

Molecular and Genetic Strategies to
Enhance Functional Expression of
Recombinant Protein in *Escherichia coli*

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The versatile *Escherichia coli* facilitates protein expression with relative simplicity, high cell density on inexpensive substrates, well known genetics, variety of expression vectors, mutant strains, co-overexpression technology, extracytoplasmic secretion systems, and recombinant protein fusion partners. Although, the protocol is rather simple for soluble proteins, heterologous protein expression is frequently encountered by major technical limitations including inefficient translation, formation of insoluble inclusion bodies, lack of posttranslational modification mechanisms, degradation by host proteases, and impaired cell physiology due to host/protein toxicity, in achieving functional expression of stable, soluble, and bioactive protein.. In this thesis, model protein expression systems are used to address the technical issues for enhancing recombinant protein expression in *E. coli*.

When yellow fluorescence protein (YFP) was displayed on *E. coli* cell surface, the integrity of the cell envelope was compromised and cell physiology was severely impaired, resulting in poor display performance, which was restored by the coexpression of Skp, a periplasmic chaperone. On the basis of monitoring the promoter activities of *degP*, *rpoH*, and *cpxP* under various culture conditions, it was demonstrated that the cell-surface display induced the σ^E extracytoplasmic stress response, and $P_{degP}::lacZ$ was proposed to be a suitable “sensor” for monitoring extracytoplasmic stress.

Intracellular proteolysis has been recognized as one of the key factors limiting recombinant protein production, particularly for eukaryotic proteins heterologously expressed in the prokaryotic expression systems of *E. coli*. Two amino acids, Leu149 and Val223, were identified as proteolytically sensitive when *Pseudozyma antarctica* lipase (PalB) was heterologously expressed in *Escherichia coli*. The functional expression was enhanced using the double mutant for cultivation. However, the recombinant protein production was still limited by PalB misfolding, which was resolved by DsbA coexpression. The study offers an alternative genetic strategy in molecular manipulation to enhance recombinant protein production in *E. coli*.

To overcome the technical limitations of protein misfolding, ineffective disulfide bond formation, and protein instability associated with intracellular proteolysis in the

functional expression of recombinant *Pseudozyma antarctica* lipase B (PalB) in *Escherichia coli*, an alternative approach was explored by extracellular secretion of PalB via two Sec-independent secretion systems, i.e. the α -hemolysin (Type I) and the modified flagellar (Type III) secretion systems, which can export proteins of interest from the cytoplasm directly to the exterior of the cell. Bioactive PalB was expressed and secreted extracellularly either as HlyA fusion (i.e. PalB-HlyA via Type I system) or an intact protein (via Type III system) with minimum impact on cell physiology. However, the secretion intermediates in the intracellular fraction of culture samples were non-bioactive even though they were soluble, suggesting that the extracellular secretion did mediate the development of PalB activity. PalB secretion via Type I system was fast with higher specific PalB activities but poor cell growth. On the other hand, the secretion via Type III system was slow with lower specific PalB activities but effective cell growth.

Functional expression of lipase from *Burkholderia* sp. C20 (Lip) in various cellular compartments of *Escherichia coli* was explored. The poor expression in the cytoplasm was improved by several strategies, including coexpression of the cytoplasmic chaperone GroEL/ES, using a mutant *E. coli* host strain with an oxidative cytoplasm, and protein fusion technology. Fusing Lip with the N-terminal peptide tags of T7PK, DsbA, and DsbC was effective in boosting the solubility and biological activity. Non-fused Lip or Lip fusions heterologously expressed in the periplasm formed insoluble aggregates with a minimum activity. Biologically active and intact Lip was obtained upon the secretion into the extracellular medium using the native signal peptide and the expression performance was further improved by coexpression of the periplasmic chaperon Skp. The extracellular expression was even more effective when Lip was secreted as a Lip-HlyA fusion via the α -hemolysin transporter. Finally, Lip could be functionally displayed on the *E. coli* cell surface when fused with the carrier EstA.

Keywords: *Escherichia coli*, recombinant protein expression, YFP, lipase, cell-surface display, protein secretion, inclusion bodies, proteolysis, mutagenesis, chaperone, fusion tags, cell physiology, stress pathways.

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Chapter 1

Introduction

1.1 Overview

The term “genetic engineering” was first coined in Jack Williamson’s science fiction novel *Dragon’s Island* in 1951, about two years prior to James Watson and Francis Crick discovered that ‘the double helix DNA is the secret of life’; the medium for transmission of genetic information. The 1960s witnessed the “Green Revolution” when genetically improved seed was developed to meet food production targets in economically backward countries and earned the originator, Norman Borlaug, the Nobel Prize in 1970. Along with “recombinant DNA technology”, biotechnology has impacted all aspects of modern society in the twenty-first century including food, agriculture, health/medicine, pharmaceuticals, alternative bio-fuel sources, industrial enzymes, biodegradation and bioremediation, cosmetics, biosensors, bioinformatics, and advancement of knowledge. It has enabled scientists to extract and transfer specific genes from one species and overproduce in another host. Among the various expression systems such as yeast, insects, bacteria, and mammalian cells, the gram negative bacterium *E. coli* still remains as the most popular host for high level production of recombinant proteins from both prokaryotic and eukaryotic sources due to its many advantages (93, 173, 206). The past 25 years have seen enormous progress in the understanding of recombinant protein expression in *E. coli* including vast number of cloning vectors and mutant strains, improving mRNA stability, codon-optimization, coexpression of chaperones, post-translational modifications, use of secretory pathways, minimizing including bodies, improving solubility, and decreasing proteolysis (171).

Studies in our lab have focused on enhancing the functional expression of recombinant proteins in *E. coli*. While most of the proteins are expressed in the cytoplasm, extracytoplasmic expression such as periplasm, outer membrane, and extracellular medium can offer several advantages and the related technical issues have been emphasized (57). Penicillin acylase (PAC), the hetero-dimeric periplasmic protein with a unique posttranslational pathway (164) that formed insoluble inclusion bodies when expressed in *E. coli* (259), served as a model system, demonstrated that DegP, a periplasmic heat shock

protease with chaperone function at lower temperatures significantly improves *pac* expression (162). Not only did its coexpression reduce the inclusion body formation, but it also improved the cell growth and the PAC activities significantly (196, 214). It was also discovered that arabinose-induction of *lac* derived promoters for *pac* expression significantly reduced the inclusion bodies, enhanced cell growth and improved PAC activities (195).

Microbial cell-surface display has been extensively explored due to its significant impact on various biotechnological and industrial applications, such as vaccine development, biosensor design, high-throughput screening of macromolecular libraries, and preparation of whole-cell biocatalysts (20, 159). The passenger protein is often expressed as a fusion with a carrier protein that serves as an anchoring motif on the outer membrane. Several studies have reported the use of various carrier domains, including lipoproteins, outer membrane proteins, autotransporters, porins, fimbria and flagella proteins, S-layer proteins, etc. (260). Up to now, most studies relevant to microbial cell-surface display focused on discovering novel carrier systems; however, another critical issue limiting display performance is physiological impact on the growing cells, has been seldom addressed. Cells with a high-level display tend to activate the extracytoplasmic stress pathways (2, 4, 232, 240) that sense and respond to perturbations within the cell envelope. The issue of concern is, how best to deal with this problem.

Any protein is rarely enriched when produced in the natural host; recombinant protein overproduction is frequently the sole applicable procedure. PalB (Lipase B from *Pseudozyma antarctica* previously known as *Candida antarctica*) is the most popular among the available lipases due to its interesting properties and important applications in organic synthesis and biotechnology (145). Our interest in PalB arises from its potential for biotransformation as opposed to chemical conversion of vegetable oils for biodiesel production. Previously PalB was produced in the heterologous hosts such as *Aspergillus oryzae* (122), *Pichia pastoris* (237), and *Saccharomyces cerevisiae* (313). Recent studies identified the disulfide bond formation as a serious impediment to producing bioactive PalB in *E. coli* cytoplasm. Though this issue was overcome by using the *trxB* mutant strain of *E. coli* (Origami) and N-terminal fusion tags, technical limitations related to protein aggregation, and impaired cell physiology

still prevailed. Furthermore, PalB was extremely unstable and highly susceptible to proteolysis when targeted to in the periplasm; the *E. coli* compartment with oxidizing environment that favors disulfide bond formation. Although periplasm has fewer proteases than cytoplasm, proteolytic degradation is substantial and usually a major impediment to heterologous protein expression in *E. coli*. The functional expression of bioactive PalB resistant to proteolysis in *E. coli* is a major challenge for improving the bioprocess for recombinant PalB production.

PalB is an extracellular enzyme produced in *Pseudozyma antarctica* with three intermolecular disulfide bonds that determine the development of protein structure and bioactivity (287). The gram negative bacterium *E. coli* has very few mechanisms for extracellular secretion of proteins. Type I secretion system, known as the ABC transporter, secretes *E. coli* toxins and exoenzymes via a one-step process across both the cytoplasmic and outer membranes without a periplasmic intermediate (24). On the other hand, Type III secretion system represents a specific mechanism associated with the secretion of pathogenesis factors or flagellar proteins from the cytoplasm, bypassing the periplasm, directly to the exterior of the bacteria (278). Methodological approaches have also been developed to secrete periplasmic proteins to the culture supernatant of *E. coli*. They include (1) the use of leaky mutant host strains (233) and cell-wall-less L-form *E. coli* (105), (2) chemical or biochemical permeabilization of outer membrane (52, 251), and (3) coexpression of protein release factors such as bacteriocin release protein (BRP) (288), colicin E1 lysis protein (Kil), *tolAIII* and *out* genes (294). Given the technical limitations of intracellular production of PalB, its extracellular secretion appears to be a plausible exploration. Specific secretion of the foreign gene products to the extracellular medium also provides significant advantages in terms of least proteolysis, improved disulfide bond formation and protein folding, enhanced protein stability, and simpler downstream processing.

Lipases from *Burkholderia* are interesting enzymes due to their fine characteristics of thermal stability, alkaline pH and organic solvent tolerance, high activity for various substrates, and transesterification activity (215). However studies on overexpression of *Burkholderia* lipase are very rare in literature. Lipase gene in *Burkholderia* is described to be

present in an operon along with its lipase-specific foldase (Lif) (228). Attempts to express *Burkholderia* lipase in *E. coli* resulted in very low amounts of active soluble lipase, with the protein recovered as aggregates despite the coproduction of Lif. Active enzyme was recovered only by in vitro folding. Another study reported the expression of lipase in the form of inclusion bodies and the soluble protein variants were obtained by mutagenesis (104). The functional expression of *Burkholderia* Lip using various strategies available for heterologous protein expression in *E. coli* is worth exploring to identify the factors limiting its functional expression.

1.2 Research Objectives

The overall objectives of this thesis are as follows:

- i. Investigation of the cell physiology and culture performance for *E. coli* cell-surface display by manipulation of a periplasmic chaperone Skp. Quantification of extracytoplasmic stress using *lacZ* reporter gene fusions with the promoters of *degP*, *cpxP*, and *rpoH*.
- ii. Isolation of a stable PalB variant against intracellular proteolysis for improvement of protein production bioprocess.
- iii. Functional expression of PalB by extracellular secretion to circumvent the limitations of intracellular misfolding and proteolysis.
- iv. Exploration and systematic study of various strategies of heterologous protein expression in *E. coli* for achieving functional expression of lipase (Lip) from *Burkholderia* sp. C20.

1.3 Outline of the Thesis

This thesis consists of four manuscripts preceded by an introduction, literature review, and followed by conclusions. The scope of each chapter is listed as follows:

Chapter 1 gives an introduction to the thesis, including an overview of recombinant protein expression systems used in this thesis and the technical limitations. The hypothesis, objectives and the scope of the thesis are also given in this chapter.

Chapter 2 reviews the previous progress on understanding recombinant protein production in *E. coli*, including gene expression systems, regulation at transcriptional and translational levels, targeting the gene to different compartments, protein folding and role of molecular chaperones, stress regulatory pathways, fusion protein technology, and optimal cultivation conditions for high-level protein production. A brief introduction to the proteins used as model systems in this research is also given in this chapter.

Chapter 3 investigates the physiological impact and culture performance of yellow fluorescent protein (YFP) cell-surface display in *E. coli*, the effect of coexpression of the periplasmic chaperone Skp, and the associated extracytoplasmic stress using *lacZ* reporter gene.

Chapter 4 describes the isolation of a stable PalB mutant resistant to intracellular proteolysis, the effect of DsbA chaperone coexpression in minimizing inclusion bodies, improving solubility, and bioactivity of the overexpressed protein.

Chapter 5 presents the characterization of extracellular secretion of PalB using the Type I α -hemolysin transporter, and the Type III modified flagellar apparatus. Soluble and bioactive PalB was secreted to the culture supernatant with minimum contamination from host proteins, while minimizing the effect of proteolysis, and having a negligible impact on cell physiology.

Chapter 6 describes the construction of *Burkholderia* Lip expression vectors and presents a systematic study of its expression in *E. coli*. The effect of targeting *lip* gene to various compartments, coexpression of folding factors, and fusions with soluble tags are investigated.

Chapter 7 presents the conclusions of this study, contributions of this research, and recommendations for future work.

Chapter 2

Literature Review

Heterologous protein expression has had a great impact on industrial applications, medicine and research. Earlier, the protein was isolated from their natural sources with low yields due to loss of protein and/or activity during complex purification steps and low expression levels in native organism, making them expensive and unattractive for large-scale applications. Itakura *et al.* reported the first heterologous production of a protein, a small peptide somatostatin (14 amino acids) in 1977 in *Escherichia coli* (129). The possibility to isolate the desired gene, overexpress in a heterologous host, and purify the pure protein has paved the way to investigate protein-protein interactions, enzyme kinetics, functional assays, structural analysis including crystallization, protein structure and NMR, antibody generation, and large scale production of biocatalysts and therapeutic proteins etc. The choice of an expression system for recombinant protein production depends on many factors including cell growth characteristics, gene expression levels, posttranslational modifications, regulatory issues concerning therapeutic proteins, process, design, scalability and other economic considerations (63), (121, 248). Among the various expression systems such as yeast, bacteria, insects, and mammalian cells; *Escherichia coli* still retains its popularity as the most versatile host because of its widely characterized genome, availability of an increasingly large number of cloning vectors, and mutant strains, its ability to grow rapidly, and reach high density on inexpensive substrates (270), (173), (93). This was demonstrated first by Ely Lilly's rDNA insulin, then by Monsanto's bovine growth hormone (bGH). In spite of the extensive knowledge on recombinant protein production in *E. coli*, not every gene can be expressed in this organism. This may be due to the subtle features in the gene sequence, translational efficiency and stability of mRNA, misfolding and degradation of the protein by host proteases, host/protein toxicity, inability for posttranslational modifications required for eukaryotic proteins, limitations to facilitate extensive disulfide bond formation, and lack of proper secretion mechanisms for the efficient release of protein to the extracellular medium. Nevertheless, various strategies have been developed by researchers in the past for gene

expression in *E. coli*, including regulation at transcriptional and/or translational level, targeting the proteins to different compartments, protein folding and coexpression of molecular chaperones, misfolding and degradation within the cell, use of solubility enhancing fusion tags, and optimization of fermentation conditions. However, no strategy 'fits all'. Every protein is unique, various strategies are tailored together to achieve maximal production of bioactive protein without affecting the physiology of the growing cells.

2.1 Transcriptional and Translational Regulation

The typical prokaryotic expression vector (represented in Figure 1) consists of an inducible promoter, ribosome binding site, Shine Dalgarno sequence (SD), target gene, transcription terminator, a replication origin that determines the plasmid copy number, and an antibiotic resistance marker for plasmid selection and propagation. Depending on the application, the cDNA may be cloned into plasmids with 15-60 (pMB1/ColE1) copies to a few hundred copies per cell (pUC origin). The coexpression plasmids are selected for compatibility, for e.g. ColE1 derived plasmids combined with plasmids with p15A replicon (12). A strong tightly regulated promoter that can be induced in a simple and cost effective manner is usually desired to achieve high level protein synthesis. A promoter is a sequence of nucleotides that contains signals for the proper binding and subsequent activation of RNA polymerase to a form, capable of initiating the synthesis of RNA. Within or immediately adjacent to the promoter, there is an additional sequence of DNA sequence for the specific binding of repressor or activator proteins that can modulate the activity of the promoter. The gene of interest may be expressed under the control of such a regulatory element within the cell (118). When a promoter is used for high level protein expression in *E. coli*, it should be able to accumulate protein within the cell upto about 30% of the total cellular proteins. For large scale production, the cells should be able to grow to a high cell density. Tightly regulated promoters are used for expressing proteins that are toxic to the host cell. The overexpressed genes may result in misfolding of the proteins, decrease the cell growth rate, cause cell lysis, and result in the loss of the recombinant protein production.

For many years, *E. coli* lactose utilization operon discovered by Jacob and Monod in 1964 has been the example of prokaryotic regulation. Therefore *lac* based promoters have been used extensively for the production of heterologous proteins in *E. coli*. Lactose is the natural inducer for *lac* derived promoter systems. Lactose after transglycosylation gets converted to allolactose which binds to the *lac* repressor to cause induction of the promoter. However it cannot be used in *lacY* or *lacZ* cells, where it cannot be transported into the cell or be converted to allolactose respectively. The strong induction by isopropyl- β -D-thiogalactopyranoside (IPTG) for the (*lac* and *trp*) hybrid promoters *tac* (29) or *trc* (36) are widely used for basic research (Figure 2.1). However, the use of IPTG for the large-scale production of human therapeutic proteins is undesirable because of its toxicity (81) and cost. The *lacI*^(Ts) gene encodes a thermosensitive *lac* repressor (1). Two different *lac* repressor mutants that are thermosensitive as well as IPTG inducible have also been reported (296). The activity of the phage *p_L* promoter, a cold response promoter is found to be highest at 20 °C, and declines as the temperature is raised (92). The promoter of the major cold shock gene *cspA* is also active at reduced temperatures (95). However these promoters can induce several heat shock and cold shock proteins within the cell. Still the most widely used promoters for large-scale protein production use thermal induction (*p_L*) or chemical inducers (*trp*). When cold response promoters are used, protein folding will be only slightly affected at about 15 to 20 °C, whereas the rates of transcription and translation will be substantially decreased. This in turn, will provide sufficient time for protein refolding, yielding active proteins, and avoiding the formation of inclusion bodies (209). The pH promoter has been reported to be very strong for recombinant proteins produced at levels of up to 40 to 50% of the total cellular protein and is another attractive option for high level gene expression (53). However this might be just atypical as protein synthesis depends on several other factors other than promoter strength.

Another *lac* derived promoter system is the pET vector system. Here the T7 RNA polymerase is under the control of the prophage (λ DE3) encoding the IPTG-inducible *lac* UV5 promoter (272). A single copy of RNA polymerase within the cell is sufficient for gene expression. However there are certain limitations. In many cases the overproduced protein

cannot reach a biologically active conformation and tends to form insoluble inclusion bodies. In other cases high level of mRNA can be toxic to the cell. The leaky expression by T7 RNA polymerase may result in expression instability (266). The *phoA* promoter is induced by limitation of phosphate in the medium (187) and the *trp* promoter by the limitation of tryptophan (273). The *araBAD* (or P_{BAD}) promoter is induced by the sugar L-arabinose. Although it is considered to be weaker than *tac* or *trc* promoter, in many cases it has been found to be better suited for protein overexpression (107). The P_{BAD} promoter is usually characterized by fast induction, modulated induction, and tight repression by AraC. The *tet* promoter is another system with very tight transcriptional regulation under the control of *tet* repressor and convenient chemical induction by tetracycline or anhydrotetracycline (282). In this case the problems with host type genotype or influence of glucose through catabolite repression is avoided.

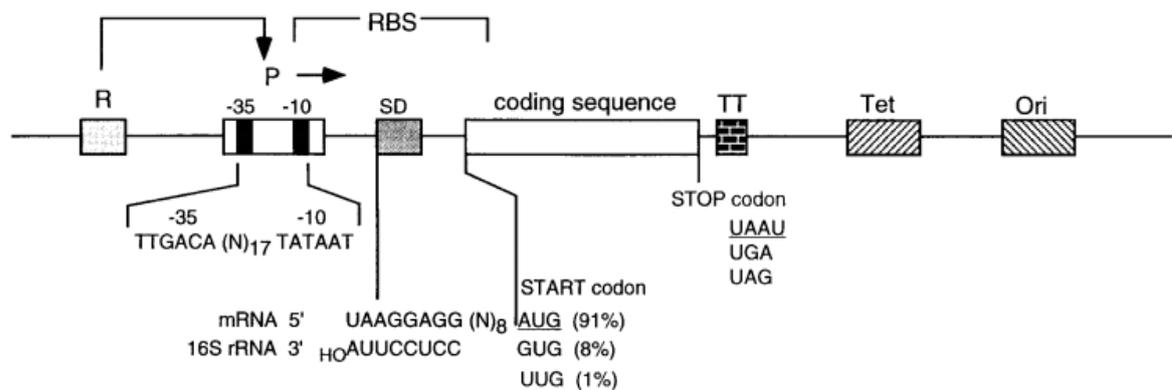


Figure 2.1 Schematic presentation of the salient features and sequence elements of a prokaryotic expression vector. Shown as an example, is the hybrid *tac* promoter (P) consisting of the 235 and 210 sequences, which are separated by a 17-base spacer. The arrow indicates the direction of transcription. The RBS consists of the SD sequence followed by an A1T-rich translational spacer that has an optimal length of approximately 8 bases. The SD sequence interacts with the 39 end of the 16S rRNA during translational initiation, as shown. The three start codons are shown, along with

the frequency of their usage in *E. coli*. Among the three stop codons, UAA followed by U is the most efficient translational termination sequence in *E. coli*. The repressor is encoded by a regulatory gene (R), which may be present on the vector itself or may be integrated in the host chromosome, and it modulates the activity of the promoter. The transcription terminator (TT) serves to stabilize the mRNA and the vector, as explained in the text. In addition, an antibiotic resistance gene, e.g., for tetracycline, facilitates phenotypic selection of the vector, and the origin of replication (Ori) determines the vector copy number (171).

