Chapter 6

Expression of antigenic candidate in L. lactis

The work presented in this chapter is published as follows:

Chapter 6. Expression of antigenic candidate in *L. lactis*

6.1 Introduction

6.1.1 *Lactococcus lactis* as an expression host

*Lactococcus lactis* (*L. lactis*), generally regarded as safe (GRAS) microorganism, produces myriad beneficial effects on human health and stimulates immune system when used as an antigen delivery vehicle. There has been recent efforts in developing *L. lactis* as a host for production of heterologous proteins of medical and technological interest (Cortes-Perez *et al.*, 2006). Several inducible expression systems have been developed for expression of heterologous proteins. Nisin Inducible Controlled Expression (NICE) is the well documented and preferred expression system for *L. lactis*. NICE comprises of PnisA promoter and *nisRK* regulatory genes, wherein bacteriocin nisin acts as an inducer. *L. lactis* NZ9000 and its derivatives are widely used with NICE system having *nisRK* gene integrated into the chromosome of *L. lactis* MG1363 (Kuipers *et al.*, 1998).

The Usp45 is commonly used signal peptide in NZ9000 and its derivatives, for extracellular secretion of proteins (Villatoro-Hernandez *et al.*, 2008). Using NICE as an inducible expression system, several proteins such as IP-10, β-glucoronidase, Aminopeptidase N, NADPH oxidase 4 and various others have been expressed in *L. lactis* (Kuipers *et al.*, 1998; Loir *et al.*, 2005).

6.1.2 Expression of OmpA in *L. lactis*

The present chapter describes the expression of Outer membrane protein A (OmpA) of *Shigella dysenteriae* type-1 as a model protein in *L. lactis* using NICE. Although, NICE system is considered to be the most suitable for recombinant membrane protein expression (Frelet-barrand *et al.*, 2010), optimization of multiple factors for efficient expression is required. The role of variable factors in optimal expression of heterologous protein using NICE in *L. lactis* has not gained much attention. Thus, our study focuses on the variable factors involved NICE using *L. lactis*.

The variants which were found to play an important role in protein expression includes concentration of inducer, protein precipitating agent and effect of host proteases.
6.1.3 *L. lactis* VEL1153 (NZ9000ΔhtrA)

In *L. lactis* NZ9000, host proteases are present intracellularly as well as extracellularly. A unique protease, High-temperature requirement A (HtrA), found in the extracellular matrix of *L. lactis*, is known to degrade the secreted protein (Cortes-Perez *et al.*, 2006). Hence, we evaluated its impact on protein expression by using *L. lactis* VEL1153 (NZ9000ΔhtrA), a variant of *L. lactis* NZ9000.

6.2 Materials and Methods

6.2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 6.2.1.1.

<table>
<thead>
<tr>
<th>Strains and Plasmids</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. coli</em> DH5α</td>
<td>ΔlacZ ΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdRI2 (K- mK+) supE44 thi-1 gyrA96 relA1</td>
<td>Lab Source</td>
</tr>
<tr>
<td><em>L. lactis</em> MG1363</td>
<td>Wild type, Plasmid free</td>
<td>Gasson 1983</td>
</tr>
<tr>
<td><em>L. lactis</em> NZ9000</td>
<td>MG1363 (nisRK genes into chromosome), plasmid free</td>
<td>(Kuipers <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td><em>L. lactis</em> VEL1153 (NZ9000 ΔhtrA)</td>
<td>NZ9000, functionally inactive HtrA (by Site Direct Mutagenesis)</td>
<td>Foucauld-Scheunemann and Poquet 2003</td>
</tr>
<tr>
<td><em>L. lactis</em> NZ9000 (pSEC:Nuc)</td>
<td>Cm*, NZ9000 harboring pSEC:Nuc</td>
<td>This work</td>
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<td><em>L. lactis</em> NZ9000 (pSEC:OmpA)</td>
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<td>This work</td>
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6.2.2 Construction of pSEC:OmpA vector

For construction of pSEC:OmpA, *ompA* sequence of *S. dysenteriae* type-1 was retrieved from NCBI (Gene accession number: 3799631) and was commercially synthesized from GenScript (GenScript, Hong Kong Ltd) in pUC57 cloning vector between *Nsi* I and *EcoR* V RE sites. pSEC:Nuc vector which was used as a backbone vector, has a
combination of NICE system and usp45 secretory signal. As depicted in Figure 6.1, ompA gene was cloned in place of nuc gene by using Nsi I and EcoR V RE. After ligating the digested products, it was used to transform E. coli DH5α. Transformants were screened by colony PCR using ompA specific primers (Forward 5’ GTTCCTACCGTTTCGGTC 3’ and reverse 5’ TGCGCACTGAGAAGAAGAGA 3’) and verified by restriction sequence analyses.

