Construction of pNMO6 an expression vector for *Lactobacillus* Sp.

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ABSTRACT

The aim of this study was to develop an expression vector for Lactobacillus strains. The expression vector pNMO6. containing the strong constitutive promoter of the Lactobacillus sakei L-ldh gene , Lactococcal signal peptide of Usp45 protein, followed by H and W domain of PrtB from Lactobacillus delburueckii subsp. bulgaricus as an anchoring matrix, was constructed based on the lactobacillus shuttle vector pDJO4. The Lactococcal pepN gene was amplified and cloned into the pNMO6 downstream the PrtB domain, then introduced into both E. coli and Lactobacillus gasseri, the pepN activity showed a high activity compared to the parental type. This expression vector provides a good tool for introducing extracellular and surface-bound proteins or antigens into lactobacillus strains for vaccination and other therapeutic purposes. This system is currently exploited for the production of bacterial vaccine antigens for use in humans.

Key words: *lactobacillus, expression vector, ldhL promoter, signal peptide.*

INTRODUCTION

Lactobacilli have great potentials as food-grade cell factories and as delivery vehicles for interesting proteins, such as antigens, antibodies and growth factors (Pouwels *et al.*, 1996, 2001; Pavan *et al.*, 2000; Kruger *et al.*, 2002; Scheppler *et al.*, 2002). Thus, there is considerable interest in the development of genetic tools for efficient and controllable gene expression in lactobacilli (de Vos, 1999; Mercenier *et al.*, 2000). Generally in bacteria, most proteins that are secreted *via* the Sec pathway are synthesized as precursors containing the mature protein and an N-terminal SP (von Heijne 1990) that is essential for precursor secretion. All SPs display a common tripartite structure including a positively charged N terminus, a hydrophobic core and a neutral or negatively charged C terminus containing the SP cleavage site. The Usp45 signal peptide (SPUsp) has a more consensual structure. The N-terminal region of Usp45 (including SPUsp and, in some cases, several amino acids of the mature protein) has already been used to drive secretion of heterologous proteins in *L. lactis*, e.g., a-amylase (van Asseldonk 1993), bovine plasmin (Arnau 1997), ovine interferon-omega (Bermúdez-Humarán *et al.*, 2003).

There has been interest in the development of methods for the expression of heterologous protein expression in lactic acid bacteria and their relatives (Pouwels *et al.*, 1998). In many of these studies, the heterologous molecules have been designed to be presented at the cell surface. Most reported

methods have made use of either the C-terminal cell wall anchoring motif LPXTG or S-layer subunits (Stahl *et al.*, 2000; Kahala and Palva 1999). The promoters and regulatory genes from the *Lactococcus lactis* nisin gene cluster have been used to develop regulated gene expression systems for lactococci (de Ruyter *et al.*, 1996), lactobacilli (Pavan *et al.*, 2000; Sorvig *et al.*, 2003) and other Gram-positive bacteria (Eichenbaum *et al.*, 1998; Bryan *et al.*, 2000). While being efficient and well regulated in lactococci, the plasmid-based nisin-controlled expression (NICE) systems often exhibit significant basal activity (i.e. activity without induction) in lactobacilli (Pavan *et al.*, 2000; Sørvig *et al.*, 2003). This problem can be circumvented by integrating the histidine kinase and response regulator genes in the chromosome (Pavan *et al.*, 2000), thus limiting the expression systems to specially designed host strains.