Differences in the translation efficiencies of different mRNAs predominantly depend on the 5' end of the mRNA species. However there is no universal sequence for the efficient initiation of translation, nevertheless general guidelines have been suggested. Initiation of translation requires a Shine Dalgarno sequence of consensus 5'-UAAGGAGG-3' (250) complementary to the 3' end of 16S rRNA, followed by an initiation codon. The most common and efficient one (two to three folds) is the AUG codon followed by GUG (8%), and rarely UUG (262) and AUU (41). Several translational enhancer sequences that markedly improve the expression of heterologous genes have been identified in the past. The g-10L sequence compared to the SD sequence caused a 40 to 340 fold increase in the expression of several genes (205). When placed upstream of SD for overexpression of *lacZ*, β -galactosidase activity increased by 110 folds (207). U-rich sequences upstream of 5' untranslated region acted as translation enhancers for the expression of the *E. coli atpE* gene (177), human interleukin-2, human interferon beta (243), and RNaseD (312). An interesting study by Sprengart et al. (258) demonstrated that sequences termed the downbox (DB), immediately downstream of the start codon also play an important role in the translation. Thus, in addition to the SD sequence and the start codon, other sequences in the mRNA also affect the translational efficiency. The mRNA stability can affect expression rates. RNase E, RNase K, RNase II, RNase III, and polynucleotide phosphorylase (PNPase) are the common enzymes identified to participate in mRNA degradation (19). 5' UTR regions of mRNAs (16) and the stem-loop structures from the 3' UTRs and intercistronic regions (117) are known to

stabilize mRNAs in *E. coli*. The 5' UTR of *ompA* prolonged the half-life of a number of heterologous mRNAs. Wong and Chang demonstrated that the addition of 3' UTR regions that can form stem loop structures to penicillinase (*penP*) and the human interleukin cDNA, increased the half life of the mRNAs and enhanced the production of the corresponding polypeptides. They also suggested that gene expression might be enhanced by the use of host strains deficient in specific RNases, however mutant strains were often inviable (70).

2.2 Expressing Genes in Different *E. coli* Compartments

2.2.1 Cytoplasmic Expression

For most applications, it is desirable to achieve maximal production of the target protein in the cytoplasm where the nascent polypeptides are first synthesized, problems related to the formation of insoluble inclusion bodies is a significant barrier in the expression of genes in the cytoplasm. However the inclusion bodies offer certain advantages (50) as they facilitate the isolation of protein in high purity and concentration for refolding in vitro (239); the target protein is also protected from the proteases in the host. Proteins that are toxic to the cell when in the active form can also be produced first as inclusion bodies and then refolded separately (115). However, the protein folding in vitro is an arduous task and therefore not an attractive alternative. It is also not certain if the refolded protein would be able to regain its biological activity. The inclusion body formation of human T-cell receptor has been modeled on the parameters: charge average, total number of residues, turn forming residues, cysteine and proline fraction (1).

Several strategies have been developed to minimize the formation of inclusion bodies in the cytoplasm. The most common approach is to reduce cultivation temperature during fermentation (244) thereby reducing the rate of transcription and translation. This also reduces the hydrophobic interactions that are responsible for protein misfolding. Several alternative to this end have been investigated; 1) using a cold inducible promoter (209), 2) selection of suitable host strain (142), 3), supplementation of non-metabolizable sugars for improving the protein solubility (32), 4) alteration of pH (268) etc. The reducing environment of *E. coli* still poses a grave problem; the best example is of the protein alkaline

phosphatase which is active only in the periplasm (67). However the disulfide bonds can now be formed in the cytoplasm of a *trxB* mutant *E. coli* strain (68). These mutations inactivate the *trxB* gene that encodes thioredoxin reductase which contributes to the sulfhydryl reducing potential of the cytoplasm (11). In a recent study from our lab, it was demonstrated that the *Pseudozyma antarctica* lipase B (PalB) was functionally expressed only in the cytoplasm of *trxB* and *gor* mutant *E. coli* strains Origami B(DE3) or by the coexpression of the disulfide bond chaperone DsbA, whereas it folded through the non productive pathway to form insoluble aggregates in the cytoplasmic expression of wild-type *E. coli* (305).

Any protein when expressed in the cytoplasm requires an initiation codon AUG (for Methionine). Although this extra amino acid might not affect the protein synthesized, but there are specific cases in which the extra Methionine had profound consequences. For example, the conformation of human hemoglobin is altered by an N-terminal Methionine residue (141). Moreover, the immunological properties of pharmaceutical proteins may change due to the presence of an extra amino acid residue. The coexpression of Methionine aminopeptidase that can remove the N-terminal Methionine is an alternative (119). Finally, the purification of recombinant protein from some 3000 gene pool (271) of intracellular proteins is another arduous task.

2.2.2 Protein Targeting to Periplasm

In contrast to the cytoplasm, the periplasm offers several advantages for protein targeting. The oxidising environment of the periplasm that contains Dsb-enzymes catalyzing the formation of disulfide bonds facilitates the proper folding of the proteins, and the protein degradation in the periplasm is also less extensive (269). Only 5 % of the total cellular proteins are present in the periplasm (200); therefore the recombinant protein can be efficiently concentrated and the purification is considerably less difficult. All the periplasmic proteins in *E. coli* require the secretion of their corresponding precursors across the cytoplasmic membrane mediated by a signal sequence. A wide variety of signal peptides, such as PelB (22), PhoA (281), OmpA (302), and SpA (189), have been adopted successfully as a genetic strategy to secrete recombinant proteins for expression in the periplasm or outer membrane of *E. coli*. The transport of proteins to the bacterial periplasm is an incompletely

understood process and the presence of a signal peptide does not always ensure efficient protein translocation through the inner membrane. For example, despite the correct cleavage of the signal peptide the T-cell receptor protein could not be detected in the periplasm (202). Coexpression of the periplasmic transport components like signal peptidase I (289), *secE*, and *prlA4* genes (222), and use of *prlF* mutant strains (256) are certain strategies for improving the translocation of proteins.

2.2.3 Extracellular Secretion

Secretion of recombinant proteins to the extracellular medium of *E. coli* has several advantages over intracellular expression. They include simpler downstream processing and purification, least proteolysis, improved protein folding and disulfide bond formation in the oxidizing environment outside the cell, enhanced solubility and bioactivity of the secreted protein, and N-terminus authenticity of the expressed polypeptide. Unfortunately very few proteins are secreted from *E. coli* due to the barrier from the outer membrane. The known secretion pathways in gram negative bacteria are Type I, II, III, IV, and V with varying mechanisms. The Type I, II, and III mechanisms are most commonly used in *E. coli* for secretion of toxins, degradative enzymes, and pathogenicity factors to the exterior of the cell.

The Type II secretion system is the commonly known Sec-dependant translocation of the proteins from the cytoplasm across the inner membrane to the cell envelope. This class includes most of the periplasmic and outer membrane proteins that are synthesized as a propeptide in the cytoplasm with a cleavable signal peptide (discussed in periplasmic expression of proteins). Genetic strategies have been developed in the past to secrete the intracellular proteins extracellularly including the use of leaky mutant strains (233) and cell wall-less L-form *E. coli* (105), outer membrane permeabilization by chemical or biochemical agents including addition of magnesium, calcium, TritonX, EDTA, and lysozyme (enzymatic) (52, 251). Coexpression of protein release factors such as bacteriocin release protein (BRP) (288), colicin E1 lysis protein (Kil), *tolAIII* and *out* genes (294) have been successfully used to secrete recombinant proteins such as penicillin amidase, β -glucanase, β -lactamase, phytase etc. However this approach leads to contamination by the host proteins and often deteriorates the growth of the producing cells.

The Type I secretion pathway comprises of the ABC family of transporters that secretes *E. coli* toxins from the cytoplasm to the exterior of the cell bypassing the periplasm with the help of ATP. *E. coli* α -hemolysin transporter is by far the most popular among the several Type I family secretion systems. The inner membrane protein HlyD trimer and HlyB form a complex with the help from ATP and interact with the outer membrane protein TolC to form the export conduit to transport the HlyA toxin/HlyA-fusion protein to the culture supernatant. It has been successfully used to secrete heterologous proteins, including single Ig domains (28), single chain Fv (79), and Shiga-like toxin Ile (286), that are otherwise hard to express intracellularly. Another study reported the modified flagellar apparatus for secretion of recombinant proteins including D1-D3 repeats from *Staphylococcus aureus*, the Peb1 adhesin from *Campylobacter jejuni*, α -enolase from *Streptococcus pneumoniae*, and the eukaryotic green fluorescent protein (170).

2.2.4 Bacterial Surface Display

Bacterial surface display is becoming an increasingly important research area for creating libraries of biomolecules that can be screened for desired properties, antibody production, bioadsorbents for removal of harmful chemicals and heavy metals, whole cell biocatalysts, vaccines, and biosensors. For a heterologous protein to be displayed on the outer membrane, it has to be fused to a carrier protein that can insert into the outer membrane of *E. coli*, and a signal peptide that can translocate the fusion protein across the cytoplasmic protein. The passenger protein may be fused with the carrier at the N-terminal, C-terminal, or as a sandwich fusion (Figure 2.2). The first example was the display of short gene fragments inserted into the genes for the *Escherichia coli* outer membrane proteins LamB, OmpA and PhoE, and the gene fusion products were found to be accessible on the outer surface of the recombinant bacteria (159). Since then, not only outer membrane proteins, but also *E. coli* lipoproteins Lpp (84, 85) and TraT (45), lipoprotein pullulanase from *Klebsiella pneumoniae* (147), *E. coli* adhesion protein AIDA-I autotransporter (134), EaeA intimin from pathogenic *E. coli* (297), fimbria proteins FimA and FimH, and flagellar protein FliC (298), have been employed to achieve heterologous surface display on *E. coli*. Functionally inactive esterase from *Pseudomonas aeruginosa* has been used to display lipases on the cell surface membrane

of *E. coli* (18). The ice nucleation protein from *Pseudomonas syringae* has been reported to accommodate large size heterologous protein for *E. coli* surface display (135) (150) (161). An interesting alternative is described by Hoischen et al. about a novel system that allows the display of recombinant proteins Staphylokinase protein on the cytoplasmic membrane using the L-form cells of *E. coli* (124). Cell-surface display has not been useful for high level gene expression to date. It has also been associated with deteriorated cell physiology resulting from outer membrane perturbation due to insertion of heterologous proteins.

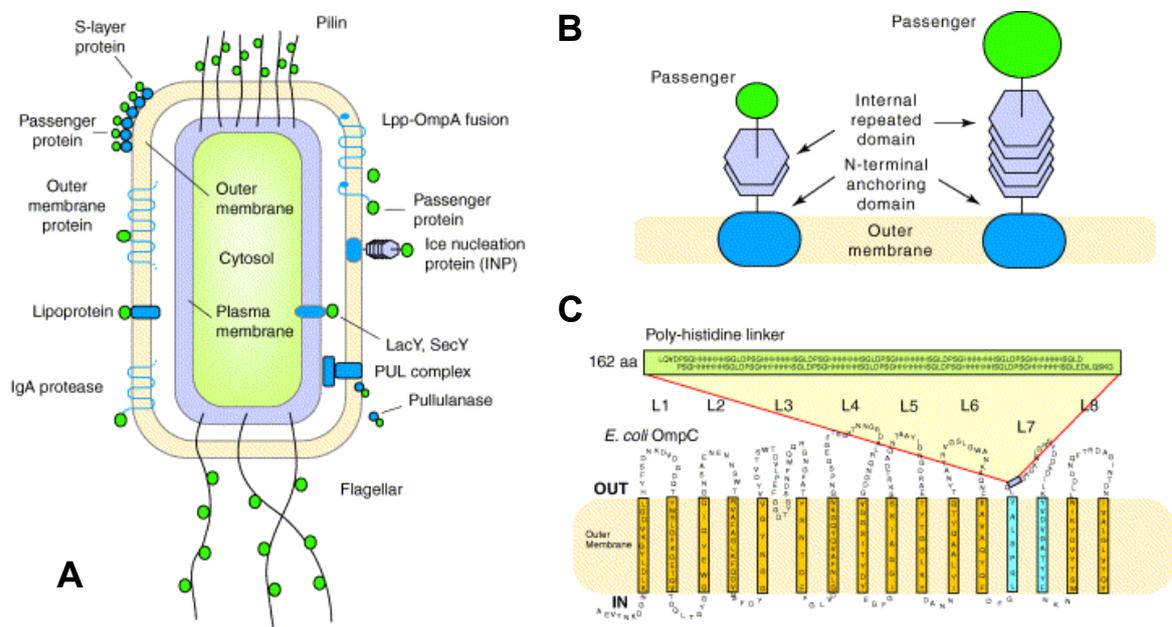


Figure 2.2 Cell surface display systems in gram negative bacteria (A) surface display systems (B) cell surface display using ice nucleation protein (INP) (C) surface display using *E. coli* OmpC (159).

2.3 Recombinant Protein Folding and Misfolding

Protein misfolding and degradation are linked as catabolism is linked to anabolism to conserve the cellular resources. This process recycles improperly folded proteins into their constituent amino acids. Protein misfolding happens as a result of failure of a polypeptide to

reach its native conformation, premature termination of translation, or by environmental stress. To cope with this situation cells have evolved its own mechanism to favour folding of a new polypeptide, refold partially folded proteins, dissolve aggregates, and dispose damaged proteins. Molecular chaperones are a class of folding modulators that perform such a function in the cell (83). They are constitutively expressed in the cell but are sometimes up regulated by increase in temperature or other stress to the cell (including heterologous protein expression) (123). Therefore they are also referred to as heat shock or stress proteins. The mechanism of functioning of a chaperone is to bind to the nonpolar region that is buried within the core of a substrate (15). They target on hydrophobic amino acids that have basic residues in their vicinity. Therefore, although there is some degree of substrate specificity but generally they are less selective.

2.3.1 Folding in the Cytoplasm

The protein folding in the cytoplasm (Figure 2.3) is modulated by three chaperone systems: DnaK-DnaJ-GrpE, trigger factor (TF), and GroEL-GroES (112). The trans-conformation of peptide-proline (X-Pro) is energetically favoured over the cis-conformation in proteins. Therefore this rate limiting trans to cis isomerization is catalyzed by a class of proteins called peptidyl prolyl isomerase (PPIases). TF is a cytoplasmic PPIase that binds to the ribosomes at the peptide exit site and are therefore related in the folding of short nascent chains (219). It has been reported that TF overexpression prevents the aggregation of endostatin, human oxygen regulated protein ORP150, and human lysozyme (198). However it should be noted that TF overproduction can cause cell filamentation. Longer nascent polypeptides are captured by DnaK. DnaJ is the cochaperone that ensures tight substrate binding by hydrolysing the DnaK bound ATP. The substrate pool for TF and DnaK pften overlap each other (69). GrpE catalyzes the substrate ejection by ADP/ATP exchange (153). They may be recaptured by DnaK-DnaJ for additional cycles of interaction or may be transferred to the GroEL-GroES system. GroEL is a large protein (800 kDa) and binds to both protein substrates and GroES. Like DnaK, GroEL favours hydrophobic and basic residues, and the trapped protein is able to fold in a hydrophilic environment within the molecule. The process does not involve dissolution of preformed recombinant inclusion bodies but is related to

improve folding of newly synthesized protein chains (112). However DnaK and GroEL can refold protein that can become unfolded by the environmental stress, when assisted by another class of chaperones called holdases. IbpA-IbpB, Hsp33 and Hsp31 are the identified holdases in *E. coli* (249). When the cell is under stress, these proteins bind the unfolded protein species and once the stress abates, transfer them to DnaK-DnaJ or GroEL-GroES. Thus they also prevent overloading on the folding chaperones. In previous studies from our lab, the cytoplasmic chaperones trigger factor, DnaK-DnaJ-GrpE, and GroEL-GroES individually or their cocktail was coexpressed with penicillin acylase (PAC) in the cytoplasm. Trigger factor could both solublize proPAC aggregates and increase PAC activity. However PAC activity was not simultaneously increased with proPAC solubilization when GroEL-GroES or DnaK-DnaJ-GrpE was coexpressed (304).

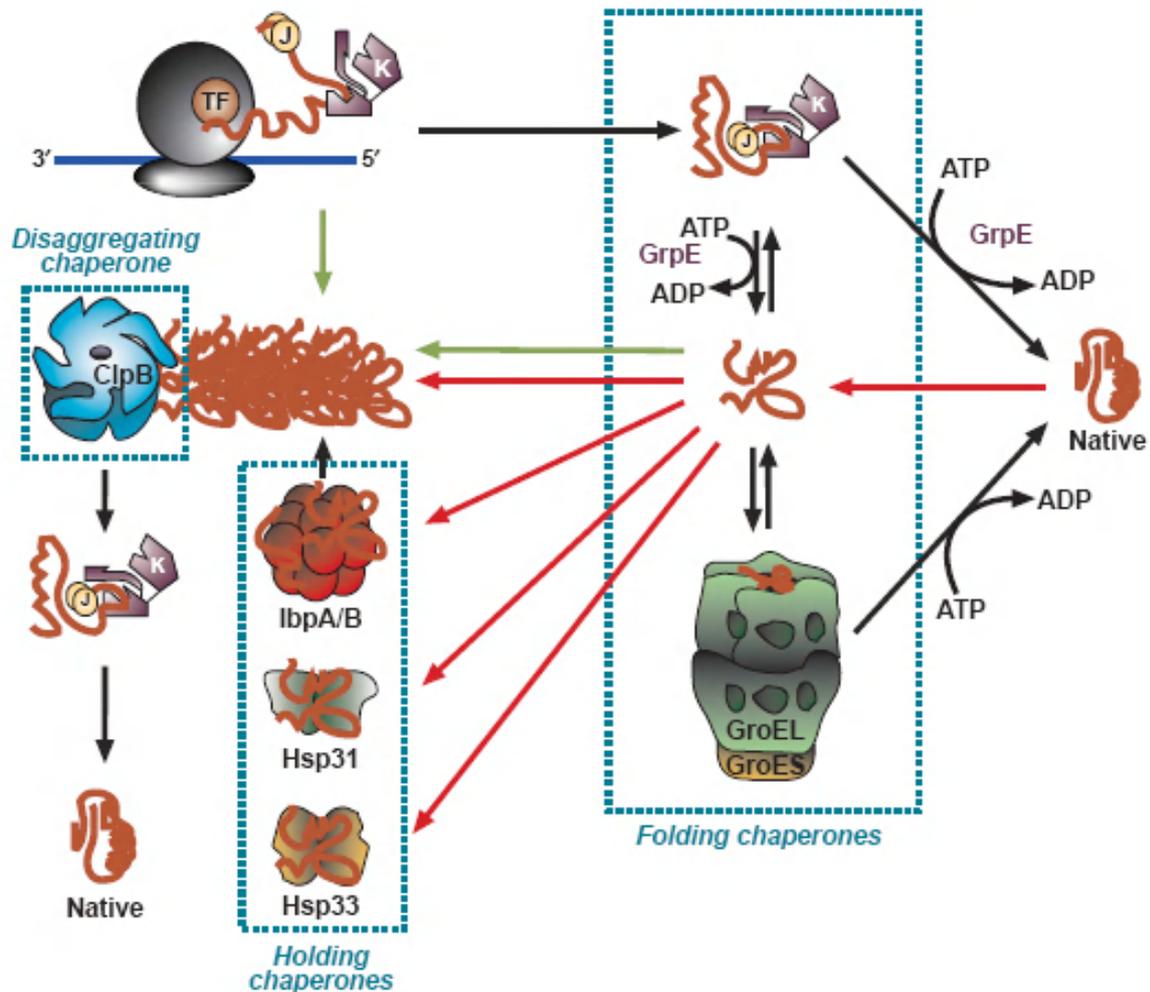


Figure 2.3 Chaperone assisted protein folding in the cytoplasm of *E. coli* Trigger factor (TF) and DnaK/J engage solvent exposed hydrophobic amino acids of nascent polypeptides to shield them from both solvent and each other. The polypeptides released from TF or GrpE mediated DnaK/J release, either they attain a native conformation, recycles to DnaK/J, or transfers to the GroEL/ES chamber for folding at infinite dilution. In times of stress (red arrows), thermolabile proteins unfold and aggregate. IbpB binds partially folded proteins on its surface to serve as a reservoir of unfolded intermediates until folding chaperones become available and intercalates

within large aggregates. The holding chaperones Hsp33 and Hsp31 become important under oxidative and severe thermal stress, respectively. ClpB promotes the shearing and disaggregation of thermally unfolded host proteins and cooperates with DnaK-DnaJ-GrpE to reactivate them once stress has abated. Recombinant proteins that miss an early interaction with TF or DnaK/DnaJ, that undergo multiple cycles of abortive interactions with folding chaperones or titrate them out, accumulate in inclusion bodies (green arrows) (15).

2.3.2 Periplasmic Folding Modulators

Unlike the cytoplasm, the bacterial periplasm is separated from the medium only by a porous membrane and is therefore directly affected by its variation. To date, certain groups of proteins are known to be involved in the protein folding in the periplasm. These include enzymes for formation and isomerization of disulfide bonds, PPIases, and foldases (Figure 2.4). The rate limiting disulfide bond formation in the periplasm step (137) is catalyzed by a set of thiol disulfide reductases known as Dsb proteins. The DsbA which is maintained in oxidised state by the four cysteines in DsbB, transfer the disulfide bond to proteins. DsbC is known to correct the incorrect disulfide bonds in the proteins. DsbC is in turn maintained in the reduced state by DsbD at the expense of NADH oxidation in the cytoplasm.

DegP/HtrA is an ATP independent protease present in the periplasm. Transcription of *degP* is induced by heat shock and is essential for the survival of bacterial cells above 42 °C. However at lower temperatures it switches from protease function to chaperone function (257). In another study from our lab, the amount of inclusion bodies of periplasmically expressed PAC reduced significantly upon DegP coexpression (196). Not only did coexpression of *degP* improve the PAC activity significantly but it also improved the cell physiology by reducing the level of cell lysis, relieving the inhibition of cell growth. Unlike DegP, the DegP mutant could not suppress the physiological toxicity caused by PAC overproduction. Neither could other periplasmic proteases DegQ or DegS had effect in reducing the physiological toxicity caused by *pac* overexpression. When *degP* was fused to *lac Z* on the λ attachment site of *E. coli* MC4100, it was expressed upon *pac* overexpression

which suggests that DegP is a heat shock protein induced in response to *pac* overexpression (214).