Figure 6.1  Schematic representation of pSEC:OmpA vector construction

Expression cassette for Outer membrane protein A (OmpA) using the nisin inducible promoter (PnisA) and signal peptide usp45. (a) OmpA sequence of S. dysenteriae type-1 was commercially synthesized from GenScript in pUC57 cloning vector between Nsi I and EcoR V RE sites. (b) pSEC:Nuc vector which has a combination of NICE system and usp45 secretory signal was used as a backbone vector (c) OmpA gene was cloned in place of nuc gene by using Nsi I and EcoR V RE sites and ligating the digested products. Arrows indicate presence of nisin inducible promoter PnisA. Gray box indicate presence of signal peptide of usp45 gene. Small empty box structure indicates trpA transcriptional terminator.

6.2.3 Transformation of L. lactis

L. lactis NZ9000 and VEL1153 (NZ9000ΔhtrA) were transformed with pSEC:OmpA by electroporation. Transformed cells were plated onto GM17 agar containing chloramphenicol and incubated at 30° C for 24 hours. Transformants were screened by colony PCR using ompA and PnisA specific primers and preserved as glycerol stocks at −80 °C. Primers sequence for PnisA; Forward 5’ TGCGATAACCGCGACGATAA 3’ and Reverse 5’ TCGAAACGATACGAAATCCAA 3’.
6.2.4 Expression of OmpA in *L. lactis*

For expression of OmpA, *L. lactis* strains harbouring pSEC:OmpA were sub-cultured in fresh GM17 broth containing respective antibiotics and were grown statically at 30°C till an optical density at 600 nm was reached to 0.4-0.6. Cultures were then induced with different concentrations of nisin 2, 5, 10 and 15 ng/ml and were grown for 3 hours. Halt Protease Inhibitor Cocktail (Thermo Scientific, IL USA) was added immediately after induction. Cells were harvested by centrifugation at 6000 g for 10 min at 4°C. Bacterial cell pellets as well as supernatant were processed as mentioned previously (Villatoro-Hernandez et al., 2008) with minor modifications. Briefly, cell pellet was resuspended in TES lysis buffer and incubated at 37°C for 1 hour. To break the cells, 50 µl of 20% SDS was added and stored at -20°C till further analysis. For precipitation of protein in supernatant two different precipitating agents; Trichloroaceticacid (TCA) and methanol were used. For precipitation by TCA method, 10% TCA as a final concentration was added to the supernatant and incubated in ice for 30 minutes followed by centrifugation at 12,000 g for 20 minutes at 4°C. In case of methanol, supernatant was precipitated with three volumes of methanol for two hours at 0°C followed by centrifugation at 12,000 g for 20 minutes at 4°C. The protein pellet was recovered in 5× SDS gel loading dye and stored at -20°C till further analysis.

6.2.5 RNA Isolation and detection of OmpA specific mRNA by Reverse Transcriptase PCR (RT-PCR)

RNA was isolated from *L. lactis* strains by RNA sure mini kit (Nucleo-pore, Genetix, India) using manufacturer’s protocol. RT-PCR was performed using 1 µg of total RNA with Maxima cDNA synthesis kit (Thermo Scientific, USA). OmpA transcripts were detected with *ompA* specific primers (Forward 5' GTTTCCTACCGTTTCGGTC 3' and reverse 5' TGCGCACTGAGAAGAAGA 3').

6.2.6 Western blot analysis

For western blot analysis, protein samples were separated by using 12% SDS-PAGE and transferred to a PVDF membrane (Immobilon-P, Millipore) by electro-blotting. The membrane was blocked for 1 hour at room temperature in Phosphate Buffered Saline (PBS) containing 5% skimmed milk. Membrane was then incubated with 1° Antibody raised against outer membrane proteins of *Shigella flexneri* [Outer membrane protein A of *S. flexneri* and *S. dysenteriae* type-1 are having 95.98% similarity. For detailed description, refer Figure 6.2] (1:500 dilutions) for 1 hour,
followed by three 5 minutes washes in PBS. Peroxidase conjugated goat anti-rabbit (Calbiochem, USA) was added at 1:1000 dilutions to the membrane and incubated for 1 hour at room temperature followed by three 5 minutes PBS washes. For detection of antigen, TMB substrate was used (Invitrogen, Carlsbad, USA) and blot was visualized by using Gel Doc™ XR+ System (Bio Rad Laboratory, USA).