The aim of this study was to construct a constitutive cell-surface expression vector for *Lactobacillus* Sp., to facilitate expression the heterologous proteins into these important species for therapeutic purposes. To construct the cell-surface display pNMO6 vector, a derivative of pDOJ4 *Lactobacillus* shuttle cloning vector (Lee *et al.*, 2007), a combination of constitutive lactate dehydrogenase promoter (ldhLp) from *Lactobacillus sakei*, Usp45 lactococcal secretion signal sequence, the H and W domain of the cell surface proteinase PrtB from *Lactobacillus bulgaricus* (Kim *et al.*, 2008) were used. The new system, designed for cell-surface display of recombinant proteins on *Lactobacillus* Sp. was confirmed by sequencing, then was evaluated by the expression of the PepN protein of *Lactococcus lactis* as a reporter gene into *Lactobacillus gasseri*. The expression of the PepN protein by the transformed cells was determined by assaying its activity compared to the parental type.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains and plasmids are listed in Table (1). *Escherichia coli* were used for the construction and propagation of plasmids and were grown in Luria Bertani medium at 37°C. *Lactobacillus* strains were grown at 37°C in Mann Rogosa Sharpe (MRS, Difco) medium (De Man *et al.*, 1960). *Lactococcus lactis* were cultivated at 30°C in M17 broth (Difco) supplemented with 0.5% glucose.

Table (1): Strains used in this study.

Strains / plasmids	Relevant characteristics	Source
Bacterial strains		
Escherichia coli		ATCC25922
Lb. bulgaricus		milk isolates
Lb. gasseri		ATCC
Lb. sakei		milk isolates
L. lactis		milk isolates
NME09	*E. coli* harbouring pNMO6 vector, Cm^r	Current work
NME10	*E. coli* harbouring pNMO6:*pep*N vector, Cm^r	Current work
NMg11	*Lb. gasseri* contaning pNMO6 vector, Cm^r	Current work
NMg12	*Lb. gasseri* contaning pNMO6:*pep*N vector, Cm^r	Current work
Plasmids		
pDOJ4	*E. coli-L. delbrueckii* shuttle cloning vector, Cm^r; *lacZ*; MCS	Lee *et al.*, 2007
pNMO6	Expression vector $P_{ldhL}SP_{Usp45}$PrtB, Cm^r	Current work
pNMO6:*pep*N	pepN gene expressed under $P_{ldhL}SP_{Usp45}$PrtB; Cm^r	Current work

DNA isolation, primers, and PCR conditions

Genomic DNA from pure cultures was extracted using AxyGEN BIOSCIENCES DNA extraction kit according to manufacturer's instructions. Plasmid DNA was isolated using the Wizard plasmid kit from Promega according to manufacturer's instructions. Primers used in this study were purchased from Sigma and are listed in Table (2). All polymerase chain reaction amplifications were performed with the *Taq* DNA polymerase kit (Fermentas). Reaction mixtures consisted of 20 mm Tris–HCl (pH 8.4), 50 mm KCl, 3 mm $MgCl_2$, 50 mm each of the four deoxynucleoside triphosphates (dNTP), 1 U *Taq* polymerase, 50 pmol each primer and 1 µl of template DNA in a final volume of 50 µl . Samples were amplified in a GeneAmp polymerase chain reaction System 2700 (Applied Biosystems). Polymerase chain reaction products were quantified by electrophoresis on a 1% (w/v) agarose gel containing ethidium bromide. The molecular weight markers; 100 bp, 1 kb (Promega) were used to estimate the size of the DNA fragments. The correct nucleotide sequence of all PCR-generated inserts was verified by DNA sequencing. The PCR products were purified before use by AxyGEN PCR purification kit according to manufacturer's instructions.

Table (2): List of the primers used in this study.

Primer name	Primer sequence
Ldh1	5'ATGC**CCCGGG**TACTGAGAAGTTGCTCTC-3'
Ldh2	5'AGCT**GGCC**TCGCCGACGAGGATAACT-3'
Usp5	5'TCTA**CCGG**GAGCGCCTACACTTTTGCTC3'
Usp6	5'GT**ATGCAT**AAACACCTGACAACGGGG 3
An377	5'-AAGGATGCATCCGCCCAGCCGGAATTGAAG-3'
An366	5'-ATATTACCTAGGATTGAATAGATTGCCGGA-3'
Sip32	5' GAT **CCC ATG**GCTGTAAAACGTTTAATTG 3'
Sip41	5'GTA **CCT CGA GTC TA**G ACT ACA ATT TTT CAG CAA TAT C 3'

DNA manipulation and transformation

Nucleic acid manipulation and cloning procedures were performed according to standard procedures (Sambrook *et al.* 2001). *E. coli* and *Lactobacillus* electrocompetent cells were prepared and transformed as described by Berthier *et al.* (1996) and Dower *et al.* (1988). The transformants were selected on the selective media supplemented with 10 µg/ ml of chloramphenicol. Restriction endonucleases and T4 DNA ligase were purchased from Promega and were used according to the recommendations of the manufacturers.

