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Cloning and Expression of Listeriolysin O in *Lactobacillus plantarum*

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Abstract

Background: The protein listeriolysin O (LLO) encoded by *hly* gene, is one of the most important virulence factors of *Listeria monocytogenes*, responsible for phagosomal membrane disruption and bacterial escape to the cytoplasm, stimulation of CD8+ T cells and Th1 response. Recently pathobiotechnological vaccination using probiotic bacteria have been proposed. One of these strategies is expression of LLO in non-pathogenic bacteria such as lactic acid bacteria as delivery strains.

Objective: In the current study, we aimed to clone hly gene in a *Lactobacillus* species via pNZ8110, an inducible expression vector which is specific for *Lactococcus* species.

Materials and Methods: *Hly* gene was amplified by polymerase chain reaction (PCR) and inserted into pNZ8110 by restriction enzymes cutting and ligation method. After transformation and propagation in Escherichia coli MC1061 intermediate host, it was successfully electrotransformed into *Lactobacillus plantarum*.

Results: Gel electrophoresis of colony PCR, extracted plasmids and restriction analysis along with sequencing confirmed the transformation. After induction with supernatant of nisin producer, strain *Lactococcus lactis* NZ9700, expression of LLO was confirmed by SDS-PAGE and western blot.

Conclusion: Here, we employed a nonpathogenic probiotic strain, *L. plantarum* for the first time to express *hly* gene of *L. monocytogenes* in order to propose a new vaccine candidate.

Objective: T

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Background

Lactobacilli are attractive candidates for use in recombinant vaccine technology besides their consumption in fermented food.[1] A number of these gram-positive bacteria normally live in gastrointestinal or urogenital tracts as microflora.[1,2] These bacteria are not only generally nonpathogenic and safe but also have some benefits especially in promoting the health; so they are called probiotics.[1]

It has been recently shown that some lactobacilli have also immunomodulatory properties such as induction of inflammatory response against infection, suppression of pro-inflammatory cytokines, stimulation of IgA production, activation of monocyte lineages, regulating balance of Th1 and Th2 pathways and adjuvanticity.[2-7]

Based on the evidence about the characteristics of lactic acid bacteria, they have been recently proposed as live vaccine vectors carrying heterologous genes.[1,6] Although their safe and nonpathogenic status is an advantage, since there is no need to construct attenuated mutants, due to their non-invasive nature, they may be less effective compared to invasive bacteria in accordance with vaccine delivery to antigen-presenting cells.[8]

The idea for delivery of antigens to the cytoplasm of cells for presentation to MHC class I is promising in vaccine technology. Enhancing the Th1 response and influencing the cell-mediated immunity is considered to be a consequence of this approach .[9]

Furthermore, intracellular survival of *Listeria monocytogenes* is known to be greatly due to the expression of listeriolysin. This protein mediates the escape of *Listeria* from phagosome and entry to the cytoplasm, where its replication is occured.[10,11]

There are several studies in which cloning and expression of *L. monocytogenes* listeriolysin O (LLO) alone or as co-expressed with some other antigens, in other bacteria as

live vaccine vectors have been investigated.[9,12-15]

The objective of the present work was to study the cloning and expression of LLO in *Lactobacillus* using nisin-controlled gene expression system (NICE) expression vector, pNZ8110. This approach to use *Lactobacillus* strain for the expression of LLO may offer it as a vector for developing vaccines inducing cell-mediated immune response for cancer therapy or against viral or intracellular pathogens including *Listeria* and/or other microorganisms.

Materials and Methods

Bacterial Strains and Plasmid

Lactobacillus strains were obtained from Shiraz Faculty of Agricultural Science. *Escherichia coli* intermediate host strain and nisin inducible expression vector (pNZ8110) were provided by NIZO Food Research and their stocks were preserved in the School of Veterinary Medicine, Shiraz University. Bacterial strains and plasmid are shown in Table 1.

Preparation of Gene-Vector

To construct the expression vector, we designed primers to amplify the full length of hly gene lacking the signal sequence from *L. monocytogenes*.