6.3 Results

6.3.1 Construction, Transformation and Expression

To facilitate the controlled secretion of antigenic protein OmpA by *L. lactis*, we have constructed *E. coli* - *L. lactis* shuttle vector pSEC:OmpA. Construction of pSEC:OmpA is schematically represented in Figure 6.1. The *ompA* gene of *S. dysenteriae* type-1 is flanked by *Nsi I* and *EcoR V* in pUC57:OmpA. Both the vectors; pUC57:OmpA and pSEC:Nuc were double digested with *Nsi I* and *EcoR V* resulting in the restriction fragments of 1071bp and 3275bp corresponding to *ompA* gene and pSEC backbone respectively as depicted in Figure 6.3. Ligation of digested products were then used to transformed *E. coli* DH5α. Obtained clones were screened by colony PCR showing amplicon of 252bp corresponding to presence of *ompA*. Clones were verified by restriction sequence analysis. Transformation in *L. lactis* strains NZ9000 and VEL1153 (NZ9000ΔhtrA) was done by electroporation and confirmed by colony PCR using *ompA* and *PnisA* specific primers as shown in Figure 6.4(a, b). Further verification of pSEC:OmpA was done by restriction sequence analysis as shown in Figure 6.4(c). The *ompA* transcripts were confirmed by the presence of 151 bp amplicon as shown in Figure 6.5. Evaluation of OmpA expression was then carried out by western blot.
Figure 6.2  Multiple sequence alignment of OmpA protein of *S. flexneri* and *S. dysenteriae* type-1 using Clustal 2.1 and Uniprot

For protein sequence similarity of Outer membrane protein A (OmpA) between *S. flexneri* and *S. dysenteriae* type-1, sequence alignment was performed using bioinformatics tools such as ClustalW 2.1 and Uniprot. A 95.98% similarity was found between OmpA sequences of aforesaid strains. Hence, it was used for further analysis.

Confirmation of pSEC:OmpA in r-L. lactis NZ9000 by colony PCR and restriction sequence analysis. PCR amplicons and RE digested fragments were analyzed using 1% agarose gel electrophoresis. (a) PCR amplification using primers specific to PnisA. Lane-1: GeneRuler 50 bp Ladder, Lane-2: Amplicon of pUC57:OmpA (Negative control), Lane: 3 to 7: Amplicon of different L. lactis transformants. (b) PCR amplification using primers specific to ompA. Lane-1: GeneRuler 50 bp Ladder, Lane-2: Amplicon of pSEC:Nuc (Negative control), Lane: 3 & 4: Amplicon of different L. lactis transformants. 151 bp and 252 bp amplicons confirmed the presence of pSEC:OmpA in L. lactis NZ9000 and L. lactis VEL1153 (NZ9000ΔhtrA). (c) Restriction sequence analysis of selected pSEC:OmpA clone. Lane-1: Fragments of pSEC:OmpA digested with BamH I and Eco RV, Lane-2: λ DNA digested by BstE II. Fragment of 1077 bp corresponding to ompA gene confirms the pSEC:OmpA in r-L. lactis NZ9000.
Figure 6.5  Detection of OmpA transcripts by RT-PCR in r-L. lactis NZ9000(pSEC:OmpA)

Detection of OmpA transcripts by RT-PCR in r-L. lactis NZ9000 (pSEC:OmpA), Lane-1: GeneRuler 50bp Ladder, Lane-2 to 4: Amplicon of induced r-L. lactis NZ9000(pSEC:OmpA) at 10 ng/mL nisin, Lane-5: Amplicon of uninduced r-L. lactis NZ9000, Amplicon of 151 bp confirms the expression of OmpA at the stage of transcription.

6.3.2 OmpA expression

Expression of heterologous protein was described by Ribeiro et al., (Ribeiro et al. 2002), we followed the same for OmpA expression with minor modification in nisin concentration i.e. 10 ng/ml. In order to determine, whether the OmpA protein secreted out or remain inside the cell, protein was precipitated from the cell pellet and supernatant that were further analyzed by Western blot. When proteins were precipitated with TCA method, as anticipated, band of OmpA was not detected in cell pellet. To our surprise, it was also absent in supernatant as shown in Figure 6.6. Here, OmpA protein of S. dysenteriae type-1 purified from r-E. coli BL21 (DE3)::pET28-OmpA was used as positive control (Figure 6.7). To resolve the ambiguity of OmpA expression, optimization of the factors involved in its expression and detection were assessed.
Figure 6.6  Comparison of Methanol and TCA as precipitating agent for expressed OmpA by Western blot

Detection of expressed OmpA by comparing methanol & TCA as precipitating agent upon induction at 10 ng/mL. Lane-1: Cell pellet of induced r-L. lactis NZ9000 (pSEC:OmpA) precipitated with methanol, Lane-2: ProSieve color protein marker, Lonza Rockland, Lane-3: Supernatant of induced r-L. lactis NZ9000 (pSEC:OmpA) precipitated with methanol, Lane-4: Supernatant of induced r-L. lactis NZ9000 (pSEC:OmpA) precipitated with TCA, Lane-5: Precision plus protein standard, BIORAD.