The ATP independent Skp captures unfolded proteins when they emerge from the Sec translocation apparatus (113). However its primary function is to assist the folding and membrane insertion of outer membrane proteins. On the other hand, Skp has been shown to improve the folding of recombinant proteins or antibody fragments in the periplasm of *E. coli* (31, 113, 160, 175). FkpA is known to exhibit both PPIase and chaperone function (10). They help in making the C-terminal domains of any protein accessible to prolyl bond isomerization. Outer membrane proteins are specific to SurA as it preferentially recognizes Ar-X-Ar motif (where Ar is an aromatic ring and X an amino acid residue) (25). SurA is a periplasmic protein required for the proper assembly of several outer membrane proteins. The *surA* gene was discovered during the investigation of mutant *E. coli* cells that are able to survive in stationary phase (155). The amino acid sequence in SurA is similar to parvulin and therefore is considered a peptidyl prolyl isomerase. On further investigation it was found that SurA is expressed in both exponentially growing and stationary phase cells and is required for growing at a higher pH, presumably to allow nascent outer membrane proteins to fold. Lack of SurA interferes with an early folding step in LamB maturation, negatively effects the expression of OmpC, OmpF and OmpA. *surA*⁻ cells have a defective cell envelope with increased sensitivity to SDS and EDTA. It was also found that SurA binds to the peptide that has an Ar-X-Ar motif which is common in outer membrane proteins (25).

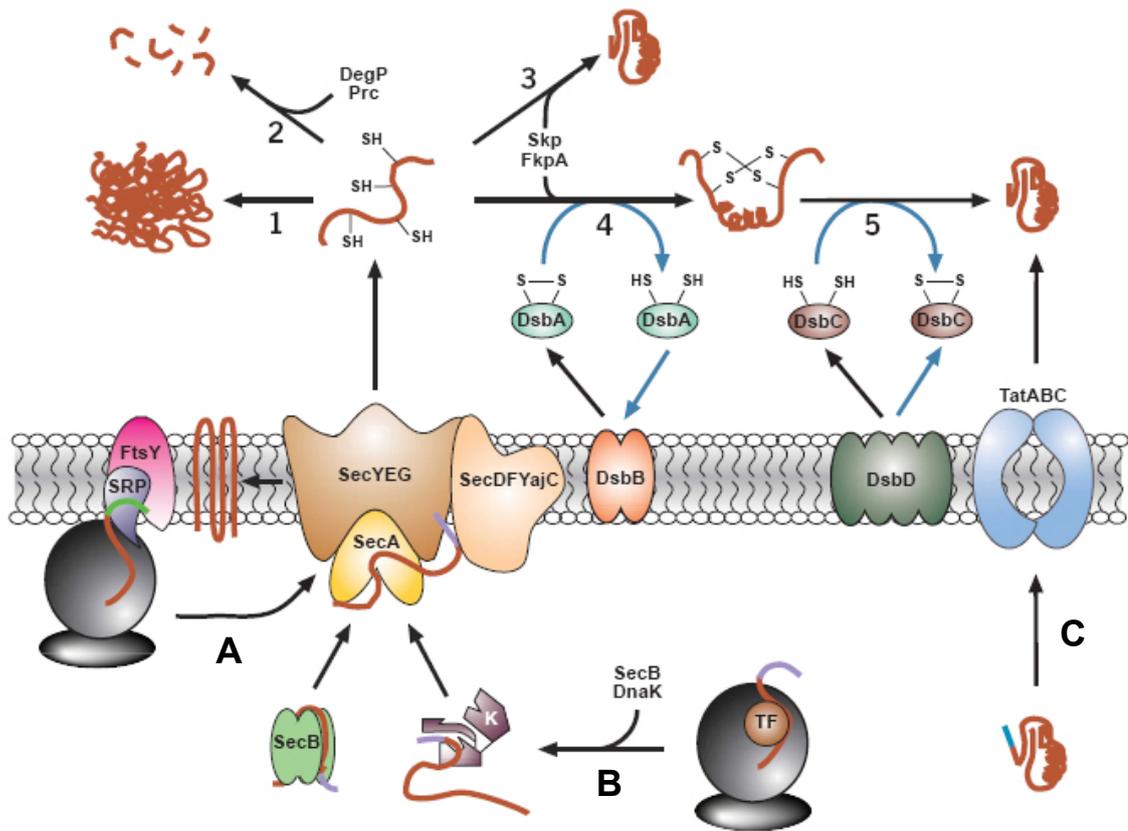


Figure 2.4 Export and periplasmic folding pathways. Proteins destined for export can be translocated across the inner membrane in three different fashions. (A) Preproteins with highly nonpolar signal sequences (green) or transmembrane segments of inner membrane proteins are recognized by SRP which, along with TF, scans nascent chains. SRP-dependent export involves delivery of the ribosome-nascent chain complex to FtsY and subsequent translocation through the SecYEG-SecDFYajC translocon. (B) The vast majority of preproteins have less hydrophobic signal sequence (lavender) and undergo Sec-dependent export. TF associates with the nascent polypeptide, halting cotranslational folding. As the chain grows, TF dissociates and the polypeptide is transferred to SecB or DnaK that maintain it in an extended conformation. Delivery to SecA and ATP-dependent translocation through SecYEG completes the process. (C) Preproteins with signal sequences containing the twin-arginine motif (cyan) are

exported via the Tat-dependent pathway in a folded form. After cleavage of the signal sequence, partially folded periplasmic proteins may aggregate (1), undergo proteolysis (2) or reach a native conformation, possibly with the assistance of folding modulators (3). Cysteine pairs in proteins containing disulfide bonds are oxidized by DsbA (4) whereas incorrect disulfides are isomerized by DsbC (5). These oxidoreductases are reactivated by DsbB and DsbD, respectively. Black arrows show products obtained after each step, whereas blue arrows represent electron flow (15).

2.4 Protein Degradation

Under prolonged or severe stress conditions, the above said chaperone-systems are unable to prevent protein aggregation. These protein aggregates are dealt with a set of molecules in *E. coli*. Among these are ClpA, ClpB, ClpX and ClpY. ClpB is known to solublize the aggregates while others are more or less involved in proteolysis (157). There are some less characterized proteins such as HtpG, HscA, HscC, and SlpA whose functions are unclear, but may play some role in reduction of protein misfolding (15). Though the periplasm contains fewer proteases than the cytoplasm, protein degradation by cell extracytoplasmic proteases is still considered as a serious impediment to the production of secreted proteins. DegP, Tsp, protease III, and OmpT are four common extracytoplasmic proteases. DegP is a periplasmic heat-shock protease (165, 263). The outer membrane protease OmpT specifically targets dibasic amino acid residues such as Arg-Lys and (74). Tsp has been shown to recognize and cleave C-terminal residues (254). The substrate specificity of Protease III is not known, though it can digest secreted fusion proteins (13).

2.5 Stress Regulatory Pathways in *E. coli*

Stress response pathways that sense protein misfolding, heat shock, or other cellular damage are highly conserved amongst living organisms (37, 231, 308). Both prokaryotic and eukaryotic cells synthesize a set of proteins called heat shock proteins (HSPs) to counteract the accumulation of damaged proteins (38, 91, 102, 311). Most HSPs are either proteases or chaperones for degrading or refolding misfolded proteins. In eukaryotic cells, protein

misfolding signals are sensed in the endoplasmic reticulum and transduced to the nucleus of the cell by a process called the unfolded protein response (UPR) (216). The stress response in the gram negative bacterium *Escherichia coli* has been compartmentalized into distinct cytoplasmic and extracytoplasmic sigma factors. The cytoplasmic stress response coordinated by the cytoplasmic sigma factor σ^{32} (103, 151) is well characterized with a set of chaperone members including DnaK/J and GroEL/ES (17, 264, 265). The stress response in the extracytoplasmic space (including the inner membrane, periplasm, and outer membrane) differs from the cytoplasmic one in that the stress signals are generated in the periplasm or outer membrane and transduced to the cytoplasm. For example, it can be induced by excessive amounts of unfolded/misfolded recombinant proteins in the periplasm, unfolded outer membrane proteins, heat shock, change in medium pH, etc. (23, 178, 184). The extracytoplasmic stress response is controlled by at least two different but partially overlapping stress regulons, i.e. the extracytoplasmic sigma factor σ^E (178, 230) and the Cpx two-component system (59).

The σ^E stress regulon induces the expression of proteases, foldases, envelope localized chaperones, and genes involved in lipid biogenesis, etc. (62) and is essential not only for stress response but also for cell viability under abnormal growth conditions (56). The σ^E regulon consists of several genes that can be categorized as periplasmic folding factors, degradases, outer membrane biogenesis, sensor proteins etc. It is encoded by the sigma factor *rpoE* or σ^E (238). Normally, σ^E is sequestered in its inactive state by the binding of an anti-sigma factor of RseA which is an inner membrane spanning protein with its periplasmic domain bound to RseB (a periplasmic protein that stabilizes RseA) and its cytoplasmic domain bound to σ^E (55, 56, 185). Upon experiencing heat shocks, RseA is rapidly degraded to release σ^E that can bind to RNA polymerase for transcription of the stress response genes in the regulon (3). Since RseA plays a central role in regulating the σ^E pathway, studies have been conducted to identify the factors responsible for RseA degradation. It has been shown that DegS is a periplasmic serine protease responsible for the degradation of RseA (3, 6). Misfolded proteins bind to the PDZ domain of DegS to relieve its inhibited protease domain and to initiate the degradation of the periplasmic domain of RseA

(293). However, the cytoplasmic domain of RseA is still sufficient to inactivate σ^E (42). Another inner membrane protein, YaeL, is a member of the σ^E regulon and has been shown to degrade the cytoplasmic domain of RseA after its periplasmic domain is degraded by DegS (5). Several genes, including *rpoH*, *degP*, *yaeL*, and *fkpA*, etc., are regulated by σ^E . The *rpoH* gene comprises of three promoters: P1, P3, P4 (71). The promoters P1 and P4 are found to be active at exponential phase, however the transcription of *rpoH* is found to be completely dependent on P3 at higher temperatures. Studies on the regulation of *rpoHP3* led to the identification of σ^E at higher temperatures (75, 295).

The Cpx envelope two-component regulatory system consists of a sensory inner membrane kinase (CpxA) and a cytoplasmic response regulator (CpxR) (120). CpxA has a periplasmic component and a cytoplasmic signaling domain. Upon detection of stress it autophosphorylates at a conserved histidine residue with the help of ATP. It then transfers this phosphate to an aspartate residue at the amino terminal of the CpxR. The CpxR-phosphate then activates several proteins that are involved in the protein folding, degradation etc. in the cell envelope (232). The Cpx regulon is positively autoregulated and negatively feedback inhibited by a periplasmic protein CpxP. (60). CpxP is present in the periplasm under non stress conditions associated with the periplasmic component of CpxA. Thus the Cpx regulon remains in 'shut down' condition (58). However when cells encounter misfolded proteins/inclusion bodies in the periplasm, CpxP complexes with these protein aggregates. Once the protein aggregate-CpxP complexes are formed, they are available for proteolysis in the cells. Thus in the presence of misfolded protein aggregates, CpxP is titrated by DegP during the process of degradation of misfolded proteins (128). Once the CpxA-CpxP complex is relieved, the Cpx stress regulatory pathway becomes positively regulated for the expression of several genes including *degP* and *cpxP*.

2.6 Fusion Protein Technology

Although recombinant protein expression and purification have been well addressed, they often exhibit variability in expression, solubility, stability, and functionality; thereby becoming difficult targets for large scale production and analyses. Although fusion tags were

first designed to facilitate affinity-purification of proteins, soon they were recognized as invaluable tools for improving the expression, solubility, and production of biologically active proteins, especially for difficult-to-express proteins. Although the reason for difficulties for expression and solubilization of certain proteins is not known, two factors that might contribute are the rate at which translation and protein folding occurs, which is much faster in *E. coli* as compared to other eukaryotic systems (299). Today, there are a number of common fusion tags namely maltose binding protein (MBP) (140), glutathione S-transferase (GST) (203), ubiquitin (Ub) (211), small ubiquitin-like modifier (SUMO) (172), thioredoxin (TRX) (154), disulfide bond enzymes (DsbA/C) (72), NusA (66), seventeen kilodalton protein (Skp) (47), T7 protein kinase (T7PK) (47), solubility enhancing tag (SET) (314), Flag (Sigma) etc. that are used to express proteins in *E. coli*. MBP and GST have the advantage of affinity purification simultaneous to improvement in solubility and therefore are heavily used. While TRX, SUMO, NusA, and MBP have gained popularity as solubility enhancers, Skp and T7PK are used to produce hard-to-express proteins. The most probable reason for improved folding and stability of the fusion proteins are that the fusion partner efficiently reaches the native conformation as it emerges from the ribosome.

However all the fusion tags suffer from the same problem; none of them can equally improve the expression and solubility of all proteins. Although, the protein fusions can be directly used in many applications, the removal of fusion tags is necessary in other cases especially when large fusion tags have been fused. The fusion tag is removed in almost all cases with cleavage by a protease. The most common of them are TEV (139), thrombin, fXa, and EK (132). The principle concerns about the use of protease are the non-specific digestion of the target protein, incomplete cleavage, expensive proteases, and additional steps for protein purification. Another concern is the reversal of solubility upon removal of the fusion tag (192). The most widely accepted hypothesis for this phenomenon is that the proteins exist as soluble aggregates in solution that precipitate as soon as the solubilizing partner has been removed. On the other hand, addition of fusion tags has been reported to be associated with loss or alteration of protein's biological activity, changes in protein conformation, and

toxicity of the target protein (76). Nevertheless, no other technology has been as effective as addition of fusion tags in improving the expression, solubility, biological activity, stability, and purification of heterologous proteins.

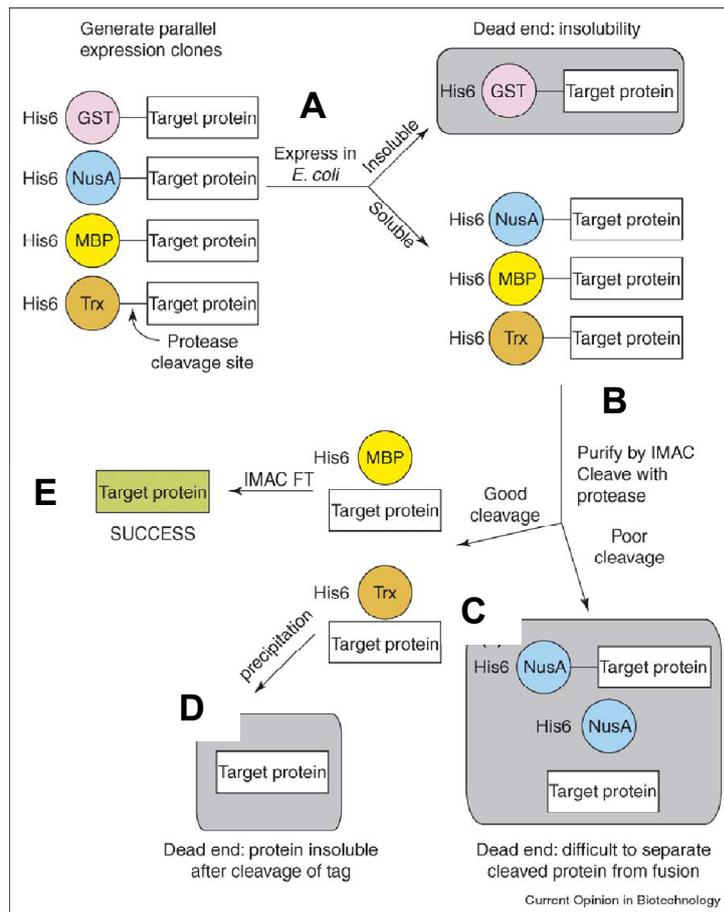


Figure 2.5 Schematic representation of the pathway from protein expression to purification using solubility tags. Four arbitrary tagged versions of the protein of interest are generated in *E. coli*. (A) After expression, some fusions will remain in the insoluble fraction and be lost from the pathway. (B) Soluble fusions are purified by IMAC (immobilized metal affinity chromatography) using the attached His6 tag. A protease is then used to cleave the fusion tag from the partner protein. (C) Some fusions

will not cleave efficiently, and will leave behind a mixture of cleaved and uncleaved proteins that cannot be easily separated. (D) Other fusions will cleave efficiently, but when separated from the solubility tag the partner protein will become insoluble and precipitate. (E) However, a well-behaved fusion will remain in solution and can be purified by a second IMAC step to remove the His6-tagged solubility tag and protease, leaving only the target protein in the flow through (IMAC FT) (76).

2.7 Fermentation Conditions

Last, but not the least, the fermentation conditions often has significant impact on the metabolism of the growing cells and protein production. Nutrient composition, temperature, pH, and oxygen availability are important parameters that can affect the production levels and proteolytic activities. However high-cell-densities can often result in severe drawbacks including low oxygen levels, inefficient mixing, and heat generation to activate the stress response mechanism in the cells that induce the expression heat shock proteases in the cell and might result in proteolysis of the target protein (14, 74, 309). The translation of mRNAs is greatly affected by temperature as well as changes in the culture medium (130). Supplementation of sugars such as arabinose, sorbitol, and glycine causes many-fold increase in the solubility and activity of the produced proteins (26, 195). By using appropriate batch, fed-batch, and continuous culture fermentations, high cell densities upto 100g/L can be achieved, and therefore can be cost-effective for production of recombinant proteins (158).

2.8 Proteins Used in this Study

2.8.1 Green Fluorescent Protein

The 27 kDa green fluorescent protein (GFP) originally isolated from the jellyfish *Aequoria victoria* is the most commonly used tool as a biological marker in molecular biology, cell biology and medicine (223, 284). It has an excitation and emission maximum at 395 nm and 509 nm respectively, which is the lower green portion of the visible spectrum. The fusion of protein to GFP does not alter its biological activity and therefore is highly useful as an expression reporter. It is highly stable to heat, alkaline pH, high pressure, photobleaching,

chaotropic and organic salts, and resistant to many proteases. It does not require a cofactor for development of activity, and is non-toxic in most cases (315). Many different GFP mutants have been engineered to meet the evolving needs of researchers. The first mutant reported was the Ser65Thr mutant of EGFP with increased fluorescence intensity and photostability (114). The mutant Phe64Leu of GFP folded with high efficiency. Later a series of mutations yielded the superfolder GFP that can fold rapidly even when fused to peptides with poor folding efficiency (277). Many colour mutants were derived including blue fluorescent protein (BFP with Tyr66His), cyan fluorescent protein (CFP with Tyr66Trp), and yellow fluorescent protein (YFP with Thr203Tyr), that find applications in fluorescence resonance energy transfer (FRET) experiments (284). Recently an infrared fluorescent protein was reported (252). Most fluorescent proteins absorb UV-visible range light, therefore the fluorescent protein in the infrared range allows more photos to travel without being bounced by electrons, thus more information can be derived from living specimens.

2.8.2 Lipase

Lipases have emerged as an important group of enzymes in the swiftly growing biotechnology industry due to its interesting properties and great potential for use in various industrial applications including food, detergent, cosmetics, and pharmaceuticals. They are ubiquitously present in plants, animals and microorganisms. Lipases are by and large produced from microbial sources including yeast, fungi, and bacteria.

2.8.2.1 *Pseudozyma antarctica* Lipase B

Lipase B (PalB) from *Pseudozyma antarctica* (originally known as *Candida antarctica*) belongs to the class of α/β hydrolases that have evolved divergently from various enantiomers, to catalyze reactions as various as hydrolysis of esters, thioesters, peptides, epoxides, and alkyl halides, or cleavage of carbon bonds in hydroxynitriles (125). The active site of PalB contains the catalytic triad, Ser105-His224-Asp187, common to all serine hydrolases. The active site lies buried in a cavity, but unlike most other lipases does not have a lid to cover the active site and does therefore not show interfacial activation (287). It is a triacylglycerol lipase and hydrolyzes triacyl glycerol to fatty acids, diacyl glycerol, monoacyl glycerol, and glycerol. It possesses a great regio- and stereo-selectivity, which is used in the

resolution of preparation of high priced pure enantiomers (46, 204). It shows a high stability in organic solvents, which together with its high stereoselectivity gives it properties which are difficult for many other catalysts to reach (145). Our interest in PalB arises from its potential as a biocatalyst for the production of biodiesel. The use of PalB as the target protein in this study significantly facilitates the experimental demonstration since it has a phenotype convenient for selection and screening purposes. Previously, recombinant PalB was expressed heterologously in fungi and yeasts, such as *Aspergillus oryzae* (122), *Pichia pastoris* (237), and *Saccharomyces cerevisiae* (313). Given the popularity of *E. coli*, functional expression of PalB in this bacterium was not demonstrated until recently (27, 167, 303, 305, 306), but the expression performance stands improvement due to various technical limitations. Since there are three intramolecular disulfide bonds in PalB and their formation can potentially affect the development of protein structure and bioactivity, functional expression of PalB in the oxidative periplasm of *E. coli* was conducted (303, 306). However, heterologous PalB targeting in the periplasm appeared to be extremely sensitive to intracellular proteolysis, resulting in its fast and complete degradation. Such proteolysis could be prevented by expressing PalB with N-terminal fusion tags (306), or with coexpression of periplasmic chaperones (303), or in the oxidative cytoplasm of genetically modified *E. coli* (i.e. Origami™ strains) (305).

2.8.2.2 Burkholderia Lipase

The most important lipase-producing bacterial sources are *Arthrobacter*, *Alcaligenes*, *Bacillus*, *Chromobacterium*, *Burkholderia*, and *Pseudomonas* (106). The fine characteristics of thermal stability, alkaline pH and organic solvent tolerance, high activity for various substrates, and transesterification activity make *Burkholderia* lipase an important biocatalyst (215) for a broad range of application in prospective industries like pharmaceuticals (80) and bioenergy production (201). Lipase gene in *Burkholderia* is described to be present in an operon along with its lipase-specific foldase (Lif) (228). Attempts to express *Burkholderia* lipase in *E. coli* resulted in very low amounts of active soluble lipase, with the protein recovered as aggregates despite the coproduction of Lif. Active enzyme was recovered only

by in vitro folding. Another study reported the expression of lipase in the form of inclusion bodies and the soluble protein variants were obtained by mutagenesis (104).