According to *L. monocytogenes* EGD-e *hly* sequence (NCBI Reference Sequence: NC_003210.1) and restriction enzyme cutting sites of expression vector pNZ8110, after checking for correct enzymes using NEBcutter program, forward primer with Nae1 cutting site and reverse primer with Kpn1 cutting site were designed (Table 2).

DNA of *L. monocytogenes* was extracted using DNP kit (Cinagen, Iran) according to the manufacturer protocol. Polymerase chain reaction (PCR) mixture consisted of 1X final concentration of PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs mix, 0.5 µM of each primer (Lonae and Lokpn), 1.5 U Taq DNA polymerase and 2 µL of extracted DNA of *L. monocytogenes* and distilled water to final volume of 25 µL.

PCR was carried out in the gradient Eppendorf thermocycler (Germany) under the following conditions: 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 30 minutes, and extension at 72°C for 2 minutes. The cycles were preceded by 95°C for 5 minutes and followed by 72°C for 5 minutes.[16] PCR product was electrophoresed on 1% agarose gel. The purified PCR product and the vector pNZ8110 were double digested with Nae1 and Kpn1 restriction enzymes (Jena Bioscience, GmbH, Germany). The fragments were connected to each other by T4 DNA ligase (Vivantis, South Korea).

Table 1. Bacterial Strains and Plasmid

Bacterial Strain and Plasmid	Description
E. coli MC1061	Intermediate host, plasmid free
Listeria monocytogenes EGD-e serovar 1/2a	Wild type *Listeria monocytogenes*
Lactococcus lactis NZ9700	Nisin producer strain
Lactobacillus plantarum	ATCC 14917
Lactobacillus reuteri	DSMZ0010
Lactobacillus acidophilus	ATCC 4336
Lactobacillus gasseri	ATCC 33323
Lactobacillus casei	ATCC 334
pNZ8110	*E. coli-Lactococcus lactis* shuttle vector containing PnisA promoter with downstream secretion signal of Usp45 protein

Cloning of pNZ8110+*hly*

Escherichia coli MC1061 was grown in LB broth (Himedia, India) at 37°C overnight. A subculture was done to prepare competent cells by modified Hanahan procedure usingpre-cooled CCMB80 buffer (80 mM $CaCl_2$, 20 mM $MnCl_2$, 10 mM $MgCl_2$, 10 mM KOAc pH 7.0, 10% glycerol, pH= 6.4).[17] The ligation mixture was used to transform competent *E. coli* MC106 according to Sambrook and Russel.[16] Transformed colonies on LB agar containing 20 µg/mL chloramphenicol (Sigma Aldrich, USA) were investigated by colony PCR using primers vec-f (forward) and vec-r (reverse), respectively (Table 2). These primers were designed according to 22 bp sequences in the plasmid pNZ8110 flanking the gene insert site which were selected by Primer3 software. PCR mixture and program was performed as mentioned in the previous section except for $MgCl_2$ concentration which was 1.5 mM. Plasmid extraction from transformant was performed by mini plasmid extraction kit (Bioneer, South Korea), and confirmed by enzyme restriction analysis and sequencing (Bioneer, South Korea). The sequencing of both extracted plasmid and PCR amplicon by 2 forward and reverse vec primers were done by Bioneer Company in the South Korea.

Electrotransformation of *Lactobacillus*

Lactobacillus casei, Lactobacillus reuteri, Lactobacillus gasseri, Lactobacillus acidophilus and *Lactobacillus*

Table 2. Oligonucleotide Primers[a]

Primer	Sequence 5′-3′
Lonae (forward)	GGGGCGCCGGCGATGCATCTGCATTCAATAAAG
Lokpn (reverse)	CGGGGTACCTTATTCGATTGGATTATCTAC
vec-F	GCATAATAAACGGCTCTGATTA
vec-R	AACTGCTGCTTTTTGGCTATCA

[a] Enzyme restriction sites are underlined.