Sequencing and sequence analysis

DNA sequencing was performed by Sigma. DNA homology searches were performed with the Basic Local Alignment Search Tool (BLAST) at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Assay for PepN activity

Lb. gasseri and *E. coli* strains harbouring pNMO6 vector with *pep*N was grown overnight in the appropriate broth supplemented with the chloramphenicol. Cells were harvested, resuspended in a buffer consisting of 0.01 M KCl, 0.05 M NaHPO4 and 0.001 M MgSO4, and disrupted by sonication, (van de Guchte *et al.* 1991). The resulting cell-free extracts were used to assay aminopeptidase activity using L-lysine p-nitroanilide (Sigma) as substrate. PepN activity was conducted at 30°C in 0.1 M

Tris/HCl, pH 8.5 and was determined according to the protocol described by Exterkate (1984). Protein concentrations were determined using the Bio-Rad Protein Assay with BSA (Sigma) as standard.

RESULTS AND DISCUSSION

Plasmid constructions

The ldhL promoter fragment (240 bp) was obtained by PCR amplification using *Lb. sakei* DNA as template (Fig. 1). The primers used, designed from the sequence (GenBank accession no. AF054624), were ldh1 and ldh2. Restriction sites *Sma*I and *Msp*I (underlined) were added at the 5'-end of each primer, respectively the PCR was performed as described in the Methods. A 280 bp fragment containing the ribosome binding site and the signal peptide of the Usp45 was PCR amplified from *L. lactis* using primers usp5 and usp6 (Le Loir *et al*., 2001) *Mps*I and *Nsi*I sites were inserted respectively. The two amplified PCR fragments were combined together using a recombinant PCR reaction as described by Higuchi (1990). The subsequent reaction was conducted with primers ldh1 and usp6. The final PCR product ldhp::USP45 was purified and confirmed by sequencing. Then this fragment was subjected to digestion by *Sma*I and *Nsi*I followed by purification using PCR Clean kit.

PCR amplification of the H and W domain of PrtB was performed with the An 377 Forward and An 366 Reverse primers (Kim *et al*., 2008) using genomic DNA of *Lb. bulgaricus* as a template. The oligonucleotides were designed to introduce a *Nsi*I and *Bam*HI restriction site (underlined) at the 5' and 3' end respectively. The 1.5 kb amplified fragment PrtB H-W was confirmed by sequencing, then digested with *Nsi*I and *Bam*HI enzymes. The ligation reaction was performed between the *Sma*I/*Nsi*I fragment (*ldhp:rbs:usp*) and the *Nsi*I/*Bam*HI fragment (PrtB-H W).

The ligated fragment (*ldhp:rbs:usp:PrtB-H W*) *Sma*I/ *Bam*HI was cloned into the pDOJ4 vector by ligation into the corresponding sites on the vector and transformed into *E. coli* JM109 plated onto LB agar containing chloroamphenicol and incubated two days at 37°C. The chloroamphenicol resistance (Cm^R) colonies were picked for analysis by PCR using primers ldh1 and An366. The positive ones gave band in the expected size. One colony of them was chosen for further analysis and named NME09. Sequencing analysis of the recombinant constructs showed that the H and W domain of the C-terminal region of the PrtB were successfully cloned in-frame downstream of the secretion signal sequence Usp45 under control of ldhL promoter into the pDOJ4 vector. Fig. (2) shows a map of the newly constructed vector, pNMO6.