Figure 6.7  Western blot analysis of purified OmpA of S. dysenteriae type-1 from r-E. coli BL21 (DE3)::pET28-OmpA using 1° Antibody raised against outer membrane proteins of Shigella flexneri as a positive control

Lane 1: Pre-stained protein ladder, Thermo Scientific, Lane 2-4: Different elusion fractions of OmpA from r-E. coli BL21 (DE3)::pET28-OmpA. A band of 37 kDa indicates the binding specificity of 1° antibody raised against outer membrane proteins of Shigella flexneri towards OmpA of S. dysenteriae type-1 and hence, it can be used for further analysis.
6.3.3 Optimization of factors

6.3.3.1 Protein precipitating agent

Precipitation of protein by TCA method has been very well documented in case of *L. lactis* expression system (Cortes-Perez *et al.*, 2006; Zellner *et al.*, 2005). However, we failed to see any band in cell pellet and even in supernatant when TCA was employed for precipitation; hence TCA was replaced with methanol. As depicted in Fig. 2, ~74 kDa protein band which is twice the molecular weight of desired protein was observed along with the degraded protein adducts when precipitated with methanol.

6.3.3.2 Inducer (Nisin)

Inducer (nisin) activates *nisRK* regulator, a two component system, followed by activation of promoter PnisA cascade. For controlled expression of several heterologous proteins, nisin concentration as low as 0.025 ng/ml (Kuipers *et al.*, 1998) and as high as 500 ng/ml has been reported (Oddone *et al.*, 2009a). Hence, it provides a wide arena for optimisation of nisin concentration in accordance to the expressed protein.

To start the optimisation 2, 5, 10 and 15 ng/ml nisin concentrations were tested initially to attain the optimum expression of OmpA. As described in Fig. 6.8, lane 3 illustrates aggregated protein product of ~74 kDa, at 2 ng/ml nisin concentration. In Lane 4, the aggregated ~74 kDa protein band and degraded protein products ranging from ~15 kDa were observed after induction at 10 ng/ml nisin concentration. Lane 1 exhibits the lower molecular weight protein bands indicating the degradation caused by induction at higher concentration of nisin i.e. 15 ng/ml. With increase in inducer concentration, there is concomitant increase in degradation of protein product.
6.3.3.3 Proteases in protein expression

Proteolytic degradation is one of the limiting factor for stable production of heterologous protein by NICE system (Rigoulay et al., 2004). Till date, ClpP (intracellular) and HtrA (extracellular) have been identified as major proteases in *L. lactis* strains (Oddone et al., 2009b). To prevent protein degradation, we incorporated protease inhibitor cocktail during the expression of OmpA, as a foremost measure to prevent protein degradation.

As shown in Figure 6.9(A), the expressed protein of desired size, devoid of any degraded protein products was observed. Indeed, the presence of ~35 kDa band just below the desired protein band of 37 kDa, demonstrates the inefficient prevention against proteases.

HtrA is a trypsin like serine protease which degrades misfolded protein at cell surface (Oddone et al., 2009b). Therefore, the existing strategy was improvised by expressing OmpA in HtrA deficient strain to combat the inevitable degradation. As shown, in Figure 6.9(B), sharp band of desired size 37 kDa was obtained with r- VEL1153 (NZ9000Δhtra). This result indicates the role of HtrA in degradation of OmpA along with other intracellular proteases. Optimum expression of heterologous protein in *L. lactis* thus depends on critical factors as mentioned previously.
Figure 6.9 Effect of nisin concentrations, protease inhibitor cocktail and ΔhtrA strain on protein expression

(a) M: ProSieve color protein marker, Lonza Rockland, Lane 1: Uninduced r-NZ9000 (pSEC:OmpA), Lane 2 to 5: r-NZ9000 (pSEC:OmpA) induced respectively with 2, 5, 10 and 15 ng/ml nisin and protease cocktail. (b) Lane M-a, M-b: Precision plus protein standard, BIORAD, Lane-1: Empty, Lane-2: r-VEL1153 (pSEC:OmpA) induced with 5 ng/mL, Lane-3: r-VEL1153 (pSEC:OmpA) induced at 5 ng/mL with protease cocktail, Lane-4: Uninduced r-VEL1153 (pSEC:OmpA).