plantarum were cultured in De Man, Rogosa and Sharpe (MRS) medium (Himedia, India) and prepared for electrotransformation as recommended by Alegre et al.[18] Briefly, the fresh subcultured cells were harvested at OD 600 of 0.5, washed twice with $MgCl_2$ 10 mM, followed by washing once with 0.5 M sucrose and 10% glycerol. The pellets were then resuspended in 0.5 M sucrose and 10% glycerol buffer and kept on ice. Electroporation was done using Bio-Rad gene pulser apparatus (BioRad, USA), by addition of pNZ8110+hly plasmid DNA to 100 µL of electrocompetent cells in an electroporation cuvette (0.2 cm gap, Bio-Rad, USA) at a pulse of 7000 to 13 000 V, 200 Ω and 25 µF. The electroporated cells were resuspended in MRS broth plus 80 mM $MgCl_2$, followed by incubation at 37°C and plated onto the MRS agar supplemented with 7.5 µg/mL chloramphenicol. Plates were incubated in microaerophilic condition at 37°C for 48 hours. Obtained colonies were investigated for the presence of recombinant plasmid by colony PCR and subsequent sequencing of the amplicon and the extracted plasmid.

Expression of Listeriolysin O

The recombinant *L. plantarum* was cultured in MRS broth containing 7.5 µg/mL chloramphenicol. Induction was according to modification of methods published by Bahey-El-Din et al and Pavan et al.[13,19] Overnight culture was inoculated 1/50 (v/v) in the fresh MRS medium. After 2 hours of incubation at 37°C, the medium was complemented with 2% v/v culture supernatant of nisin producer strain *Lactococcus lactis* NZ9700 in M17 medium, for nisin induction and was allowed to grow for a further 5 hours under incubation.

Culture supernatants of recombinant *L. plantarum* were collected using cold trichloroacetic acid (TCA) precipitation (15% w/v final concentration) as previously described.[4,12,20] After 40 minutes incubation on ice, the pellets were collected by centrifugation at 14 000×g for 10 minutes at 4°C, then resuspended in cold acetone followed by centrifugation and evaporation of excess acetone.

The dried pellets were then resuspended in 1x alkaline sample buffer (50 mM Tris pH = 8, 2% SDS, 100 mM DTT and 10% glycerol), boiled for 5 minutes before being subjected to SDS-PAGE.[12,20]

SDS-PAGE and Western Blot

For SDS-PAGE, 12% polyacrylamide separating gel and 4% stacking gel were used. The staining of polyacrylamide gels was performed using Coomassie Blue followed by de-staining with 10% acetic acid.

For western blotting, SDS PAGE gels were blotted onto nitrocellulose membranes (Sigma, USA) and the membrane was blocked overnight at 4°C in 1% BSA. Primary rabbit polyclonal anti-listeriolysin O antibody (Abcam, USA) and secondary anti rabbit antibody conjugated with HRP (Sigma, USA) were used at 1/1000 and 1/4000 dilutions in PBS buffer, respectively. Western blot detection was performed using H2O2 as substrate and DAB as chromogen. After visualization of bands (about 4 to 5 minutes), the reaction was stopped by washing the nitrocellulose membrane with distilled water for several times.

Results

Gel electrophoresis of PCR product for amplifying the hly gene showed a band of 1535 bp in which 1515 bp belonged to the gene sequence and 20 bp to nucleotide overhangs incorporated in the primers (Figure 1a). The transformed colonies of *E. coli* MC1061 grown on LB agar containing chloramphenicol showed positive results by colony PCR. The amplicon length in colony PCR of desired transformants was 1810 bp (Figure 1e). The extracted plasmids, restriction enzyme confirmation and colony PCR are also given in Figure 1. The length of PNZ8110 plasmid without the insert was 3459 bp. The length of plasmid+ insert (pNZ8110+*hly*) reached 4932 bp. Digestion of pNZ8110+*hly* by 2 restriction enzymes NaeI and KpnI showed 2 bands corresponding to the insert and plasmid.

Among the *Lactobacillus* species, *L. plantarum* was successfully electrotransformed. The results of colony PCR using vec primers confirmed the presence of gene and plasmid. Plasmid extraction and restriction analysis also produced the corresponding bands.

Sequencing results of extracted plasmid and colony PCR amplicon using vec reverse and forward primers and their alignments confirmed the integrity of *hly* gene sequence and correct reading frame.