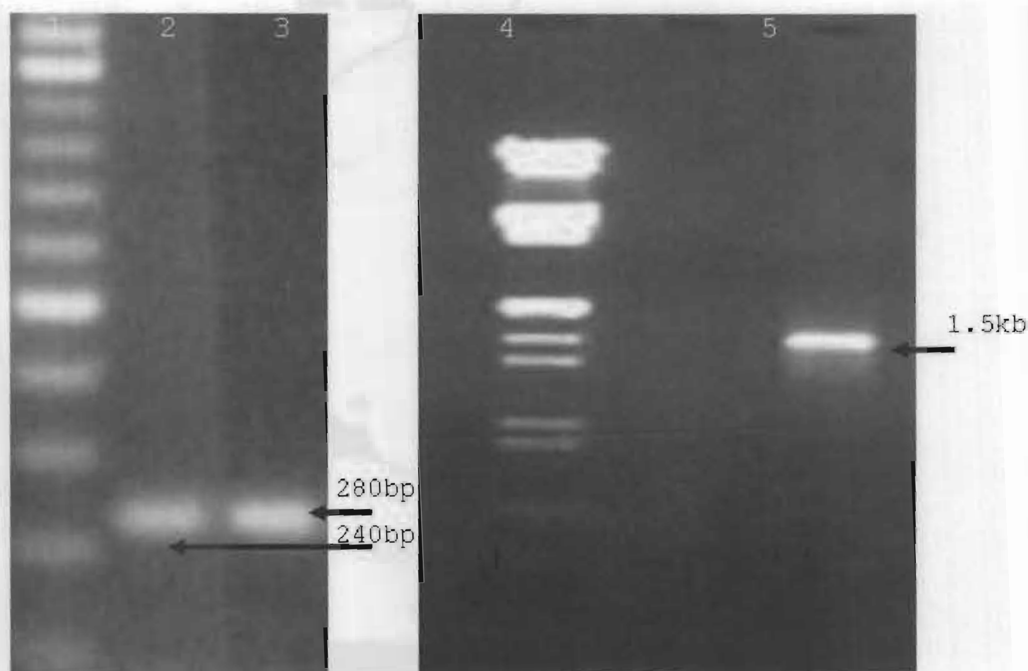

Fig. (1): PCR amplifications. Lane 1, 100 bp marker; lane 2, fragment containing the ldhL promoter; lane 3, usp45 fragment; lane 4, 1 kb marker; lane 5, H & W Domain of prtB fragment.

Cloning the pepN gene into the expression vector pNMO6

To further analyse the potential of the pNMO6 expression system, the aminopeptidase encoded by *pep*N gene was amplified from *L. lactis* using primers Sip32 and Sip41 (Table 2) (Sorvig *et al.*, 2005). *Bam*HI and *Pst*I restriction sites were inserted respectively. Thus, the generated fragment could easily be introduced/ exchanged using the *Bam*HI and *Pst*I restriction sites present in the pNMO6 vector (Fig. 2). The 1 kb *pep*N fragment was cloned into pNMO6 downstream the prtB H-W Fragment and its expression was controlled by the ldhL promoter. The final plasmid pNMO6:*pep*N was introduced into *Lb. gasseri* and *E. coli*. The presence of the vector in transformants was confirmed by plasmid isolation as described above. The confirmed strains were named NMg12 and NMc10, respectively.

Assay the pepN activity

The pepN activity was determined as mentioned in the Methods. Cells were harvested at OD600 ~1.8 to ensure maximum or near-maximum expression levels. Fig. (3) shows the results with the pNMO6:*pep*N vector in the strains *Lb. gasseri* (NMg12) and *E. coli* (NMc10). Expression activity measurements showed a high difference value in PepN activity in the modified strains (NMg12 and NMc10) compared to strains (NMc09 and NMg11) and harbouring the pNMO6 vector without pepN fragment. The parental type without any vector was also subjected to the assay (Fig. 3).