6.4 Discussion

Accomplishing successful construction of pSEC:OmpA, expression of OmpA in L. lactis has been explored using NICE. Although it is widely used, knowledge about variable factors and their integrative effects in modulating expression of heterologous protein is scarce. Despite the presence of OmpA specific transcripts, which provided the experimental evidence for the transcription of OmpA, challenge remained at the level of detection of the expressed protein. As protein expression at post-transcriptional level is affected by several factors (Desai et al., 2010), we have evaluated the crucial factors viz. protein precipitating agents, inducer concentration and presence or absence of host proteases in expression of OmpA.

When we attempted precipitation of OmpA protein with TCA method, we failed to see any band in Western blot. This might be due to the degradation of expressed protein (Zellner et al., 2005), loss of protein during acetone wash and/or incomplete solubilization of precipitated proteins (Fic et al., 2010; Jiang et al., 2004). However, when we switched from TCA to methanol, we observed band of ~74 kDa which is twice
the size of OmpA (37 kDa). Though, under denaturing condition, it is unlikely that protein exist in dimer or aggregated form. However, there are certain membrane proteins such as KcsA, β-Glycosidases, and KvLm K+ channels which remains in the dimer or aggregated form (Bowie, 2005; Gentile et al., 2002). These reports strengthen the observation obtained with OmpA. Along with the aggregation, degraded protein product was also found which may be the consequence of the presence of proteases in host strain NZ9000 (Oddone et al., 2009b; Zhou et al., 2006) and/or degradation of highly expressed protein (Drouault et al., 2000).

In 2006, Zhou et al. reported the linear dose dependent relationship suggesting elevated protein expression at higher nisin concentration (Zhou et al., 2006). Further, overexpression of membrane proteins also induces stress response in L. lactis, resulting in protein degradation by chaperons and proteases (Marreddy et al., 2011). Induction at 5, 10 and 15 ng/ml nisin concentrations revealed the degraded pattern of expressed proteins eliciting the possibility of degradation at higher nisin concentration resulting in increased expression and susceptibility to proteases.

Due to the presence of surface as well as intracellular host proteases, considerable degradation of highly expressed proteins has been observed (Enouf et al., 2001). Such reports lead us to explore the role of proteases in heterologous protein expression.

Existence of house-keeping proteases in L. lactis makes the heterologous proteins susceptible to degradation. Due to the consistent protein degradation observed in our results, we explored protease inhibitors as a remedial tool. Extensive usage of protease inhibitor cocktail has been demonstrated to combat protein degradation (Bodziak-Wilkowska et al., 2007) which upon incorporation in our study, prevented the degradation from broad range of proteases. However, presence of 35 kDa band reflected the need to identify the candidate responsible for degradation of our protein.

Expression of ample number of proteins viz. Staphylococcus aureus nuclease, Staphylococcus hyicus lipase, bovine rotavirus non-structural protein 4 NSP4 (Enouf et al., 2001), human papillomavirus antigen E7 (Langella et al., 2002) and Brucella abortus antigen L7/L12 (Ribeiro et al. 2002) were found to be degraded in host strain NZ9000. Protein degradation in aforesaid cases is eliminated by carrying out expression in HtrA deficient strains. When expression of OmpA in r-VEL1153 (NZ9000ΔhtrA) strain was carried out, we achieved 37 kDa band devoid of any
degraded protein product. The obtained results strengthen the role of HtrA as a key extracellular protease.

In addition to HtrA, proteases like ClpP and several other housekeeping proteases which are poorly understood (Frees & Ingmer, 1999), were inhibited by incorporation of protease inhibitor cocktail. Such findings in our study suggest the critical role of proteases in efficient protein expression.

Altogether, the present study revealed the role of protein precipitating agent, inducer concentration and intracellular as well as extracellular proteases in heterologous protein expression by L. lactis. Our result demonstrates the combination of htrA mutant strains, protease inhibitor cocktails, lower nisin inducer concentration and methanol as protein precipitating agent giving optimum expression of heterologous membrane protein. This report outlines key factors and their integrative effects in modulating expression of heterologous protein using NICE.

In nutshell, present work will contribute to the better understanding of factors affecting heterologous protein expression using NICE in L. lactis.