Chapter 3

Physiological Improvement to Enhance *Escherichia coli* Cell-Surface Display via Reducing Extracytoplasmic stress

3.1 Introduction

The advent of recombinant DNA technology has revolutionized the strategies for protein production. Due to the well-characterized genome and techniques available for genetic manipulation, *Escherichia coli* serves as the most common host for gene expression. The expressed gene products can be targeted in various cellular compartments, including cytoplasm, inner membrane, periplasm, and outer membrane (57) or can be even secreted into extracellular medium (52, 90). Recently, microbial cell-surface display has been extensively studied due to its significant impact on various biotechnological and industrial applications, such as vaccine development, biosensor development, high-throughput screening of macromolecular libraries, and preparation of whole-cell biocatalysts (20, 159).

To conduct cell-surface display in *E. coli*, a gene fusion needs to be constructed such that the gene product (i.e. precursor) expressed in the cytoplasm will consist of at least three major parts, i.e. signal sequence, passenger domain, and carrier domain. The signal sequence directs the precursor for its translocation into the periplasm. The carrier domain serves as a surface anchoring motif immobilized on the outer membrane. Depending on the type of anchoring motif, the passenger domain is linked to the carrier domain as an N-terminus, C-terminus or sandwich fusion so that the displayed passenger is exposed to the extracellular environment with its native structure and function. Various outer membrane proteins, lipoproteins, fimbria proteins, flagella proteins, S-layer proteins, and autotransporter proteins have been shown to be effective in carrying passengers for *E. coli* cell-surface display (159).

Up to now, most studies related to microbial cell-surface display focused on discovering carrier systems and developing display vectors. However, another critical factor limiting display performance is the physiological impact, which has been seldom investigated. Cells with a high-level display tend to experience a stressful condition since the

outer membrane becomes fragile or even disintegrated upon frequent insertion of passenger-carrier fusions. As a result, cells become sick and are subjected to growth arrest or even lysis. On the other hand, in order for the passenger to be exported across the outer membrane properly, the passenger-carrier fusion in the periplasm should be maintained in an unfolded or translocation-competent form (208). Premature folding or off-pathway misfolding of this fusion species in the periplasm could potentially mediate protein aggregation and induce periplasmic stress, resulting in poor display performance. Though the above physiological impacts are commonly observed particularly for *E. coli*, this bacterium is preferred to be used as a display host system due to its well-established genetic strategies and tools. Nevertheless, technical issues relevant to physiological impact could be further explored to improve display performance.

It is known that overexpression of foreign gene products in the extracellular compartment, such as periplasm or outer membrane, tends to generate local physiological stress. Recently, the responses to extracytoplasmic stress have gained much attention, particularly during the past decade. Specific stress-sensing and regulatory pathways of σ^E and Cpx, which are partially overlapping, have been well characterized (4, 62, 231, 240). The response to extracytoplasmic stress is primarily driven by the synthesis of a variety of stress-responsive proteins expressing protease activity that degrades misfolded proteins and/or chaperone activity that renatures misfolded proteins. Up to now, several periplasmic proteins, including DegP (257), Skp (31, 113), SurA (156), FkpA (10, 30), DsbC (48), and substrate-binding proteins (234), have been identified to have chaperone activities and could play a role to assist the folding and even targeting of extracytoplasmic proteins. As a practical application, these periplasmic chaperones can be potential candidates to enhance cell-surface display via relieving extracytoplasmic physiological stress and/or via assisting periplasmic transport, folding, and targeting of passenger-carrier fusions. In addition, several periplasmic chaperones are known to be associated with outer membrane proteins for the transport in the periplasm and subsequent targeting onto the outer membrane (188). As such, cell-surface display of proteins could be possibly assisted by these periplasmic chaperones via a similar mechanism.

In this study, the effect of coexpression of Skp, a characterized periplasmic chaperone in *E. coli*, on cell-surface display performance was investigated. EstA, an outer membrane esterase from *Pseudomonas putida*, and yellow fluorescent protein (YFP) were used as the carrier and passenger proteins, respectively. The use of YFP as a model protein facilitated the evaluation of display performance by measuring the fluorescence intensity of YFP-displaying cells. The study was conducted under two assumptions. First, the folding and targeting of passenger-carrier fusions are similar to those of carriers alone (i.e. outer membrane proteins). Second, the physiological stress occurring in the periplasm tends to limit the performance of *E. coli* cell-surface display and periplasmic chaperones can possibly offer a promise for resolving this technical problem. To the best of our knowledge, this is the first report demonstrating the potential applicability of genetic manipulation to enhance cell-surface display on *E. coli* via physiological improvement.

3.2 Materials and Methods

3.2.1 Bacterial Strains and Plasmids

Bacterial strains, plasmids, and oligonucleotides used in this study are summarized in Table 3.1 and are briefly described below. DH5 α and JM109 were used as the host for cloning and cell-surface display, respectively. P1 phage transduction was conducted to construct JM109-derivatives with various promoter-*lacZ* fusions using SP594, SR1458 and SR1710 as the respective donor cells and JM109 as the recipient cell. To prepare the P1 phage lysate from the donor cell, 0.2 mL of an overnight LB (5 g/L NaCl, 5 g/L Bacto yeast extract, 10 g/L Bacto tryptone) culture of the donor strain was infected with the P1 phage. The P1-infected culture was immediately mixed with 2.5 mL of warm tryptone top agar (10 g/L Bacto tryptone, 5 g/L NaCl, 6.5 g/L Bacto agar, 5 mM CaCl₂). This mixture was poured onto a fresh LB agar plate (15 g/L Bacto agar) with 5 mM CaCl₂. The plate was incubated at 37 °C for approximately 4 h until the appearance of plaques. Then, 5 mL of LB medium with 5 mM CaCl₂ was poured onto the plates for further overnight incubation at 4 °C. The P1-containing medium on top of the agar plate was collected and mixed thoroughly with 0.5 mL chloroform. After incubation at 37 °C for 30 min, the mixture was centrifuged at 6,000 \times g for

10 min. The supernatant was collected as the P1 phage lysate from the donor cell and was stored at -80 °C in 15 % glycerol. The P1 phage lysate was later used to infect the recipient cell. To do this, 25 mL of LB medium in an Erlenmeyer flask was inoculated with 1 mL of the recipient seed culture for cultivation at 37 °C and 200 rpm. The log phase culture (0.4~0.6 OD₆₀₀) at 5 mL was centrifuged at 6,000×g for 10 min. After discarding the supernatant, the cell pellet was resuspended in 2 mL of P1-adsorption medium (5 mM CaCl₂, 10 mM MgCl₂). An appropriate amount of the P1 phage lysate was added to the suspension. The bacterium-phage mixture was incubated at room temperature for 30 min. Then, the mixture was supplemented with 2 mL of LCTG broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 1 g/L glucose, 2.5 mM CaCl₂) for further incubation at room temperature for 2 h. After incubation, the mixture was centrifuged at 6,000×g for 10 min. After discarding the supernatant, the cell pellet was resuspended in 0.2 mL of LCTG broth and the suspension with an appropriate dilution was plated on an LB agar plate with 80 µg/mL X-gal for blue-white screening. The plate was incubated overnight at room temperature and then transferred to 37 °C incubation until blue colonies appeared. A few blue colonies were selected and restreaked on the LB plates with X-gal to ensure the maintenance of blue-color phenotype.

Molecular cloning was performed according to standard protocols (241). Restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). Polymerase chain reaction (PCR) was conducted in an automated thermal cycler (GeneAmp Thermocycler, Applied Biosystems, Foster City, CA, USA). Purification of plasmid DNA was performed using a spin-column kit purchased from Clontech (Palo Alto, CA, USA). Plasmid transformation was carried out using an electroporator (*E. coli* Pulser, Bio-Rad, Hercules, CA, USA) or a chemical method according to Chung and Miller (54). The plasmid, pESTKnYFP, was used to display YFP on *E. coli* cell surface. To construct the plasmid, the *yfp* gene was PCR-amplified using pEYFP as the template, YFP-Sfi-Up and YFP-Sfi-Lo as the primers, and *Pfu* polymerase (Stratagene, La Jolla, CA, USA). The PCR product was purified and digested with *Sfi*I, gel extracted and ligated to the *Sfi*I digested fragment of pEST100-FScut to obtain pESTYFP. The Kn^R marker in pESTYFP was replaced by Cm^R. To do this, pACYC177 (44) was first amplified in ER2925, a *dcm* mutant (213), to avoid the Dcm methylation at the two

StuI sites encompassing the Kn^{R} fragment. The purified plasmid was cleaved with *StuI* and the 1.3-kb DNA fragment containing the Kn^{R} gene was gel-extracted. Plasmid pESTYFP was digested with *ScaI* and the 6.6 kb DNA fragment was ligated with the 1.3-kb Kn^{R} cassette to form pESTKnYFP. Plasmid pARSkp was constructed by subcloning the *BglIII/HindIII* fragment containing the *skp* gene from pHELP1 to similarly digested pAR3. Plasmid pESTKnYFP has a pBR322 replication origin and a Kn^{R} marker and is, therefore, compatible with pARSkp having a pACYC184 replication origin and a chloramphenicol-resistant (Cm^{R}) marker.

Table 3.1 Strains, plasmids, and oligonucleotides.

Strain and DNA	Relevant genotype or phenotype	Source and Reference [#]
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ (<i>lac</i>) <i>U169</i> <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA</i> <i>deoR</i> (ψ 80 <i>lacZDM15</i>)	Lab stock (300)
ER2925	<i>Ara-14</i> <i>leuB6</i> <i>fhuA31</i> <i>lacY1</i> <i>tsx78</i> <i>glnV44</i> <i>galK2</i> <i>glaT22</i> <i>mcrA</i> <i>dcm-6</i> <i>hisG4</i> <i>rfbD1</i> <i>R(zgb210::Tn10)</i> <i>TetS</i> <i>endA1</i> <i>rpsL136</i> <i>dam13::Tn9</i> <i>xyIA-5</i> <i>mtl-1</i> <i>thi-1</i> <i>mcrB1</i> <i>hsdR2</i>	NEB (213)
JM109	F' <i>traD36</i> <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^q Δ (<i>lacZ</i>) <i>M15</i> / Δ (<i>lac-proAB</i>) <i>glnV44</i> <i>e14</i> <i>gyrA96</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> <i>thi</i> <i>hsdR17</i>	Lab stock (307)
JM1458	SR1458 (P1) \times JM109, ϕ (<i>P_{degP}-lacZ</i>)	This study
JM1710	SR1710 (P1) \times JM109, ϕ (<i>P_{rpoH}-lacZ</i>)	This study
JM594	SP594 (P1) \times JM109, ϕ (<i>P_{cpxP}-lacZ</i>)	This study
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>) <i>U169</i> <i>rpsL150</i> <i>relA1</i> <i>deoC1</i> <i>rbsR</i> <i>fthD5301</i> <i>fruA25</i> λ	CGSC (35)

SR1458	MC4100 ϕ (P _{degP} -lacZ)	S. Raina (230)
SR1710	MC4100 ϕ (P _{rpoH} -lacZ)	S. Raina (230)
SP594	MC4100 ϕ (P _{cpxP} -lacZ)	T. J. Silhavy (Silhavy collection)

Plasmid

pACYC177	A cloning vector, Ori (pACYC184), Kn ^R , Cm ^R	Lab stock (235)
pAR3	A cloning vector, P _{araB} , Ori (pACYC184), Cm ^R	J. Gutierrez (221)
pARSkp	P _{araB} ::skp, Ori (pACYC184), Cm ^R	This study
pEST100-FSCut	P _{lac} ::cut::estA, Ori (pBR322), Cm ^R	H. Kolmer (18)
pESTKnYFP	P _{lac} ::yfp::estA, Ori (pBR322), Kn ^R	This study
pESTYFP	P _{lac} ::yfp::estA, Ori (pBR322), Cm ^R	This study
pEYFP	P _{lac} ::yfp, Ori (pBR322), Ap ^R	Clontech (210)
pHELP1	P _{araB} ::secB::skp, Ori (pACYC184), Cm ^R	W. J. Harris (113)

Oligonucleotides

YFP-Sfi-Up	5' CGAGGATCCCCGGGTGGCCAGCCGGCCA TGGTGAGCAAGGG 3'	This study
YFP-Sfi-Lo	5' GCTCAGTTGGAATTCTAGAGTCGGCCCCC GAGGCCTTGACAGCTCGTC 3'	This study

CCRC: Culture Collection and Research Center, Taiwan; NEB: New England Biolabs; CGSC: *E. coli* Genetic Stock Center

3.2.2 Cultivation

Cells were revived by streaking the stock culture, stored at -80 °C, on LB agar plates. The plate was incubated at 37 °C for approximately 15 h. An isolated single colony was picked to inoculate 25 mL of LB medium, which was then incubated at 37 °C and 200 rpm in a rotary shaker for approximately 15 h to form a seed culture. The medium was supplemented with 50 µg/mL kanamycin (Kn) or 34 µg/mL chloramphenicol (Cm) when necessary. Erlenmeyer flasks containing 25 mL LB medium were inoculated with the seed culture and were shaken in a rotary shaker at 28 °C and 200 rpm. When the cell density reached approximately 0.5 OD₆₀₀, the culture was supplemented with IPTG and/or arabinose for induction. The flask cultures were further shaken under the same conditions for another 4 h. All the cultivations were conducted at least in duplicate.

3.2.3 Cell Fractionation

The culture sample equivalent to 20 OD₆₀₀ units was centrifuged at 6,000×g for 6 min at 4 °C to pellet the cells. After discarding the supernatant, the cell pellet was resuspended in 3 mL sodium phosphate buffer (0.05M, pH 7.5) and lysed by a French Press (ThermoIEC, Waltham, MA, USA). The cell lysate was further centrifuged for 15 min at 12,000×g and 4 °C. The pellet containing insoluble proteins and cell debris was washed with phosphate buffer, resuspended in TE/SDS buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA, 1% SDS), and heated to 100 °C for 5 min for dissolution. The supernatant and pellet were analyzed with SDS-PAGE as the soluble and insoluble (i.e. membrane) fractions, respectively.

3.2.4 Analytical Methods

The culture sample was appropriately diluted with an isotonic saline solution for measuring cell density in OD₆₀₀ with a spectrophotometer (DU[®]520, Beckman Coulter, Fullerton, CA, USA). To measure the whole-cell fluorescence, the culture sample was centrifuged for 6 min at 6000×g and 4 °C and the cell pellet was resuspended with sodium phosphate buffer (0.05M, pH 7.5) to reach a final cell density of 0.5 OD₆₀₀. The YFP fluorescence was determined with a spectrofluorometer (FP-6500, Jasco, Tokyo, Japan) using the excitation and emission wavelengths of 510 nm and 527 nm, respectively.

For immunogold labeling, the culture sample was centrifuged at 6000×g and 4 °C for 6 min, and the cell pellet was resuspended to 0.5 OD₆₀₀ with PBS buffer. A 100-μL drop of this cell suspension was placed on a sheet of parafilm. A formvar-coated nickel grid was placed, with the coating side facing down, on the drop surface of the cell suspension for approximately 10 min. The grid was blotted dry and placed on a drop of PBS with 0.3% skim milk powder as a blocking agent for 20 min. The grid was incubated in the blocking agent containing the primary antibody (i.e. anti GFP rabbit IgG from Clontech, diluted in 1/50) for 1 h. The grid was washed with drops of the blocking agent for 3×2 min. The grid was incubated on a drop of the blocking agent containing the secondary antibody (i.e. goat anti-rabbit IgG conjugated with gold from Sigma, diluted in 1/20) for 1 h. The grid was washed with drops of the blocking agent for 3×2 min and then with DI water for 4×2 min. The grid was stained with 2% aqueous uranyl acetate for 1 min and washed with DI water for 1 min. The grid was air-dried and observed under a transmission electron microscope (TEM; Philips CM10, Eindhoven, Netherlands). All the steps were followed by intermediate blot drying of grids on a filter paper.

To conduct trypsin digestion, the culture sample was centrifuged for 6 min at 6000×g and 4 °C and the cell pellet was resuspended with the digestion buffer (200 mM Tris-HCl, 150 mM MgCl₂, pH 7.5) containing 2 mg/mL trypsin to reach a final cell density of 0.5 OD₆₀₀. The cells were then incubated at 37 °C for an hour and polymethyl sulfonyl fluoride (PMSF), a trypsin inhibitor, was added to a final concentration of 1 μM and the fluorescence intensity was measured

For EDTA and SDS sensitivity tests, EDTA and SDS at a final concentration of 2 mM and 0.1%, respectively, were added to a 0.5-OD₆₀₀ cell suspension in a sensitivity testing buffer (Tris-HCl 200 mM, pH 7.5). The cell suspension was incubated at 37 °C. Samples were taken at different time intervals and cell density was measured.

β-galactosidase assay was conducted using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate (182).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Mini-PROTEAN[®]II electrophoresis cell (Bio-Rad, Hercules, CA, USA) using a 12.5%

polyacrylamide separating gel stacked by a 4% polyacrylamide stacking gel. The sample loading amount was 0.05 and 0.4 OD₆₀₀-units for the soluble and insoluble fractions, respectively. Electrophoresis was conducted under a constant voltage of 200 V for approximately 1 h. The gel was stained with Coomassie blue and dried in a hood. The dried gel was then scanned.

To conduct Western blotting, the proteins on the polyacrylamide gel were electroblotted to a PVDF membrane after SDS-PAGE using a Mini Trans-Blot[®] Cell (BioRad, Hercules, CA, USA) according to a standard protocol (283). The electrophoretic transfer was conducted at a constant voltage of 100 V for 1 h. Protein-antibody hybridization was performed as described by Sambrook et al. (241). The primary antibody was anti-GFP rabbit IgG (Clontech, Mountain View, CA, USA) and the secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP, Sigma, St. Louis, MO, USA). GFP-related polypeptides were detected by a colorimetric method using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as the substrate. The processed membrane was scanned.

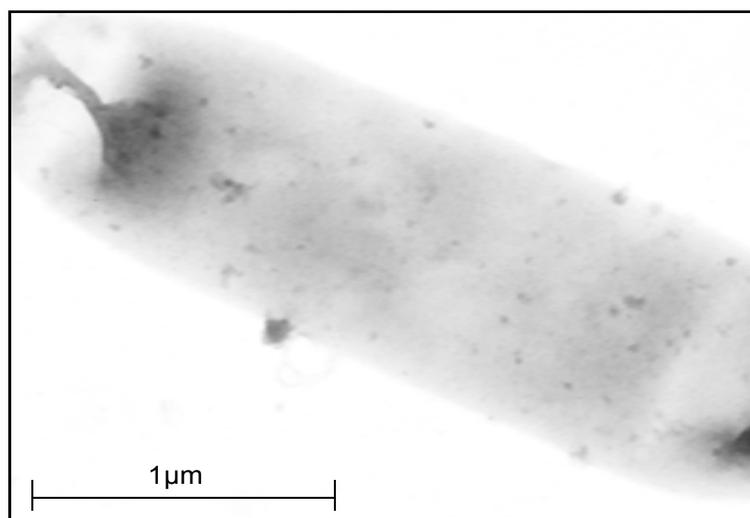
3.3 Results

3.3.1 Cell Surface Display of YFP Results in Physiological Deterioration

Using JM109 (pESTKnYFP) as the host/vector system for conducting cell-surface display of YFP under various IPTG inducer concentrations, the YFP display performance is summarized in Table 3.2. The control culture without IPTG supplementation had a cell density and a specific fluorescence intensity of 2.2 OD₆₀₀ and 4.5 U/OD₆₀₀/mL, respectively. While the specific fluorescence intensity was increased by more than two fold, indicating a certain level of YFP display, cell growth was significantly inhibited due to physiological deterioration (less than 0.5 OD₆₀₀) and, as a result, the volumetric fluorescence intensity was even reduced for all the induced cultures. With such a rapidly deteriorating cell physiology, the specific fluorescence intensity was saturated at a low IPTG concentration 0.05 mM. The result of TEM (Figure 3.1) confirms that YFP was successfully displayed on *E. coli* cell surface though at a relatively low level.

Table 3.2 YFP display performance for JM109 (pESTKnYFP) under various culture conditions.

IPTG (mM)	0	0.05	0.1	0.5	1.0
Cell Optical Density (OD₆₀₀)	2.2±0.1	0.43±0.01	0.47±0.01	0.35±0.00	0.34±0.01
Specific Fluorescence Intensity (U/OD₆₀₀/mL)	4.5±0.2	15.2±0.6	15.1±0.2	13.1±0.2	13.8±0.2
Volumetric Fluorescence Intensity (U/mL)	9.5±0.5	6.5±0.4	5.6±0.1	3.9±0.3	4.5±0.3



A

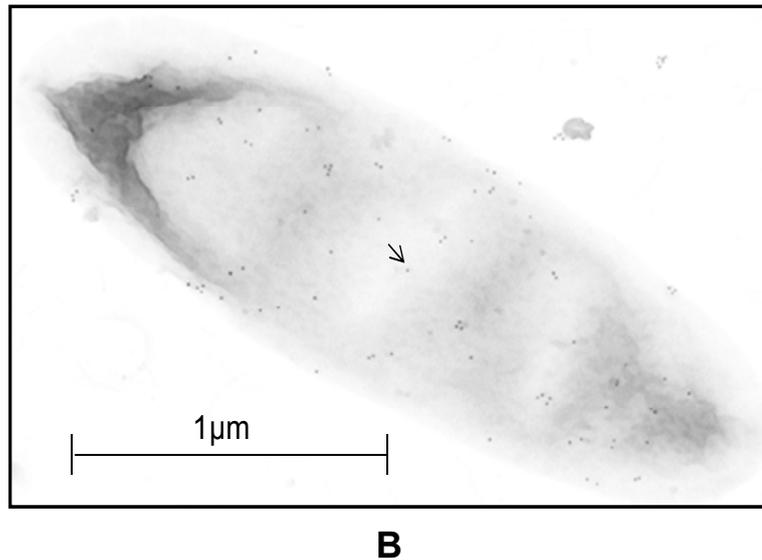
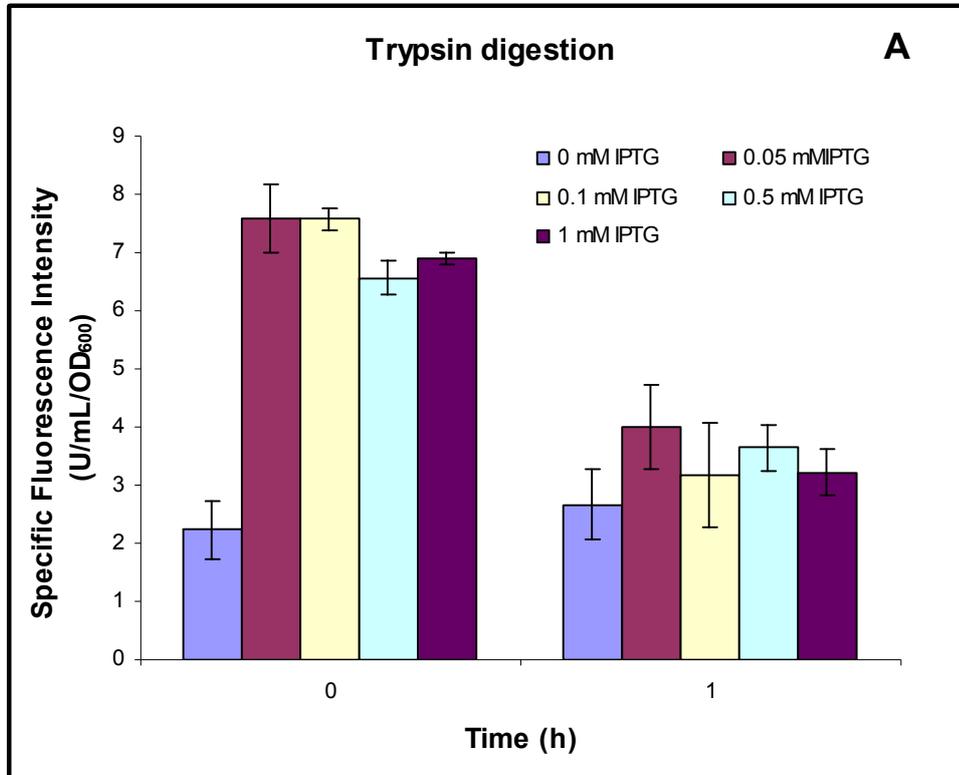


Figure 3.1 Result of TEM for immunogold labeling of displayed YFP on *E. coli* cell surface. The black dots represent the gold particles conjugated with the displayed YFP. Panel A/ JM109; Panel B/ JM109 (pESTKnYFP) induced with 0.1 mM IPTG.