Production of LLO by Engineered *Lactobacillus* Species

SDS-PAGE followed by western blotting using rabbit anti-LLO polycolonal antibody (Abcam, USA) revealed the

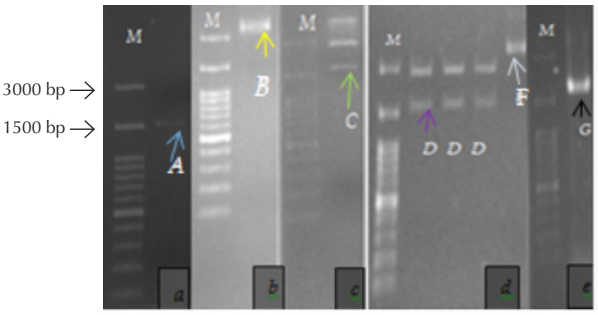

Figure 1. Representative 1% Agarose Gel Electrophoresis Representing M for Marker 100 bp Plus DNA Ladder RTU, Cat. No.PR911653 (Cinagen, Iran).
The bands of 3000 and 1500 bp of the marker are shown in the left. (a) PCR product (1535 bp) of hly gene (A); (b) pNZ8110 plasmid (3459 bp) (B); (c) partial digestion of pNZ8110+hly by 2 restriction enzymes NaeI and KpnI (C); (d) complete digestion of pNZ8110+hly by 2 enzymes (D) and pNZ8110+ hly plasmid (4932 bp) (F); (e) colony PCR of transformed bacteria using vec primers (1810 bp) (G).

production of LLO protein by engineered *L. plantarum* (Figures 2 and 3).

Discussion

During the past decades, the interest in developing genetically engineered LAB strains with desired properties has been continuously growing.[1] Among LAB strains, genetically modified *L. lactis* strains along with various genetic tools including commercial inducible expression vectors such as NICE systems have been available. In contrast to *L. lactis*, few inducible expression vectors for gene cloning have been developed in lactobacilli. Considering the versatility and diversity of lactobacilli at the species level, even specific expression vectors may represent different efficiencies.[19,21-23]

Despite high advantages of lactobacilli, the limited number of specific expression systems, low transformation efficiency, different restriction systems, different cell wall structures, acid production and some other features make the use of these bacteria as a cloning vector somehow troublesome.[24,25] We used several *Lactobacillus* species including *L. casei, L. gasseri, L. acidophilus* and *L. plantarum* for cloning of LLO using inducible expression plasmid, pNZ8110. In spite of several efforts using some different published methods and voltages (data not shown) for electroporation, no colony was recovered except for *L. plantarum*. This result is compatible with other researchers' findings, that referred to this species as a feasible expression host.[19,26,27]

In general, transformation efficiencies in lactobacilli are often lower compared to some other bacteria. Low number or lack of transformants after electroporation may be due to some factors such as various restriction (RM) systems encoded by the host, and dam/dcm methylation pattern of plasmid DNA.[25] Anyhow in our study, failure of transformation for other lactobacilli may also be due to technical errors and need more experiments with modification of methods that we used.

Lactobacillus plantarum has been used as a cloning host for expression of some heterologous antigens such as onco-fetal antigen.[26] Furthermore, this strain exhibits natural immuno-adjuanticity and more immunogenicity than *L. lactis* and *L. casei* and modulation of immune system toward Th1 responses.[2,5,28-30] On the other hand, LLO facilitates the stimulation of both the CD4+ T cell through MHC class II and CD8+ T cell through MHC class I antigen presentation.[31,32] It is mentioned that nanomer LLO 91–99, is an immunodominant epitope that induces both cell-mediated and humoral immunities.[33,34] The protective role of this potent immunogenic antigen, to some extent, against *L. monocytogenes* challenge in murine model has been shown.[12,35,36] Cloning of *L. monocytogenes hly* gene in some bacteria such as *E. coli, Bacillus, Salmonella* and *L. lactis* have been done so far.[12,36-38] Bahey-El-Din et al constructed *L. lactis* strains that secreted LLO and P60 alone or in combination. The strains secreting