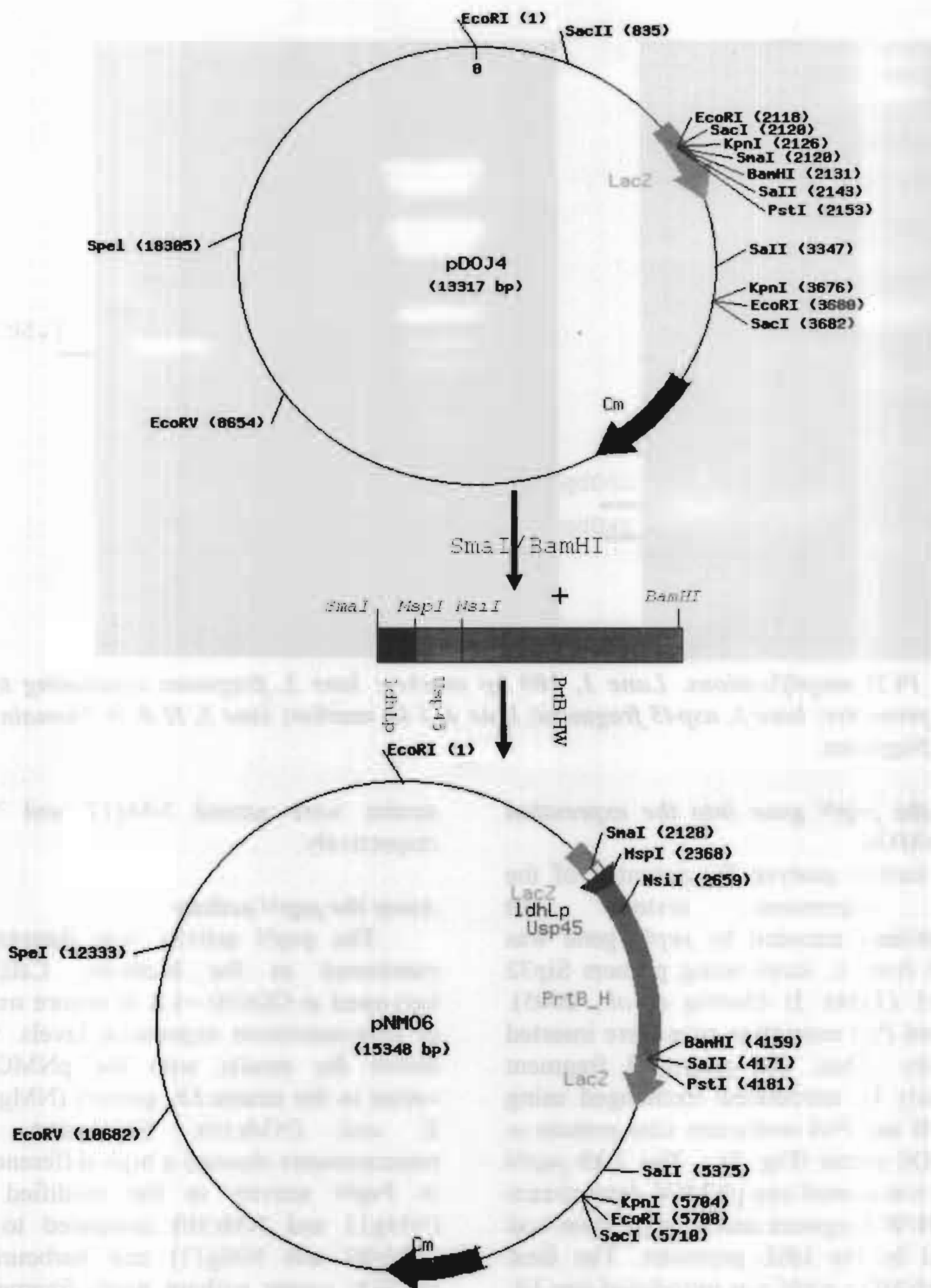

Fig. (2): Construction of the expression vector pNMO6.

