini, P. L., Genomic subpopulations within the species *Pediococcus acidilactici* detected by multilocus typing analysis: relationship between pediocin AcH/PA-1 producing and non-producing strains. *Microbiol,* 146 (2000) 2027-2038.

13. Sambrook, J. Russel, D.W. Molecular cloning: A laboratory manual, 3rd edition Cold spring Harbor Laboratory, Cold Spring Harbor, NY. (2001).

14. P170 expression system manual. Heterologous protein production using *Lactococcus lactis* Version 18-Sept-03.

15. O'Sullivan, D J, Klaenhommer, J R, High and low copy number *Lactococcus* shuttle cloning vectors with features for clone screening. *Gene,* 137 (1993) 227-231.

16. Marugg JD, Gonzalez CF, Kunka BS, Ledeboer AM, Pucci MJ, Toonen MY, Walker SA, Zoetmulder LC, & Vandenbergh PA. Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, and bacteriocin from *Pediococcus acidilactici* PAC1.0. *Appl Environ Microbiol*, 58 (1992) 82360-82367.

17. Axelsson L, Katla T, Bjornslett M, Eijsink V G H & Holck A, A system for Heterologous expression of bacteriocins in *Lactobacillus sake, FEMS Microbiol Lett*, 168 (1998) 137-143.

List of Figures

Fig. 1 Construction of recombinant plasmid pAMJAB. a, Restriction digestion and analysis of the pAMJAB plasmid. Recombinant pAMJAB digested with *Hin*d III (lane 1) and double digested with *Bgl*II/ *Pst*I enzymes (lane 2). M is 100bp ladder (MBI Fermentas). Arrow indicates the insert release of expected size. b, molecular map of recombinant & diagrammatic representation of pediocin fusion protein. Arrow indicates position of proteolytic cleavage site. c, deduced amino acid sequence and fusion protein construct of SP310mut-*ped*AB obtained upon nucleotide sequencing.

Fig. 2 Agarose gel electrophoretic analysis of the plasmid isolated from *L. lactis* MG1363. R1 and R2, two independent pAMJAB recombinants; V, vector; H, DNA isolated from host MG1363, as a control and Er, pAMJAB isolated from *E. coli* DH5α by alkaline lysis. M is a λ *Bst*II digest (MBI Fermentas). Arrow indicates the expected size of the plasmid observed.

Fig. 1a

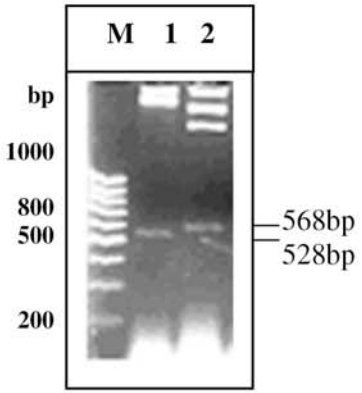

Fig. 1b

Fig. 1c

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1   gattagggta actttgaaag gatattcctc atgaaattta ataaaaaaag agttgcaata gccacgttta ttgctttgat atttgtaagt
        D  -  G    N  F  E    R  I  F  L    M  K  F    N  K  K    R  V  A  I    A  T  F    I  A  L    I  F  V  S
91  ttttttacaa tatcatcaat ccaagatgct caagcaggaa gagcagatct atactacggt aatggggtta cttgtggcaa acattcctgc
        F  F  T    I  S  S    I  Q  D  A    Q  A  G    R  A  D    L  Y  Y  G    N  G  V    T  C  G    K  H  S  C
181 tctgttgact ggggtaaggc taccacttgc ataatcaata atggagctat ggcatgggct actggtggac atcaaggtaa tcataaatgc
        S  V  D    W  G  K    A  T  T  C    I  I  N    N  G  A    M  A  W  A    T  G  G    H  Q  G    N  H  K  C
271 tagcattatg ctgagctggc atcaataaag gggtgatttt atgaataaga ctaagtcgga acatattaaa caacaagctt tggacttatt
        -  H  Y    A  E  L    A  S  I  K    G  -  F    Y  E  -    D  -  V  G    T  Y  -    T  T  S    F  G  L  I
IInd    A  S  I  M    L  S  W    H  Q  -    R  G  D  F    M  N  K    T  K  S    E  H  I  K    Q  Q  A    L  D  L


361 tactaggcta cagtttttac tacagaagca cgatactatc gaaccttacc agtacgtttt agatattctg gagactggta tcagtaaaac
        F  T  R  L    Q  F  L    L  Q  K    H  D  T  I    E  P  Y    Q  Y  V    L  D  I  L    E  T  G    I  S  K
451 taaacataac cagcaaacgc ctgaacgaca agctcgtgta gtctacaaca agattgccag ccaagcgtta gtagataagt tacattttac
    T  K  H  N    Q  Q  T    P  E  R    Q  A  R  V    V  Y  N    K  I  A    S  Q  A  L    V  D  K    L  H  F
541 tgccgaagaa acaaagttct  -
    T  A  E  E    T  K  F    -
```

Fig. 2