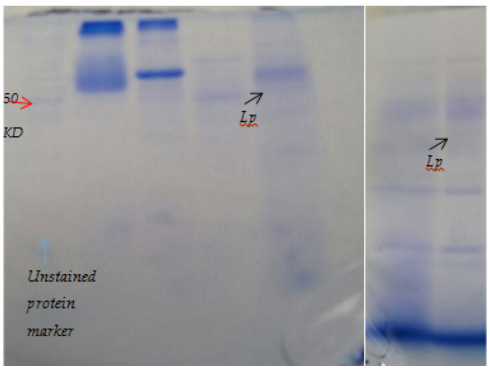

Figure 2. LLO Protein Expression detected by SDS PAGE. At the left, page Ruler Unstained Protein Ladder, 26614 (Thermo Scientific, USA) the 50 KD protein band in the ladder which has a greater intensity is shown by the red arrow. The 2 black arrows (Lp) show the band of LLO (between 50 and 60 KD) secreted from recombinant *L. plantarum*.

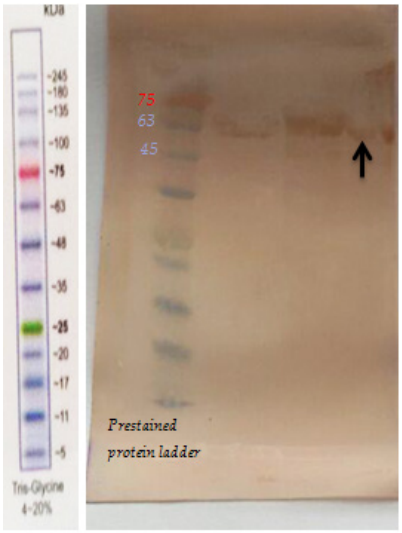

Figure 3. Western blot showing specific band of recombinant listeriolysin (black arrow). The prestained protein markers are shown in the left (BLUelf prestained protein ladder GeneDireX, Taiwan). Bands of 75, 63 and 45 kDa are shown on the left of the marker.

LLO could evoke protective immunity against Listerial infection in murine model by IP immunization.[13] *Bacillus subtilis* was genetically manipulated to express LLO and PA by Huang et al.[9] Giammarini et al,[37] Radford et al,[15] and others cloned the hly gene in *E. coli.* Cloning and expression of LLO and OVA in *E. coli* showed protective immunity against tumors in mice.[15] There is no report or at least there are few researches that used lactobacilli for this purpose.

Pavan et al used the lactococcal expression vectors pNZ8008 and pNZ8032 for nisin inducible expression of β-glucuronidase and TTFC, the C subunit of tetanus toxin in *L. plantarum* by incorporating *nisRK* gene into the chromosome of this strain. They also showed that best transformation efficiency for *L. plantarum* CD033 was achieved when non-methylated vectors were employed.[19]

Our study aimed to clone the *hly* gene in a generally

recognized as safe (GRAS) strain of *Lactobacillus*. The main reasons for selecting these bacteria for this purpose were related to using them for the first time and their potent intrinsic immunomodulatory effects .As we did not have access to any inducible plasmid for expression in *Lactobacillus* strains except plasmid pNZ8110, we applied this plasmid to examine the transformation and expression of LLO in a collection of lactobacilli. Here, the LLO was successfully expressed in L. plantarum in a secretory form and detected in the supernatant of induced cultures by SDS-PAGE and western blot.

Primary experiments showed no pathogenicity of this strain for mice groups (data not shown). Anyhow it is logical to expect more research focused on probiotics for their application in various fields including bioengineering and medicine.[39]

Conclusion

To conclude, the *hly* gene was successfully cloned and expressed in *L. plantarum*. This study can provide a recombinant strain for developing a vaccial strain against listerial infections. Moreover, this recombinant strain is proposed to accompany other heterologous antigens against tumors and other intracellular pathogens.

Authors' Contributions

This research was part of the PhD project. MH performed the laboratory work and wrote the first draft of the manuscript. SH was the first supervisor of this project, designed the study and revised the manuscript. SMT was the co-supervisor of the project, participated in coordination, revised the manuscript and also participated in the statistical analysis; SMHH was the advisor of the PhD project, and carried out the western blot. All authors read and approved the final manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

Ethical Approval

The experiment was approved by State Ethics Committee, Shiraz University, Shiraz, Iran (IACUC no: 4687/63). Additionally, the advices of European Council Directive (86/609/EC) of November 24, 1986, were fully considered in the experimental procedures.

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