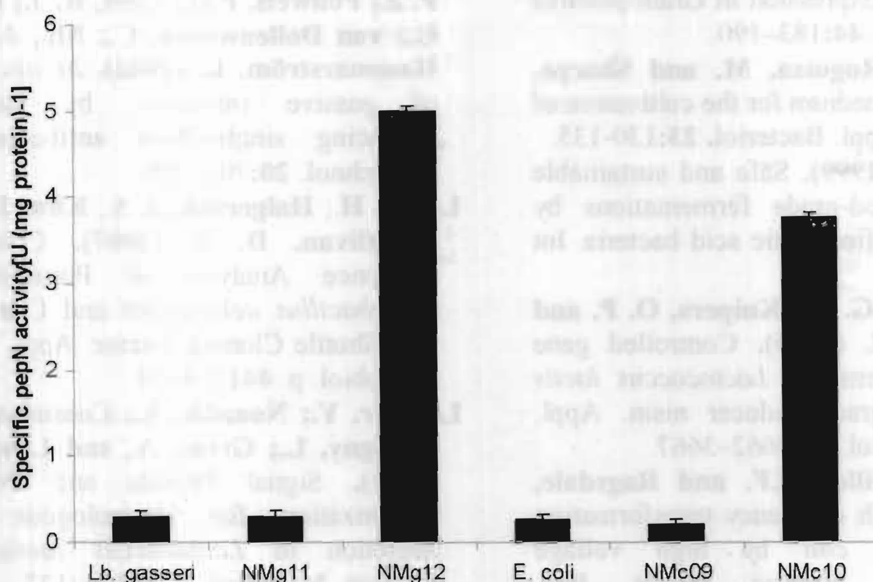

Fig. (3): PepN activity in L. gasseri and L. gasseri (NMg11) measured at OD600 1.8. One unit (U) of PepN activity represents production of one micromole p-nitroanilide per minute. All data are the mean of three independent experiments; the error bars indicate the standard deviation.

CONCLUSION

In conclusion, pDOJ4 shuttle vector was successfully modified to serve as an expression vector for *Lactobacillus* sp. The resulted expression vector was named pNMO6. The efficiency of the vector was verified by expressing the *pep*N gene in a laboratory *Escherichia coli* and the probiotic strain *Lactobacillus gasseri*. Work is now in progress to exploit the new expression systems pNMO6 for the expression of proteins of industrial and medical importance on the cell surface of *Lactobacillus* strains.

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الملخص العربي

تركيب بلازميد pNMO6 للتعبير الجينى فى سلالات اللاكتوباسيلاى

نهلة مختار منصور
قسم كيمياء المنتجات الطبيعية والميكروبية- شعبة العلوم الصيدلية
مجموعة الفاكسينات-مركز التميز العلمى للعلوم المتقدمة. المركز القومى للبحوث, شارع البحوث- الدقى-مصر

الهدف من هذه الدراسة هو تركيب بلازميد لاستخدامه فى سلالات اللاكتوباسيلاى والتى لها أهمية قصوى كمكون اساسى من الفللورا الطبيعية للانسان والتى لها من فوائد صحية مختلفة و كونها من المدعمات الحيويه. ولفوائدها المتعددة ألقى الأتجاه الى استخدام أساليب الهندسه الوراثيه لهذه السلالات للاستفاده القصوى من خواصها لأغراض علاجيه و كحامل للفاكسينات و البروتينات المختلفه. ولهذا وجب وجود أداه جيدة لتيسير هذا الاتجاه والتى تتمثل فى وجود بلازميد يتناسب مع هذه السلالات و حيث أنه غير متوفر تجاريا فقد تم تركيب بلازميد pNMO6 للتعبير الجينى على سطح الخليه. و قد اعتمد على اضافه promoter و الاجزاء الجينيه المسئولة عن اظهار البروتينات المستهدفه الى بلازميد pDJO4 والذى تم اهداؤه من Prof. Osullvan (USA). وقد تم مبدئيا اختبار كفاءه البلازميد فى التعبير الجينى. وجارى الان اختبار هذا النظام حاليا لانتاج مولدات المضادات البكتيرية.