Various tests, including trypsin digestion and EDTA and SDS sensitivity, were conducted to characterize JM109 (pESTKnYFP) for YFP display on *E. coli* cell surface and the results are summarized in Figure 3.2. The fluorescence intensity for all the induced cells (except for the non-induced cells as a control) was significantly reduced after 1-h incubation for trypsin digestion (Figure 3.2A), implying that the expressed YFP was likely displayed on cell surface to which trypsin could access. All the induced cells were much more sensitive to either EDTA (Figure 3.2B) or SDS (Figure 3.2C) than non-induced cells, indicating that the outer membrane became more fragile upon IPTG-induction for YFP display. The result of Western blotting (Figure 3.3) shows that the expressed YFP was located in the insoluble fraction of the lysate and disappeared after trypsin digestion. All the above results indicate that YFP was displayed on *E. coli* cell surface upon IPTG-induction and cell physiology was significantly deteriorating as a result of YFP display.



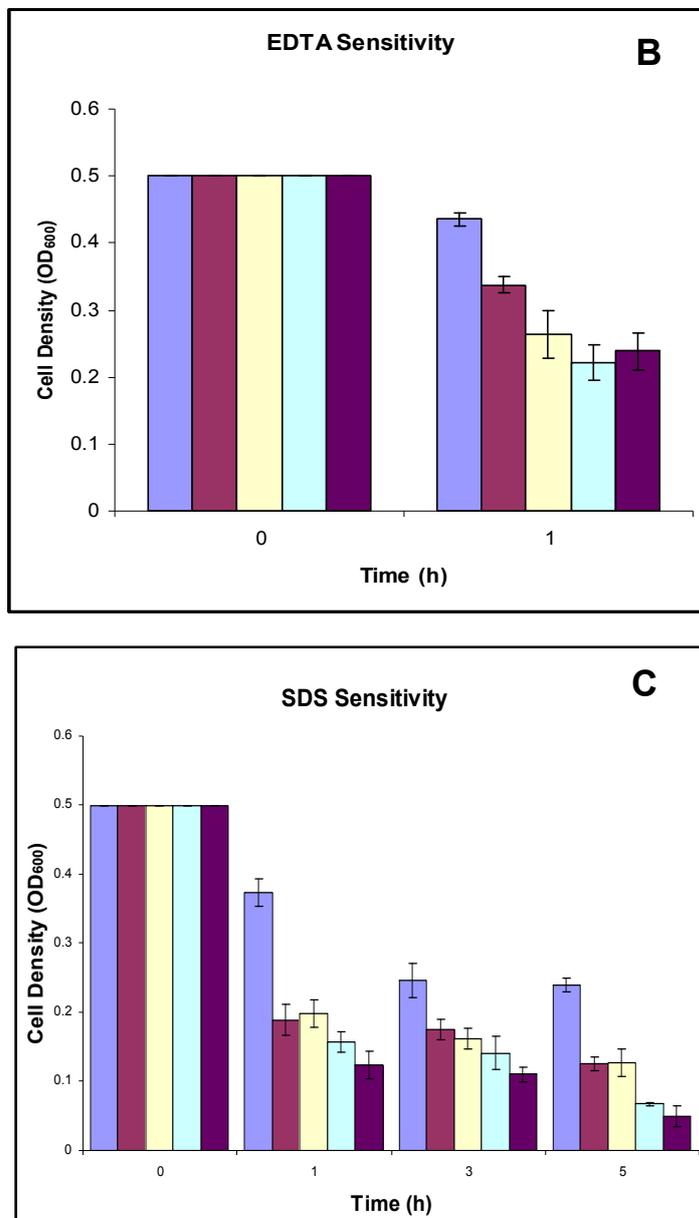


Figure 3.2 Characterization of YFP display on *E. coli* cell surface using JM109 (pESTKnYFP) as the displaying host/vector system under various culture conditions. Panel A/ trypsin digestion; Panel B/ EDTA-sensitivity test; Panel C/ SDS-sensitivity test.

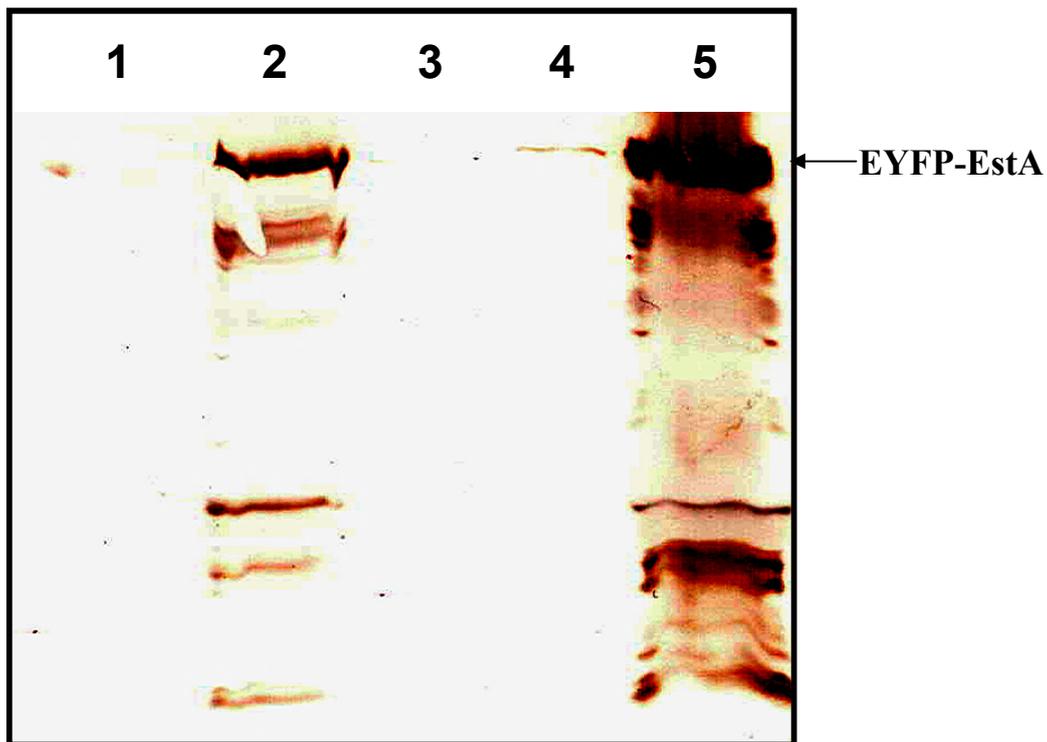


Figure 3.3 Result of Western blotting for YFP cell-surface display using JM109 (pESTKnYFP) as the displaying host/vector system. Lane 1/ whole cell extract, no IPTG; Lane 2/ whole cell extract, 0.1 mM IPTG; Lane 3/ whole cell extract , 0.1 mM IPTG, with trypsin digestion ; Lane 4/ soluble fraction, 0.1 mM IPTG; Lane 5/ insoluble fraction, 0.1 mM IPTG. The soluble fractions were also analyzed without any hybridization signals (data not shown).

3.3.2 Skp Coexpression Highly Improves YFP Display Performance

Using JM109 (pESTKnYFP, pARSkp) as the host/vector system for conducting cell-surface display of YFP under various induction conditions, the YFP display performance is summarized in Table 3.3. Compared to the single-plasmid system, i.e. JM109 (pESTKnYFP), the YFP display performance was approximately the same for non-induced cultures. However, the presence of pARSkp significantly improved cell survival and growth

for IPTG-induced cultures (e.g. 0.47 vs. 1.4 OD₆₀₀ for 0.1 mM IPTG), even without adding arabinose for Skp induction. With such improvement in cell physiology, the specific fluorescence intensity was either maintained at a similar level (i.e. 15.1 vs. 14.6 U/OD₆₀₀/mL for cultures with 0.1 mM IPTG) or was increased by approximately 30% (i.e. 13.1 vs. 17.4 U/OD₆₀₀/mL and 13.8 vs. 18.2 U/OD₆₀₀/mL for cultures with 0.5 and 1.0 mM IPTG, respectively), resulting in significant improvement in volumetric fluorescence intensity up to 5 fold (e.g. 4.5 vs. 25.4 U/mL for cultures with 1.0 mM IPTG).

Table 3.3 YFP display performance for JM109 (pESTKnYFP, pARSkp) under various culture conditions.

IPTG (mM)	0	0.1	0.5	1.0
Arabinose (0 mg/mL)				
Cell Optical Density (OD₆₀₀)	2.2±0.2	1.4±0.2	1.4±0.0	1.4±0.1
Specific Fluorescence Intensity (U/OD₆₀₀/mL)	4.9±0.2	14.6±1.0	17.4±0.5	18.2±0.3
Volumetric Fluorescence Intensity (U/mL)	11±0.5	19.8±0.1	24.9±0.4	25.4±1.2
Arabinose (0.5 mg/mL)				
Cell Optical Density (OD₆₀₀)		2.5±0.0	2.3±0.1	2.1±0.0
Specific Fluorescence Intensity (U/OD₆₀₀/mL)		23.9±0.8	30.3±1.5	33.1±0.5

Volumetric Fluorescence Intensity (U/mL)	59.3±2.0	70.3±2.0	70.0±5.0
<hr/> Arabinose (5 mg/mL) <hr/>			
Cell Optical Density (OD ₆₀₀)	2.2±0.1	2.1±0.2	2.3±0.2
Specific Fluorescence Intensity (U/OD ₆₀₀ /mL)	28.7±0.5	27.5±0.2	31.9±0.2
Volumetric Fluorescence Intensity (U/mL)	61.2±2	57.8±0.2	73.4±0.5

Compared to the cultures of either JM109 (pESTKnYFP) or JM109 (pESTKnYFP, pARSkp) induced with IPTG only, both cell growth and specific fluorescence intensity were increased when Skp was coexpressed through arabinose-induction (either at 0.5 or 5 mg/mL), resulting in a significant increase in volumetric fluorescence intensity for more than 15 fold (e.g. 4.5 for single-plasmid system with 1 mM IPTG vs. 70 U/mL for binary-plasmid system with 1 mM IPTG and 0.5 mg/mL arabinose). Note that, in contrast to the result of JM109 (pESTKnYFP) cultures in which the display level was saturated with a low IPTG concentration, the specific fluorescence intensity for JM109 (pESTKnYFP, pARSkp) cultures increased monotonically with IPTG concentration up to 1 mM due to improved cell physiology. The result of TEM shows that the density of displayed YFP for JM109 (pESTKnYFP, pARSkp) (Figure 3.4) was significantly higher than that for JM109 (pESTKnYFP) (Figure 3.1B), implying an improved display performance with Skp coexpression. The result of Western blotting (Figure 3.5A) shows that the expressed YFP was still located in the insoluble fraction of the lysate upon Skp coexpression (Figure 3.5B) and the displayed level was significantly higher than that of JM109 (pESTKnYFP) (Figure 3.3). The result of trypsin digestion (Figure 3.6A) suggests that YFP was displayed on the

cell surface. Figures 3.6B and 3.6C show that YFP-displayed cells with Skp coexpression were much less sensitive to both EDTA and SDS than YFP-displayed cells without Skp coexpression (Figures 3.2B and 3.2C), implying significantly improved cell physiology was obtained upon Skp coexpression.

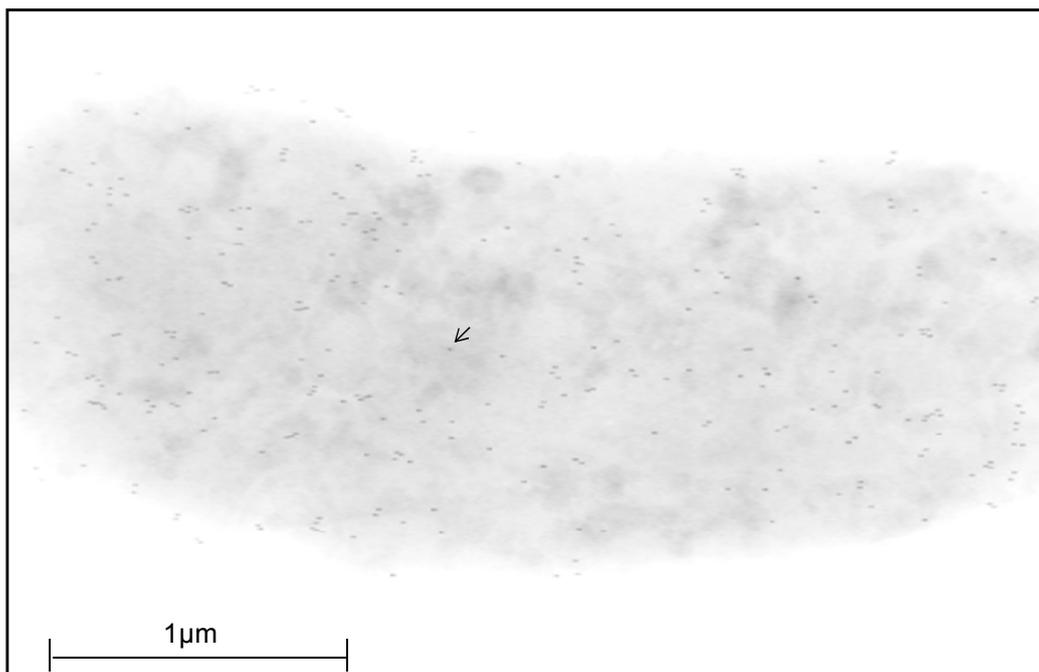
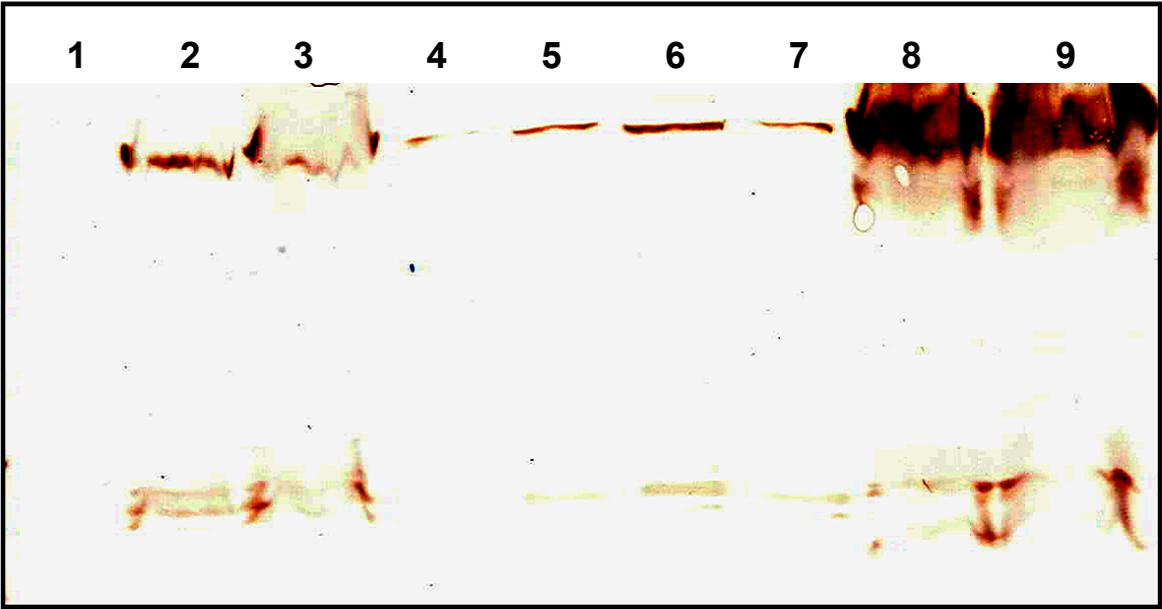
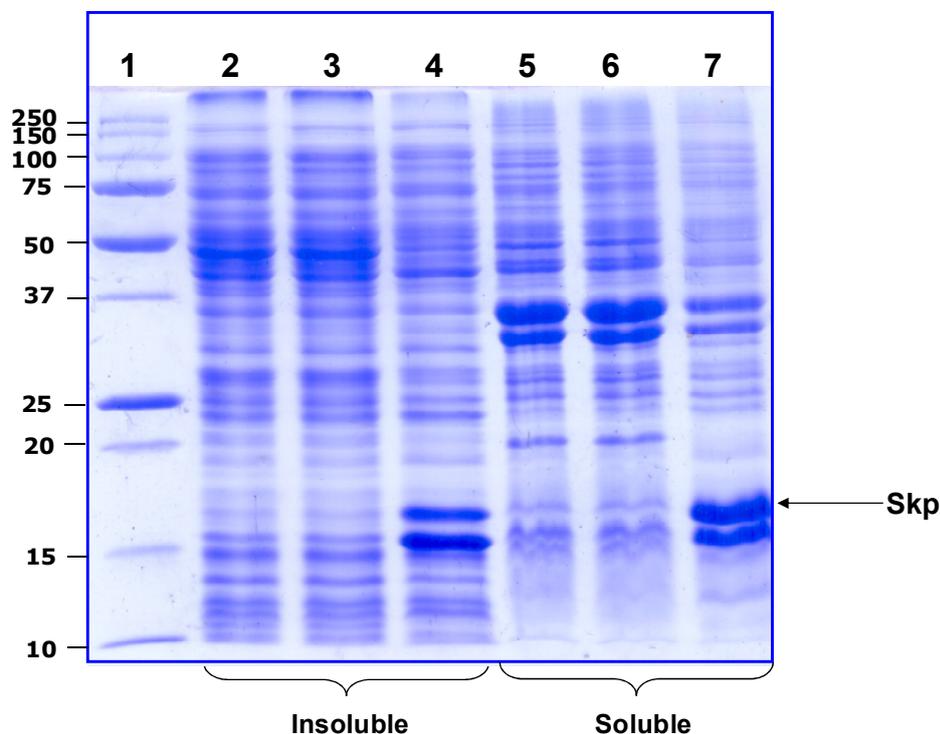


Figure 3.4 Result of TEM for immunogold labeling of displayed YFP on *E. coli* cell surface using JM109 (pESTKnYFP, pARSkp) as the displaying host/vector system. The culture was supplemented with 0.1 mM IPTG and 0.5 mg/mL arabinose. Note that the density of the black dots, representing the gold particles conjugated with the displayed YFP, is significantly higher than that of JM109 (pESTKnYFP) under the same culture condition (i.e. Figure 3.1B).

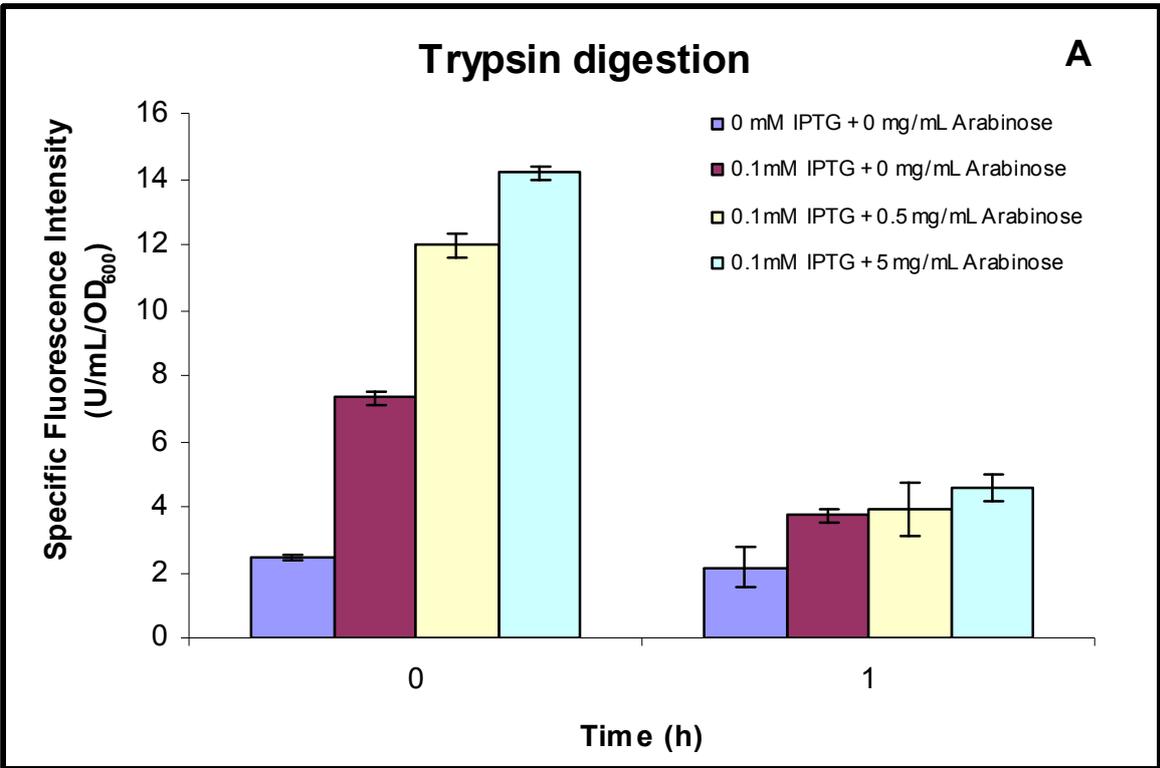


A



B

Figure 3.5 Result of Western blotting (Panel A) and SDS-PAGE (Panel B) for YFP cell-surface display using JM109 (pESTKnYFP, pARSkp) as the displaying host/vector system. Panel A: Lane 1/ whole cell extract, no IPTG; Lane 2/ whole cell extract, 0.1 mM IPTG; Lane 3/ whole cell extract, 0.1 mM IPTG, 0.5 mg/mL Arabinose; Lane 4/ whole cell extract, 0.1 mM IPTG, with trypsin digestion; Lane 5/ whole cell extract, 0.1 mM IPTG, 0.5 mg/mL Arabinose, with trypsin digestion; Lane 6/ soluble fraction, 0.1 mM IPTG; Lane 7/ soluble fraction, 0.1 mM IPTG, 0.5 mg/mL Arabinose; Lane 8/ insoluble fraction, 0.1 mM IPTG; Lane 8/ insoluble fraction, 0.1 mM IPTG, 0.5 mg/mL Arabinose. The soluble fractions were also analyzed without any hybridization signals (data not shown). Panel B: Lane 1/ Protein standard; Lane 2/ soluble fraction, no IPTG; Lane 3/ soluble fraction, 0.1 mM IPTG; Lane 4/ soluble fraction, 0.1 mM IPTG, 0.5 mg/mL Arabinose; Lane 5/ insoluble fraction, no IPTG; Lane 6/ insoluble fraction, 0.1 mM IPTG; Lane 7/ insoluble fraction, 0.1 mM IPTG, 0.5 mg/mL Arabinose.



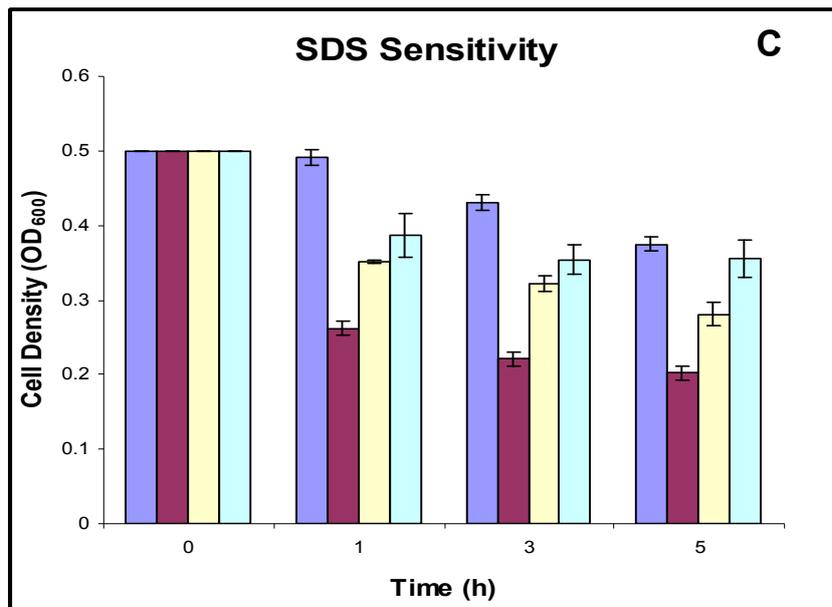
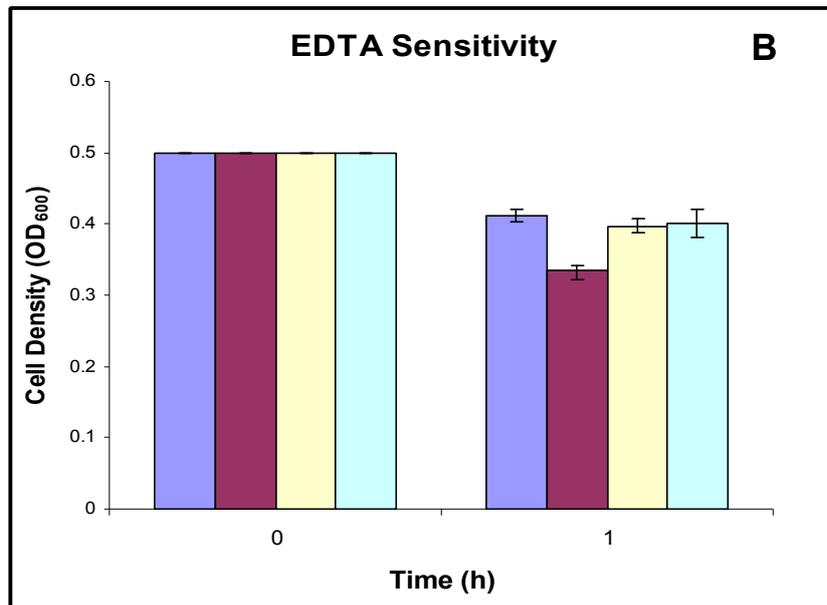


Figure 3.6 Characterization of YFP display on *E. coli* cell surface using JM109 (pESTKnYFP, pARSkp) as the displaying host/vector system under various culture conditions. Panel A/ trypsin digestion; Panel B/ EDTA-sensitivity test; Panel C/ SDS-sensitivity test.

3.3.3 Skp Coexpression Reduces Extracytoplasmic Stress

To monitor extracytoplasmic stress responses to cell-surface display, three JM109-derived strains were constructed by transduction of a *lacZ* reporter gene fused with P_{degP} , P_{cpxP} , and P_{rpoH} , respectively, into JM109. Using these three strains, the results of YFP display performance for single-plasmid (i.e. pESTKnYFP) and binary-plasmid (i.e. pESTKnYFP and pARSkp) systems are summarized in Tables 3.4 and 3.5, respectively, and they are similar to the results for the corresponding display systems using wild-type JM109 (i.e. Tables 3.2 and 3.3). Cells without Skp coexpression were subjected to growth arrest upon YFP display (Table 3.4), whereas growth was no longer inhibited and cell physiology was significantly improved upon Skp coexpression (Table 3.5).

Table 3.4 YFP display performance for JM109-derivatives harboring pESTKnYFP under various culture conditions: “-” and “+” represent “no induction” and “induction with 0.1 mM IPTG”, respectively.

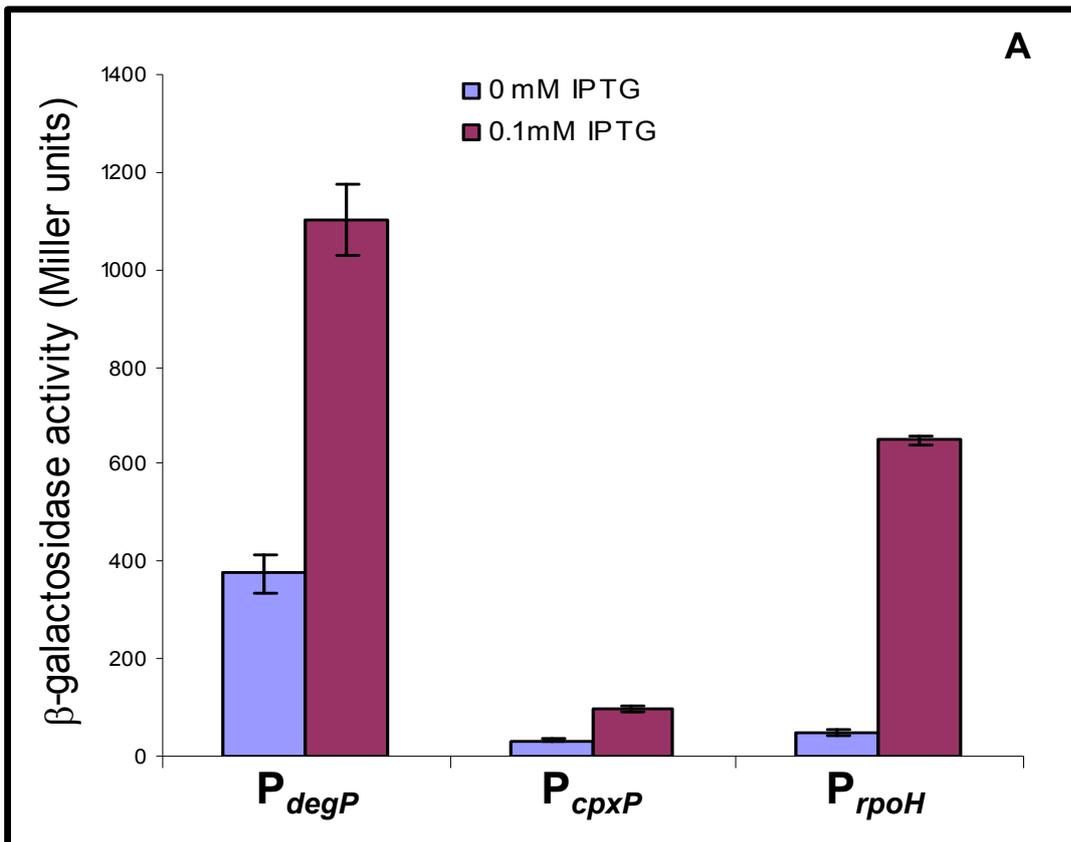
IPTG	JM1458/ $P_{degP}::lacZ$		JM594/ $P_{cpxP}::lacZ$		JM1710/ $P_{rpoH}::lacZ$	
	-	+	-	+	-	+
Cell Optical Density						
(OD ₆₀₀)	2.4±0.0	0.88±0.05	2.1±0.1	1.2±0.1	2.4±0.0	0.86±0.02
Specific Fluorescence						
Intensity						
(U/OD ₆₀₀ /mL)	4.7±0.2	13.4±0.4	4.4±0.4	14.2±1.1	5.2±1.0	14.1±0.4
Volumetric Fluorescence						
Intensity						
(U/mL)	10.9±0.4	11.8±1.0	9.0±0.6	16.9±2.0	12.7±2.5	12.1±0.4
% Cell Lysis		70		28		55

Table 3.5 YFP display performance for JM109-derivatives harboring pESTKnYFP and pARSkp under various culture conditions: “–”, “+I”, and “+A” represent “no induction”, “induction with 0.1 mM IPTG”, and “induction with 0.5 mg/mL arabinose”, respectively.

IPTG/Arabinose	JM1458/P _{degP} ::lacZ			JM594/P _{cpxP} ::lacZ			JM1710/P _{rpoH} ::lacZ		
	–	+I	+I/+A	–	+I	+I/+A	–	+I	+I/+A
Cell Optical									
Density (OD ₆₀₀)	2.4±0.1	1.1±0.0	1.7±0.1	2.3±0.1	1.3±0.1	1.9±0.1	2.4±0.0	0.88±0.02	1.6±0.0
Specific									
Fluorescence Intensity (U/OD ₆₀₀ /mL)	4.7±0.2	14.9±0.9	25.3±1.0	4.9±0.1	14.4±1.1	26.9±1.0	4.1±0.3	15.3±0.4	25.9±1.2
Volumetric									
Fluorescence Intensity (U/mL)	11.2±1.2	16.5±1.0	42.8±3.1	11.1±0.6	19.3±2.8	49.9±3.2	9.9±0.7	13.5±0.4	42.5±2.2
% Cell Lysis		32	25		27	22		48	22

The results of stress response to YFP display for single-plasmid (i.e. pESTKnYFP) and binary-plasmid (i.e. pESTKnYFP and pARSkp) systems under various culture conditions are summarized in Figures 3.7A and 3.7B, respectively. All the *degP*, *cpx*, and *rpoH* promoter activities were induced upon IPTG-induction for YFP display (Figures 3.7 and 3.8), implying that cells were experiencing extracytoplasmic stress and these stress responses were triggered via both Cpx and σ^E pathways. Note that, compared to the IPTG-induced single-

plasmid system [i.e. JM1458 (pESTKnYFP)], the *degP* promoter activity was significantly reduced in the presence of pARSkp even though Skp was not induced by arabinose supplementation (i.e. 1,100 vs. 498 Miller units). The *degP* promoter activity was further reduced upon adding arabinose for Skp coexpression (i.e. 175 Miller units). However, the *cpx* promoter activity appeared not to be affected by the presence of pARSkp, whether Skp was arabinose-induced or not. Compared to the IPTG-induced single-plasmid system [i.e. JM1710 (pESTKnYFP)], the *rpoH* promoter activity was slightly increased in the presence of pARSkp but without Skp induction (i.e. 649 vs. 823 Miller units), and was further increased upon Skp induction (i.e. 1,005 Miller units).



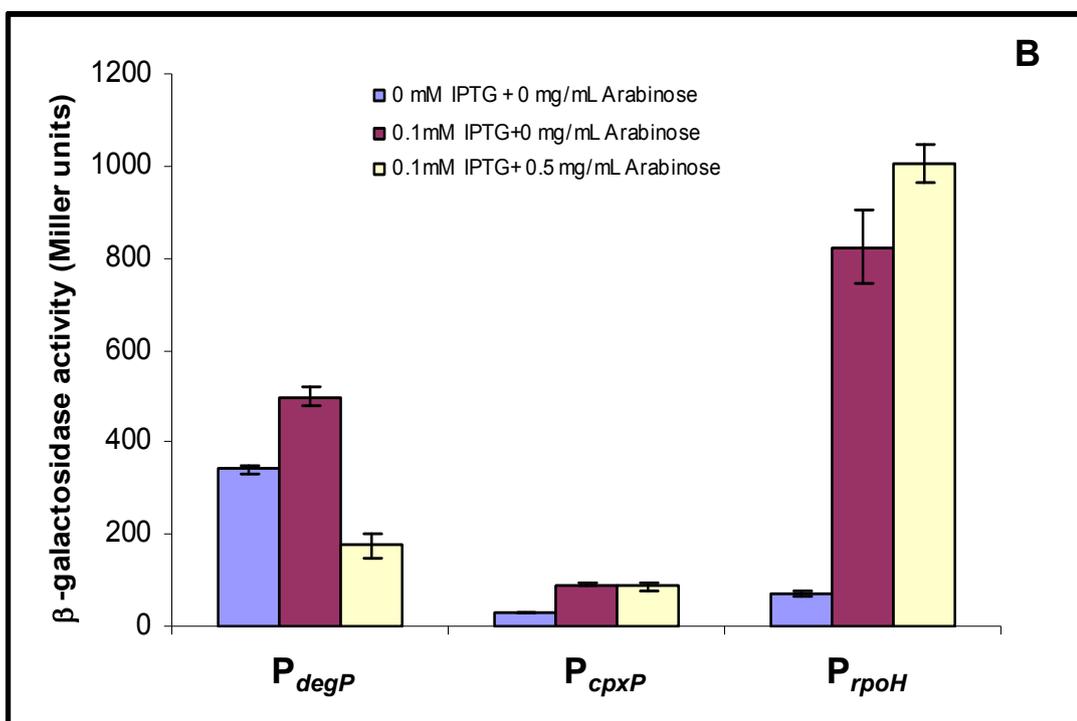


Figure 3.7 Promoter activities representing the extracytoplasmic stress in response to YFP display on *E. coli* cell surface under various culture conditions. Panel A/ JM109 (pESTKnYFP); Panel B/ JM109 (pESTKnYFP, pARSkp).

3.4 Discussion

Displaying proteins on *E. coli* surface tends to seriously affect cell physiology, resulting in growth arrest and poor display performance. Up to now, no study has systematically investigated the factors contributing to such physiological deterioration though it is generally recognized that the outer membrane fragility caused by frequent export of carrier-passenger fusions could be a primary one. Developing strategies for in-depth understanding and suppression of the physiological impact caused by cell-surface display warrants more explorative effort. Since the mechanism for targeting of carrier-passenger fusions onto the outer membrane is believed to be similar to that of carriers alone (i.e. outer membrane proteins), a multitude of problems related to physiological improvement have to be

considered to enhance the display performance, including (1) polypeptide translocation across two membranes, (2) transport of carrier-passenger fusions in the periplasm, (3) prevention of spontaneous folding and maintenance of export-competent forms for carrier-passenger fusions during the whole targeting process, and (4) blocking of the off-pathway processes that lead to aggregation, proteolysis, and perturbation of the constituents of the outer envelope and/or carrier-passenger fusions. Nevertheless, the physiological improvement cannot be convincingly demonstrated without cell physiology being properly monitored. The global stress-response regulons (240) are identified as the cell's natural defense system in response to physiological stress through regulating the synthesis of a variety of stress proteins expressing protease activity that degrades damaged proteins and/or chaperone activity that assists protein folding. As a practical application, these stress-responsive proteins can be used not only as "sensors" for monitoring cell physiology but also proper candidates to suppress the physiological stress. So far, three regulons, i.e. Cpx, σ^E , and Bae, have been discovered to be involved in sensing and suppressing extracytoplasmic stress (240), but only the first two are well characterized.

Among the stress proteins in response to extracytoplasmic stress, Skp is a characterized periplasmic chaperone that interacts with unfolded proteins translocated into the periplasm via Sec secretion system (184). In this study, coexpressing Skp to enhance YFP cell-surface display through improved cell physiology was successfully demonstrated. Cell growth was no longer inhibited and individual cells appeared to be more robust for displaying YFP on the surface. Cells with YFP display appeared to be less sensitive to EDTA and SDS and the density of surface-displayed YFP was highly increased upon Skp coexpression. It was reported that Skp plays a key role in proper biogenesis of the envelope by mediating the folding and targeting of several outer membrane proteins (39, 49, 65, 111, 242). In this regard, folding and/or targeting of carrier-passenger fusions could be potentially facilitated by Skp. On the other hand, Skp has been shown to improve the folding of recombinant proteins or antibody fragments in the periplasm of *E. coli* (31, 113, 160, 175). Such an effect could also be useful to increase the solubility and prevent the premature

folding of periplasmic carrier-passenger fusions for their export and insertion on the outer membrane.

DegP is a periplasmic heat-shock protein with both protease and chaperone activities (257) and its production can be triggered via the Cpx (60) and/or σ^E pathways (75). CpxP is a periplasmic component of the Cpx regulon and is involved in the response to extracytoplasmic stress through an interaction with CpxA, which is another Cpx component acting as a membrane integral sensor (231). CpxP can suppress the toxicity caused by overexpression of envelope proteins and its production is only induced through the Cpx pathway (58). RpoH (also known as σ^H or σ^{32}) is a heat-shock transcription factor regulating the expression of many heat-shock gene and its production is only induced through the σ^E pathway (4). Fusions of the three stress-responsive promoters (i.e. P_{degP} , P_{cpxP} , and P_{rpoH}) with a *lacZ* reporter gene were constructed so that the expression level of β -galactosidase, the reporter gene product, could be assayed for determining the level of various extracytoplasmic stresses, e.g. the stress caused by misfolding of overproduced gene products in the periplasm (127).

In this study, YFP display on *E. coli* cell-surface induced all the three promoter activities investigated (Figure 3.7), implying that cells experienced extracytoplasmic stress arising from cell-surface display. Cell growth was seriously arrested and poor display performance was obtained upon IPTG-induction (Table 3.4). The results suggest that the stress response was activated via both the Cpx and σ^E pathways. It was reported that the overexpression of envelope proteins can activate both the Cpx (255) and σ^E pathways (178). Furthermore, the σ^E pathways can be induced by perturbations to outer membrane protein folding (184). Cell-surface display of YFP with the use of an outer membrane protein as a carrier could possibly trigger the stress response via a mechanism similar to the above ones. It was reported that inner membrane anchoring is essential for DegP induction (186) and DegP is induced primarily to digest the accumulated misfolded proteins for cleaning the periplasm (138, 212). In that case, YFP display could possibly result in the misfolding of certain periplasmic protein(s) anchoring on the inner membrane and the protein misfolding

was then prevented by Skp coexpression. However, these misfolded protein(s) remains to be identified.

Interestingly, among these three promoter activities investigated here, only the *degP* one showed a stress response consistent with the phenotypical cell physiology observed under various culture conditions for cell-surface display. Namely, the *degP* promoter activity was highly induced when cell growth was significantly arrested by YFP display without Skp coexpression (Figure 3.7), whereas the *degP* promoter activity was reduced when the growth arrest was released upon arabinose-induction for Skp coexpression (Tables 3.3 & 3.5, Figure 3.8). The results suggest that the *degP* promoter activity could potentially serve as an effective “sensor” for monitoring extracytoplasmic stress and cell physiology during the course of cell-surface display. However, it is still uncertain via which of the Cpx and σ^E pathways was the *degP* promoter activity triggered. The *degP* promoter activity was previously used for similar physiological monitoring when periplasmic proteins were overproduced (127, 214). In contrast, the other two promoter activities (i.e. *cpxP* and *rpoH*) were not reduced to reflect the phenotypical improvement of cell physiology when Skp was coexpressed and the induction of the two promoter activities appeared not to be affected by the presence of pARSkp (i.e. ~3 fold for P_{cpxP} and ~12 fold for P_{rpoH}), indicating that these two stress responses were not suppressed upon Skp coexpression. Note that a significant amount of the overexpressed Skp existed in an insoluble form (Figure 3.5B), which might cause other types of extracytoplasmic stress reflected as the non-reduced *cpxP* and *rpoH* promoter activities upon Skp coexpression.

Chapter 4

Alleviation of Proteolysis to Enhance Recombinant Protein Production in *Escherichia coli*

4.1 Introduction

Given the availability of various host systems for recombinant protein production, bacterium *Escherichia coli* retains its popularity as the most common one. However, *E. coli* is a prokaryotic cell with several technical issues for heterologous expression of eukaryotic proteins, including host/protein incompatibility, codon bias, and lack of posttranslational processing abilities, etc. which can limit its practical applicability. Host/protein incompatibility appears to be rather critical since eukaryotic proteins heterologously expressed in *E. coli* are often recognized as foreign objects which can induce heat-shock responses and drive protease-mediated degradation. *E. coli* contains several proteases situated in various intracellular compartments, including the cytoplasm, periplasm, inner membrane, and outer membrane (98, 174). While these proteases normally play a pivotal role in maintaining cell physiology by clearing up abnormal proteins (94), they can potentially reduce the production yield by degrading heterologous recombinant proteins. Even though proteases are well characterized, the mechanisms associated with protein degradation and protein substrate specificity are incompletely understood. On the other hand, lack of proper posttranslational processing abilities, such as glycosylation, peptide splicing, and disulfide bond formation, can result in major structural disturbances which eventually lead to protein misfolding.

Cytoplasm is the compartment where all nascent proteins are first synthesized and most of them reside. There are at least five main heat-shock proteases, namely ClpAP, ClpXP, ClpYQ, Lon, and FtsH (97), that can be involved in degrading recombinant proteins in the cytoplasm. ClpAP and ClpXP are two-component proteases that share the common ClpP degradation subunit but have different regulatory units of ClpA and ClpX (99). While FtsH,

ClpAP and ClpXP are responsible for degradation of proteins with non-polar tails (116), ClpYQ and Lon proteases are more generic in their substrate specificity.

Alternatively, periplasm can be a proper destination for targeting of recombinant proteins due the following expression advantages (57, 180): (i) Downstream purification can be facilitated; (ii) Disulfide bonds can be formed in the oxidative periplasm; (iii) Recombinant proteins with an authentic N-terminus can be produced; and (iv) Recombinant proteins in the periplasm are less likely subjected to proteolytic attack. In addition, effective expression systems with optimal growth conditions can yield secreted proteins with an expression level as high as 30% of total cellular protein (89). Though the periplasm contains fewer proteases than the cytoplasm, protein degradation by cell extracytoplasmic proteases is still considered as a serious impediment to the production of secreted proteins. DegP, Tsp, protease III, and OmpT are four common extracytoplasmic proteases. DegP is a periplasmic heat-shock protease (165, 263). The outer membrane protease OmpT specifically targets dibasic amino acid residues (74). Tsp has been shown to recognize and cleave C-terminal residues (254). The substrate specificity of Protease III is not known though it can digest secreted fusion proteins (13).

Herein, we used a case study for functional expression of a eukaryotic protein, i.e. lipase B (PalB) from *Pseudozyma antarctica* (previously known as *Candida antarctica*), in *E. coli* to demonstrate molecular manipulation for alleviation of recombinant protein's sensitivity toward intracellular proteolysis and to better understand the relation between proteolytic specificity and substrate protein sequence. PalB is an important industrial enzyme with justified applicability (9). The use of PalB as the target protein in this study significantly facilitates the experimental demonstration since it has a phenotype convenient for selection and screening purposes. Previously, recombinant PalB was expressed heterologously in fungi and yeasts, such as *Aspergillus oryzae* (122), *Pichia pastoris* (237), and *Saccharomyces cerevisiae* (313). Given the popularity of *E. coli*, functional expression of PalB in this bacterium was not demonstrated until recently (27, 167, 303, 305, 306), but the expression performance stands improvement due to various technical limitations. Since there are three intramolecular disulfide bonds in PalB and their formation can potentially affect the

development of protein structure and bioactivity, functional expression of PalB in the oxidative periplasm of *E. coli* was conducted (303, 306). However, heterologous PalB targeting in the periplasm appeared to be extremely sensitive to intracellular proteolysis, resulting in its fast and complete degradation. Such proteolysis could be prevented by expressing PalB with N-terminal fusion tags (306) or with coexpression of periplasmic chaperones (303) or in the oxidative cytoplasm of genetically modified *E. coli* (i.e. Origami™ strains) (305).

To alleviate the proteolysis limiting functional expression of PalB in *E. coli*, a stable PalB mutant variant against the proteolysis was derived in this study using error-prone polymerase chain reaction (ep-PCR) followed by screening on tributyrin agar plates. Based on sequencing analyses and site-directed mutagenesis experiments, two amino acid residues, i.e. Leu149 and Val223, were identified as “hot spots” for PalB stabilization. Functional expression of PalB in *E. coli* was significantly enhanced by using PalB mutants that can dodge periplasmic proteolysis, and was even further improved by coexpression of a periplasmic folding factor DsbA. While most studies in protein engineering were conducted primarily to identify mutant proteins with enhanced or novel bioactivities, this is an approach to derive protein variants with less susceptibility toward intracellular proteolysis so that protein manufacturing bioprocess can be improved. On the other hand, though it has been well perceived that the *in vivo* proteolytic specificity can be intrinsically determined by the sequence of protein substrate, experimental demonstration of this is rarely reported. The current study complements the lack of such experimental demonstration by showing that the proteolytic specificity of PalB heterologously expressed in *E. coli* can be drastically altered by simple amino acid substitutions.

4.2 Materials and Methods

4.2.1 Bacterial Strains and Plasmids

Bacterial strains, plasmids, and oligonucleotides used in this study are summarized in Table 4.1 and are briefly described below. BL21(DE3) was used as the host strain for PalB expression.

Table 4.1 Strains, plasmids, and oligonucleotides.

Strain	Relevant genotypes	Source & Reference
BL21(DE3)	$F^- ompT dcm lon hsdS_B (r_B^-, m_B^-) gal \lambda(DE3[lacI ind1 sam7 nin5 lacUV5-T7 \text{ gene } 1])$	Novagen
Plasmid		
pARDsbA	$P_{araB}::dsbA$, Ori (pACYC184), Cm ^R	(303)
pARDsbC	$P_{araB}::dsbA$, Ori (pACYC184), Cm ^R	(303)
pETG	$P_{T7-pelB}::palB$, Ori (pBR322), Ap ^R	(303)
pETGM-2	A pETG derivative containing two mutations of Leu149Val and Val223Ile in <i>palB</i> (by ep-PCR with pETG and LB-P10/LB-P11)	This study
pETGM-3	A pETG derivative containing a mutation of Leu149Gly in <i>palB</i> (by SDM with pETG and P104/P114)	This study
pETGM-4	A pETG derivative containing a mutation of Leu149Val in <i>palB</i> (by SDM with pETG and P103/P113)	This study
pETGM-5	A pETG derivative containing a mutation of Leu149Ala	This study

	in <i>palB</i> (by SDM with pETG and P105/P115)	
pETGM-6	A pETG derivative containing a mutation of Leu149Met in <i>palB</i> (by SDM with pETG and P106/P116)	This study
pETGM-7	A pETG derivative containing a mutation of Leu149Ile in <i>palB</i> (by SDM with pETG and P107/P117)	This study
pETGM-8	A pETG derivative containing a mutation of Val223Phe in <i>palB</i> (by SDM with pETG and P112/P122)	This study
pETGM-9	A pETG derivative containing a mutation of Val223Met in <i>palB</i> (by SDM with pETG and P111/P121)	This study
pETGM-10	A pETG derivative containing a mutation of Val223Ala in <i>palB</i> (by SDM with pETG and P110/P120)	This study
pETGM-11	A pETG derivative containing a mutation of Val223Gly in <i>palB</i> (by SDM with pETG and P109/P119)	This study
pETGM-12	A pETG derivative containing a mutation of Val223Ile in <i>palB</i> (by SDM with pETGM-2 and P136/P137, i.e. by reverting the mutation of Val149 to Leu in pETGM-2)	This study

Oligonucleotide	DNA Sequence *	
LB-P10	5'-GGCCA <u>TGGGT</u> CTACCTCCGGTTCGG-3'	This study
LB-P11	5'- <u>CTGAATTCT</u> CAGGGGGTGACGATGCCGGAGCAGG-3'	This study
P103	5'-CCTCTCGATGCAG <u>TCG</u> CGGTTAGTGCACCC-3'	This study
P113	5'-GGGTGCACTAACC <u>GCGACT</u> GTCATCGAGAGG-3'	This study
P104	5'-CCTCTCGATGCAG <u>GCGCC</u> GTTAGTGCACCC-3'	This study

P114	5'-GGGTGCACTAAC <u>GGCGCC</u> TGCATCGAGAGG-3'	This study
P105	5'-CCTCTCGATGCA GCCGCG GTTAGTGCACCC-3'	This study
P115	5'-GGGTGCACTAAC <u>CCGCGG</u> CTGCATCGAGAGG-3'	This study
P106	5'-CCTCTCGATG <u>CCATGG</u> CGGTTAGTGCACCC-3'	This study
P116	5'-GGGTGCACTAAC <u>CCGCA</u> TGGCATCGAGAGG-3'	This study
P107	5'-CCTCTCGATG <u>CGATCG</u> CGGTTAGTGCACCC-3'	This study
P117	5'-GGGTGCACTAAC <u>CCGCA</u> TGGCATCGAGAGG-3'	This study
P109	5'-GGGCCGCTGTT CGGG ATCGACCATGCAGGC-3'	This study
P119	5'-GCCTGCATGGTCGAT CCG AACAGCGGCC-3'	This study
P110	5'-GGGCCGCTGTT CGCG ATCGACCATGCAGGC-3'	This study
P120	5'-GCCTGCATGGTC <u>GATCGC</u> GAACAGCGGCC-3'	This study
P111	5'-GGGCCGCTGTT ATG ATCGACCATGCAGGC-3'	This study
P121	5'-GCCTGCATGGTCGAT ATG AACAGCGGCC-3'	This study
P112	5'-GGGCCGCTGTT TTT ATCGACCATGCAGGC-3'	This study
P122	5'-GCCTGCATGGTCGAT AAA GAACAGCGGCC-3'	This study
P136	5'-CCTCTCGATGCA CTG GCGGTTAGTGCACCC-3'	This study
P137	5'-GGGTGCACTAAC <u>CCGCA</u> TGGCATCGAGAGG-3'	This study

* Italic: mutation nucleotides, underline: restriction site (silent mutation), bold: mutation codon

The plasmid pETG contains the leader-less *palB* gene fused with the *pelB* signal peptide and the expression of *pelB::palB* is under the regulation of the T7 promoter (306). Other plasmids designated as pETGM-n (n = 2~12) are pETG derivatives containing various mutations in *palB*. The plasmids pARDSbA and pARDSbC respectively contain the *dsbA* and *dsbC* genes whose expression was under the regulation of the *araB* promoter. Molecular cloning was performed according to standard protocols (241). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). Polymerase chain reaction (PCR) was performed in an automated thermal cycler (GeneAmp[®] PCR System 9700; Applied Biosystems, Foster City, CA, USA). Plasmid DNA was purified using a spin-column kit purchased from BD Biosciences Clontech (Palo Alto, CA, USA). Plasmid transformation was carried out according to Chung and Miller (54) or using an electroporator (Micropulser, Bio-Rad, Hercules, CA, USA). Error-prone PCR (ep-PCR) was conducted using GeneMorph II EZClone Domain Mutagenesis kit (Stratagene, Dedar Creek, TX, USA) with LB-P10 and LB-P11 as the primers and pETG as the template. Site-directed mutagenesis (SDM) was conducted using Quickchange Lightning Site-Directed Mutagenesis kit (Stratagene) with pETG or pETGM-2 as the template and various primer pairs (Table 4.1). The reaction mixtures were transformed into BL21(DE3) and selected on LB agar plates containing 1% tributyrin.

4.2.2 Cultivation

The protocol for shaker flask cultivation was described previously in Chapter 3. For bioreactor cultivations, the above described seed culture was used to inoculate a bench-top bioreactor (Omni-Culture, VirTis, Gardiner, NY, USA) containing 1-L working volume of LB medium with 10 $\mu\text{L/L}$ Antifoam 289 (Sigma, St. Louis, MO, USA). IPTG at 0.1 mM and arabinose at 0.2 g/L were added for induction purposes when the culture cell density reached $\sim 0.5 \text{ OD}_{600}$. The bioreactor was purged with filter-sterilized air at 2 L/min for aeration. The culture pH was regulated at 7.0 ± 0.1 by adding 3 N NH_4OH or 3 N HCl using a combination of pH electrode (Mettler-Toledo, Switzerland), a pH controller (PC310, Suntex, Taipei, Taiwan), and two peristaltic pumps (101U/R, Watson Marlow, Falmouth, UK). The bioreactor was operated at 28 °C and 650 rpm for approximately 8 h after induction.

4.2.3 Analytical Methods

The protocols for various analyses are described in Chapter 3. PalB activity was qualitatively evaluated by the area and transparency of the halo formed on tributyrin agar plates. To conduct this, the soluble fractions of 50- μ L cell lysates were loaded on the plate incubated at 37 °C overnight. On the other hand, PalB enzyme assay was conducted using a pH stat (Brinkman Metrohm 842 Titrando, FL, USA). An appropriate volume of the soluble fraction of cell lysates was added to 5 mL of 2% olive oil emulsion in water at 37 °C and the reaction solution was maintained at pH 8.0 by controlled addition of 0.02 N NaOH (279). One unit of enzyme activity is defined as the amount of enzyme required to liberate one μ mole of fatty acid per min.

Primary anti-PalB antibodies for probing PalB using Western blotting were raised in a rabbit intermittently immunized with the antigen of recombinant PalB expressed from *A. oryzae* (Sigma-Aldrich). It was further purified by SDS-PAGE and PalB used for immunization was obtained by polypeptide elution of the corresponding band in the polyacrylamide gel slice using an Electro-Eluter (Model 422; Bio-Rad).

4.2.4 Protein Localization

To prepare cell extracts corresponding to various intracellular compartments, cells equivalent to 20 OD₆₀₀-units were centrifuged at 2 °C and 6000 \times g for 6 min. The cell pellet was washed with 10 mL Tris-HCl buffer (10 mM, pH 8.0) and resuspended in 10 mL Tris-HCl buffer (10 mM, pH 8.0) with 0.5 M sucrose. The cell suspension was incubated with 25 μ g/mL lysozyme for 20 min at room temperature. The lysozyme spheroplast was then diluted with equal volume of Tris-HCl buffer (10 mM, pH 8.0) with 1mM EDTA and incubated at room temperature for 20 min to obtain EDTA spheroplast. The cell suspension was divided into two halves and centrifuged at 6000 \times g for 6 min at 2 °C. The supernatant containing the soluble periplasmic protein fraction was assayed for PalB activity.

The first half of the spheroplast pellet was resuspended in 2 mL Tris-HCl buffer (10 mM, pH 8.0) with 10 mM MgCl₂ and 0.3 M sucrose, and incubated with 0.1 mg/mL trypsin for 10 min at room temperature. Trypsin digestion was terminated by adding 5mM PMSF and the spheroplast suspension was centrifuged at 6000 \times g for 6 min at 2 °C. The trypsin

digested spheroplast pellet and the second half of the spheroplast pellet were each resuspended in 2 mL Tris-HCl buffer (10 mM, pH 8.0), sonicated for 4 min, and then centrifuged at 4 °C and 12,000×g for 15 min. The supernatant containing the soluble cytoplasmic fraction was assayed for PalB activity. The pellet from the spheroplast without trypsin treatment contains the insoluble cytoplasmic and periplasmic inclusion bodies, whereas the pellet with trypsin digestion treatment only contains the insoluble cytoplasmic inclusion bodies. These pellets were solublized with TE/SDS buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA, 1% SDS), heated at 100 °C for 5 min, and analyzed with SDS-PAGE and Western blotting.

4.3 Results

Results for functional expression of wild-type and various PalB mutants in the periplasm of *E. coli* are summarized in Figure 4.1 (tributylin plate assay), Table 4.2 (enzyme assay), and Figure 4.2 (Western blotting), respectively. In all the expression constructs, the PelB signal peptide was used to drive the secretion of PalB into the periplasm and the expression of translational fusions of PelB and PalB variants was under the regulation of the T7 promoter with BL21(DE3) as the expression host.

The cell lysate of BL21(DE3) (pETG) culture sample (both un-induced and IPTG-induced) did not have any detectable PalB activity (Table 4.2). Consistently, overnight incubation of the cell lysates on a tributyrin plate failed to produce any halos associated with tributyrin hydrolysis (Figure 4.1). Western blotting results revealed no PalB-related gene products in both soluble and insoluble fractions of the culture samples (Figure 4.2), excluding the possibility of protein misfolding as the reason for non-functional PalB expression.

To identify more stable PalB variants, ep-PCR was conducted using pETG as the template and the PCR product was transformed into BL21(DE3). The transformants were screened on tributyrin agar plates for colonies expressing tributyrin hydrolytic activity. A colony developed a large size of halo. The harboring plasmid (designated as pETGM-2) of this clone was purified for sequencing analysis and two mutations, i.e. Leu149Val and Val223Ile, were

identified in the expressed mutant PalB (designated as M-2). A high PalB activity at 155 U/L/OD₆₀₀ was determined for the IPTG-induced culture sample of BL21(DE3) (pETGM-2), whereas no PalB activity was detected for the un-induced one (Table 4.2). Nevertheless, halos were clearly observed for both un-induced and IPTG-induced culture samples of BL21(DE3) (pETGM-2) (Figure 4.1). Note that the halo for IPTG-induced culture was significantly larger and more transparent than the un-induced culture. Western blotting results revealed PalB-related gene products in both soluble and insoluble fractions (Figure 4.2). The bands were even visible for the un-induced culture sample, implying a potential expression issue of protein misfolding. It was however intriguing to observe such a dramatic effect on PalB stabilization and the enhancement of functional expression associated with these two simple mutations.

Table 4.2 Cultivation performance for the production of various PalB mutant derivatives.

Culture	Cell Density (OD ₆₀₀)		Activity* (U/L/OD ₆₀₀)	Mutation in PalB
	w/o IPTG	w/ IPTG		
BL21(DE3) (pETG)	3.1±0.0	3.3±0.1	ND	None
BL21(DE3) (pETGM-2)	3.4±0.1	3.3±0.0	155	Leu149Val, Val223Ile
BL21(DE3) (pETGM-3)	3.2±0.1	3.3±0.0	ND	Leu149Gly
BL21(DE3) (pETGM-4)	3.4±0.1	3.6±0.1	60	Leu149Val
BL21(DE3) (pETGM-5)	3.3±0.1	3.5±0.1	ND	Leu149Ala
BL21(DE3) (pETGM-6)	2.7±0.1	3.0±0.1	ND	Leu149Met
BL21(DE3) (pETGM-7)	3.5±0.0	3.2±0.0	38	Leu149Ile

BL21(DE3) (pETGM-8)	3.0±0.0	3.6±0.0	50	Val223Phe
BL21(DE3) (pETGM-9)	2.9±0.1	2.8±0.0	ND	Val223Met
BL21(DE3) (pETGM-10)	3.4±0.1	3.3±0.1	ND	Val223Ala
BL21(DE3) (pETGM-11)	3.5±0.0	3.5±0.1	30	Val223Gly
BL21(DE3) (pETGM-12)	3.6±0.1	3.5±0.1	57	Val223Ile

* These activities represent IPTG-induced cultures. No activity was detected for all control cultures without IPTG induction. ND: Not detected

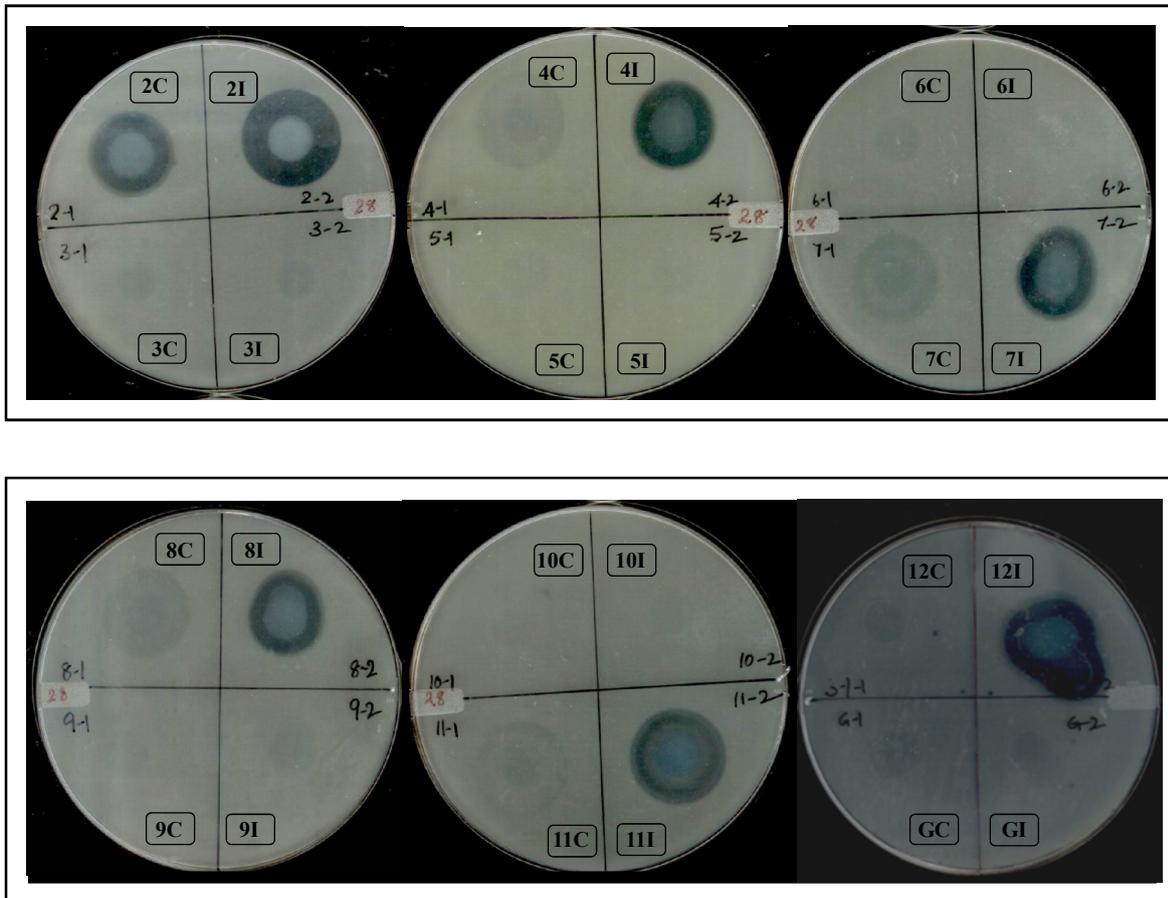


Figure 4.1 Qualitative visualization of PalB activity using tributyrin plates for the expression of various PalB mutant derivatives. The number (n = 2~12) represents the PalB mutant (M-n) with the use of BL21(DE3) harboring the expression plasmid pETGM-n summarized in Tables 4.1 and 4.2. “G” represents the control experiment using BL21(DE3) harboring pETG. “C” and “I” represent the cultures respectively without and with IPTG induction.

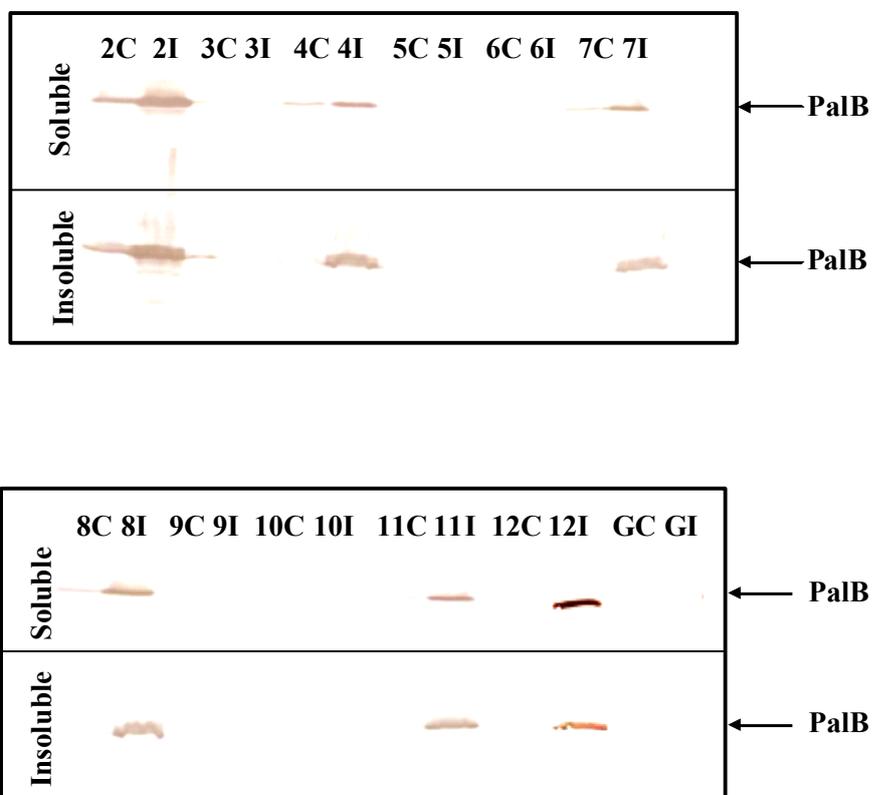


Figure 4.2 Western blotting analysis of the culture samples for the expression of various PalB mutant derivatives. Both soluble and insoluble fractions are shown. The number (n = 2~12) represents the PalB mutant (M-n) with the use of BL21(DE3) harboring the expression plasmid pETGM-n summarized in Tables 1 and 2. “G” represents the control experiment using BL21(DE3) harboring pETG. “C” and “I” represent the cultures respectively without and with IPTG induction.

Using cultivated BL21(DE3) harboring pETGM-2, protein localization experiment was conducted to locate the expressed PalB mutant and the results are summarized in Figure 4.3. Approximately 76% (i.e. 125/165) of total PalB activity was localized in the periplasmic fraction and the rest resided in the cytoplasmic fraction. On the other hand, most of the protein band corresponding to the insoluble PalB mutant disappeared upon treating spheroplasts with trypsin (lane 6 vs. 7 in Figure 4.3), implying the presence of insoluble PalB mutant in the periplasm. The results suggested that the translocation of expressed PalB mutant mediated by PelB signal peptide was rather effective. Similar results of the localization experiment were obtained using cultivated BL21(DE3) harboring pETGM-4 (data not shown).

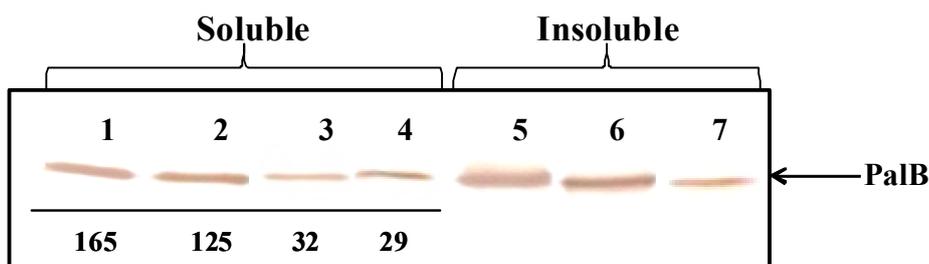


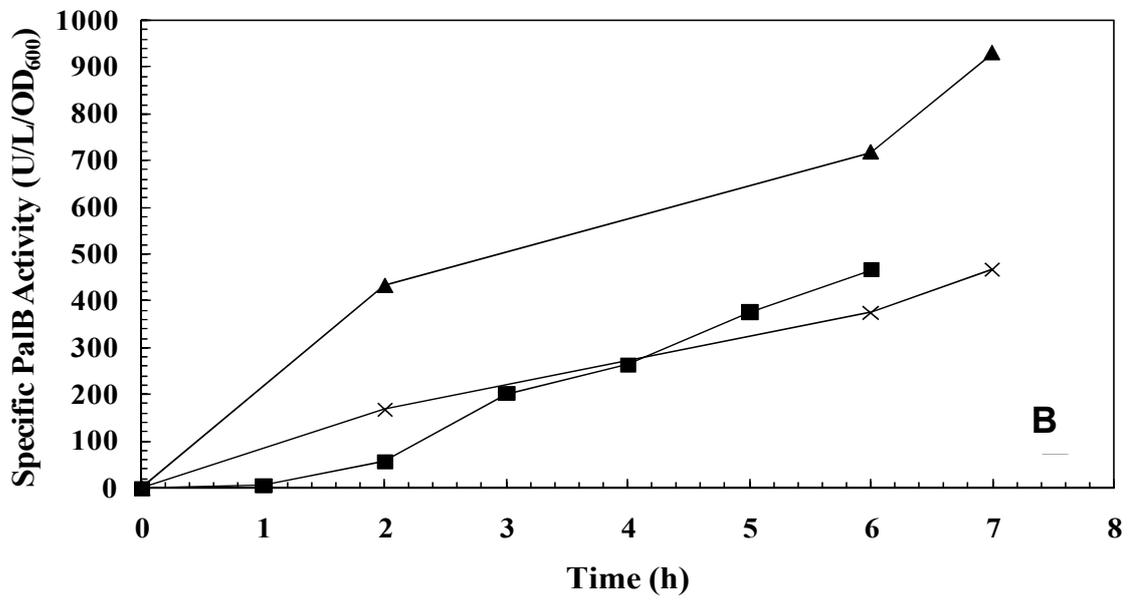
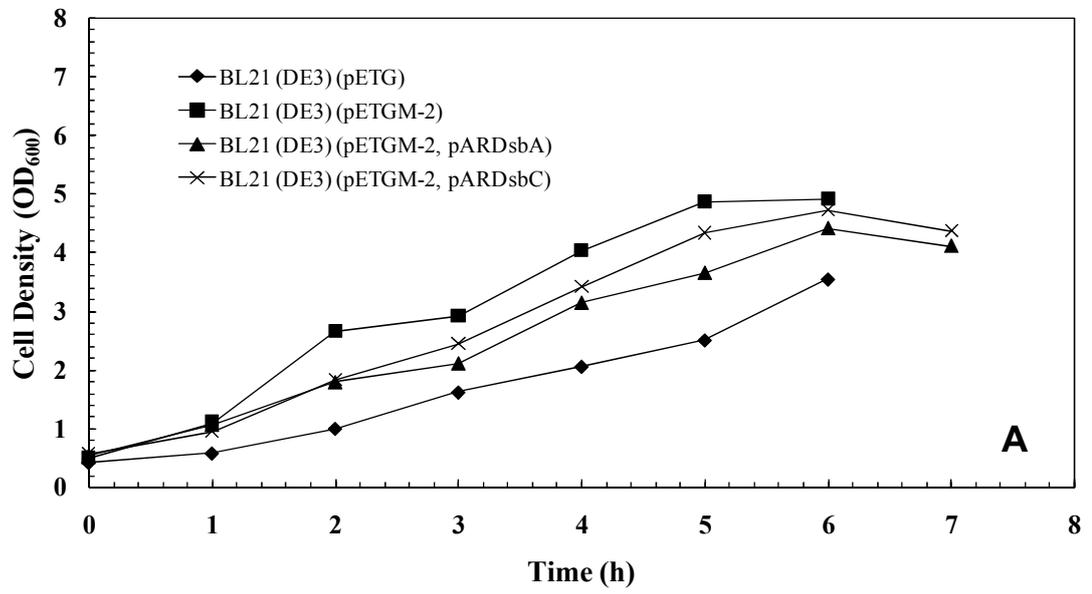
Figure 4.3 Western blotting analysis of the expressed PalB mutant (M-2) in various intracellular fractions of BL21(DE3) harboring pETGM-2. Lane 1/ Total soluble fraction; Lane 2/ Soluble periplasmic fraction; Lane 3/ Soluble cytoplasmic fraction from the spheroplast without trypsin treatment; Lane 4/ Soluble cytoplasmic fraction from the spheroplast with trypsin treatment; Lane 5/ Total insoluble fraction; Lane 6/ Insoluble fraction from the spheroplast without trypsin treatment; Lane 7/ Insoluble fraction from the spheroplast with trypsin treatment. The numbers under lanes 1~4 represent the specific PalB activity (in U/L/OD₆₀₀) of the corresponding fraction.

Apparently, Leu149 and Val223 are two amino acids critically affecting PalB's susceptibility to proteolysis in *E. coli*. To further understand this structural effect, site-directed mutagenesis was conducted to introduce various single mutations to these two "hot spots", including Leu149Gly (M-3), Leu149Val (M-4), Leu149Ala (M-5), Leu149Met (M-6), Leu149Ile (M-7), Val223Phe (M-8), Val223Met (M-9), Val223Ala (M-10), Val223Gly (M-11), and Val223Ile (M-12) and their corresponding expression plasmids are listed in Table 4.1. Note that only hydrophobic amino acid residues were selected to avoid any major disturbances in the local protein structure associated with the mutagenesis. Among the five single mutations on Leu149, only Leu149Val (M-4) and Leu149Ile (M-7) resulted in slight improvement in functional expression, whereas the others behaved similar to the wild-type in hydrolyzing tributyrin (Figure 4.1). Among the five single mutations on Val223, only Val223Phe (M-8), Val223Gly (M-11), and Val223Ile (M-12) resulted in slight improvement in functional expression, whereas the others behaved similar to the wild-type (Figure 4.1). For the above single mutants with their tributyrin-hydrolyzing behavior similar to the wild-type, neither PalB activity (Table 4.2) nor PalB-related gene product (Figure 4.2) was detectable.

Replacing Leu149 with Val or Ile had an effect in reducing PalB's susceptibility to periplasmic proteolysis and Val appeared to be more effective than Ile (Table 4.2; 60 U/L/OD₆₀₀ for M-4 vs. 38 U/L/OD₆₀₀ for M-7). Similarly, replacing Val223 with Phe, Gly or Ile had a similar PalB stabilization effect with Ile as the most effective one (Table 4.2; 50 U/L/OD₆₀₀ for M-8, 30 U/L/OD₆₀₀ for M-11, and 57 U/L/OD₆₀₀ for M-12). While it was unsurprising to observe individual improvement associated with the single mutants of Leu149Val (M-4) and Val223Ile (M-12), the two mutations appeared to have a synergistic effect in reducing PalB's susceptibility to proteolysis (Table 4.2: 155 U/L/OD₆₀₀ for M-2). Note that, similar to the double mutant M-2, PalB-related gene products were detected in the insoluble fraction of cell lysate of the single mutants expressing PalB activity, implying that protein misfolding could be another limiting step for functional expression of PalB in *E. coli*.

Various periplasmic folding factors, including DegP, FkpA, SurA, Skp, DsbA, and DsbC were coexpressed with M-2 for potential alleviation of PalB misfolding in *E. coli*.

Using shaker-flask cultivations for preliminary investigation, only DsbA and DsbC had a slightly improving effect on functional expression of PalB (data not shown). We then conducted bioreactor cultivations to characterize this effect and the results are summarized in Figure 4.4. There was neither PalB activity nor PalB-relevant polypeptides for the control culture of BL21(DE3) (pETG), even for the culture sample taken at 1-h after IPTG-induction; implying the expressed gene product was extremely unstable. Functional expression of PalB for another control culture of BL21(DE3) (pETGM-2) was improved and the specific PalB activity reached 465 U/L/OD₆₀₀, which was much higher than that of shaker-flask cultivation (i.e. 155 U/L/OD₆₀₀) possibly due to a better cell growth environment in the bioreactor. Interestingly, coexpression of DsbA significantly boosted functional expression of PalB and the specific PalB activity was more than 2-fold that of the control culture of BL21(DE3) (pETGM-2) (Figure 4.4B) with a slight reduction in the amount of insoluble PalB (lane 6 vs. 8 in Figure 4.4C). On the other hand, the cultivation performance of BL21(DE3) (pETGM-2, pARDsbC) was rather similar to that of the control culture of BL21(DE3) (pETGM-2), suggesting that the improving effect associated with DsbC coexpression was minimal.



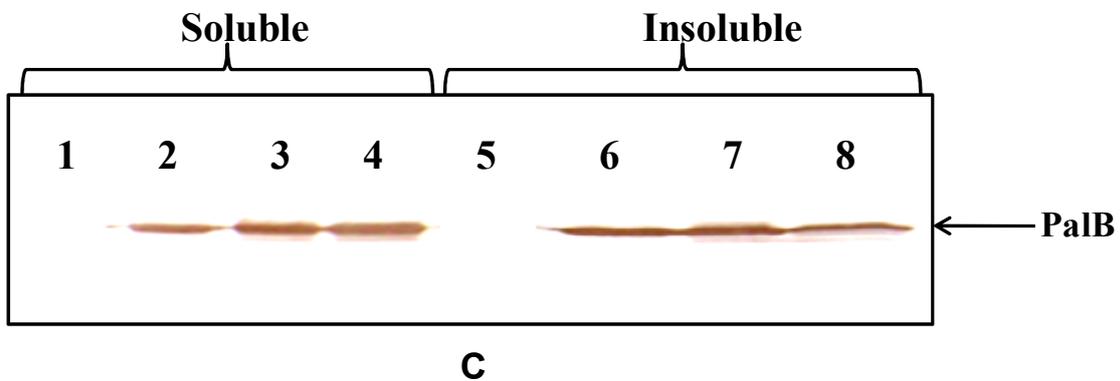


Figure 4.4 Bioreactor cultivation performance for functional expression of PalB using various host/vector systems. Panel A: Time profiles of cell density; Panel B: Time profiles of the specific PalB activity [Note that there was no detectable activity for BL21(DE3) (pETG) culture samples]; Panel C: Western blotting analysis of the final samples of the four cultures. Both soluble and insoluble fractions are shown. Lane 1/5 BL21(DE3) (pETG); Lane 2/6 BL21(DE3) (pETGM-2); Lane 3/7 BL21(DE3) (pETGM-2, pARDsbC); Lane 4/8 BL21(DE3) (pETGM-2, pARDsbA).

4.4 Discussion

While many studies based on rational mutagenesis, directed evolution, and gene shuffling are conducted to derive mutant PalBs with improved enzyme properties, such as thermostability, bioactivity or enantioselectivity (217, 218, 236, 267), this is a similar protein engineering approach to improve recombinant protein production through identification of mutant PalB variants with less sensitivity toward *in vivo* proteolysis when being overexpressed. Theoretically, functional expression of mutant PalB variants in the periplasm could be further enhanced with proper disulfide bond formation in this oxidative compartment.

The results of no detectable PalB activity and PalB-related gene product for BL21(DE3) (pETG) suggest that either translation of the *pelB::palB* fusion gene was ineffective or the translated gene product was degraded. The latter was more plausible since translation of *pelB*-free *palB* or other *palB* fusions was rather effective (303, 306) and there were not many rare codons in *palB* which would otherwise impede translation (data not shown). Our previous results showed that the wild-type PalB was subjected to potential proteolysis in the periplasm (303). Interestingly, the wild-type PalB was less degradable when being expressed in the cytoplasm which contains more proteases than the periplasm (305) or being expressed with N-terminal fusion tags in the periplasm (306).

Since intracellular proteolysis of recombinant proteins is a rather common technical issue, approaches to overcome this limitation have been extensively explored. These include the use of protease-deficient mutant strains as the expression host (179, 290), growth of production cells at a lower temperature (51), coexpression of folding factors (291, 303), protein fusion technology (33, 73, 108), elimination of protease cleavage sites (96, 247, 290), modification of the hydrophobicity of target proteins (136, 191), and manipulation of cultivation conditions (14, 158). While genetic elimination of proteolytically sensitive sequences without impacting target protein's bioactivity appears to be technically attractive, there is no effective prediction or identification of these potential protease cleavage sites.

Our present results suggest that Leu149 and Val223 are two amino acids critically affecting PalB's susceptibility toward periplasmic proteolysis in *E. coli*. Such proteolytic sensitivity was significantly alleviated by replacing Leu149 with Val or Ile and/or replacing

Val223 with Phe, Gly or Ile, but was unchanged when replacing Leu149 with Gly, Ala or Met and replacing Val223 with Met or Ala. Note that Leu149 is located at the entrance of the partially formed lid of the protein structure, whereas Val223 is situated near one of the catalytic triad His226 in the active site. However, these amino acid substitutions with positive effects were expected to convert the overall protein conformation into a less proteolytically sensitive form without changing the enzyme mechanism or kinetics. In addition, only hydrophobic amino acids were selected for mutagenesis to avoid major disturbances in the local protein structure. It was interesting to observe positive effects by replacing Val223 with either a bulky Phe or a small Gly, though replacing Val223 with Ile had the best improving effect; implying that the molecular size of the amino acid was not a critical factor associated with the proteolytic sensitivity. Note that, though the cultivation performance for functional expression of PalB was significantly improved with the use of the double mutant species (i.e. Leu149Val and Val223Ile), protein bands corresponding to PalB were present in the insoluble fraction, implying that the improved functional expression was limited by another limiting step of protein misfolding.

The majority of the expressed PalB mutants (e.g. M-2 and M-4), either soluble or insoluble, was detected in the periplasm, implying translocation mediated by PelB signal peptide was effective. However, it was a bit surprising that certain PalB activity was detected in the reductive cytoplasm which is supposedly unsuitable for disulfide bond formation. Interestingly, functional expression of PalB M-2 variant was significantly enhanced upon the coexpression of DsbA, but not DsbC. The results suggest that the initiation but not isomerization associated with disulfide bond formation in the periplasm became limiting. The chaperone activity associated with DsbA could also assist the folding of the expressed PalB double mutant. It was previously reported that functional expression of PalB in the oxidative cytoplasm of *E. coli* Origami strains could be limited by disulfide bond formation (305).

Although many proteases have been identified in *E. coli*, their proteolytic mechanisms, recognition sequences, and substrate specificity remain incompletely characterized. This study appears to identify new protease-sensitive sequences since further investigation of the amino acid sequence near Leu149 and Val223 (i.e. Leu₁₄₆-Asp₁₄₇-Ala₁₄₈-Leu₁₄₉-Ala₁₅₀-

Val₁₅₁-Ser₁₅₂ and Pro₂₂₀-Leu₂₂₁-Phe₂₂₂-Val₂₂₃-Ile₂₂₄-Asp₂₂₅-His₂₂₆) did not reveal any sites previously reported as proteolytically sensitive. However, proteolytically sensitive sequences might not be specific to certain proteases and various factors affecting protease binding, secretion, folding, solubility or steric hindrance, e.g. neighboring domains or amino acids (190), can contribute to the sensitivity of potential proteolytic sites, making such sequence manipulation for alleviation of proteolysis more difficult. Since the proteolytic sensitivity of PalB remained unchanged when replacing Leu₁₄₉ or Val₂₂₃ with several other amino acids, a protease-recognition pattern other than amino acid sequence might be involved in PalB degradation.

Mutant *E. coli* strains deficient in a selection of genetic loci associated with protein degradation were constructed to enhance the production of labile recombinant proteins (13, 179). Since BL21(DE3) used in this study is deficient in *ompT* encoding an envelope protease OmpT which cleaves periplasmic proteins at dibasic amino acid sequences such as Arg-Lys (101), OmpT was not responsible for PalB degradation. Precise identification of the protease(s) responsible for PalB degradation could assist the construction of proper mutant strains for functional expression. Nevertheless, the specific alleviation of target protein's sensitivity toward intracellular proteolysis proposed herein offers an alternative to enhance recombinant protein production since the knockout in protease genes can potentially impair the physiology, growth properties, and even viability of the production strains and subsequently affect recombinant protein yield.

Chapter 5

Enhancing Functional Expression of Heterologous Lipase B in *Escherichia coli* by Extracellular Secretion

5.1 Introduction

Escherichia coli retains its popularity as the most versatile host for recombinant protein production due to many expression advantages (93, 173, 206). Given the extensive knowledge associated with heterologous protein expression in *E. coli*, technical limitations are still encountered, particularly including posttranslational processing, protein misfolding and aggregation, proteolysis, and recombinant protein toxicity (171).

Cytoplasm is the intracellular compartment where all nascent proteins are first synthesized and most of them reside. However, it might not serve as a suitable destination for recombinant protein production because of the liability to protein proteolysis (274), the inability to form disulfide bonds (225), the tendency to protein misfolding (149), and the complicated downstream processing (271). These drawbacks can be potentially overcome by secretion of foreign gene products to the periplasm or extracellular medium (181).

All the periplasmic and outer membrane proteins in *E. coli* require the secretion of their corresponding precursors across the cytoplasmic membrane. The major mechanism driving such translocation is the Sec-dependant Type II secretion pathway, which is mediated by a cleavable signal peptide at the N terminus (52). A wide variety of signal peptides, such as PelB (22), PhoA (281), OmpA (302), and SpA (189), have been adopted successfully as a genetic strategy to secrete recombinant proteins for expression in the periplasm or on the outer membrane of *E. coli*.

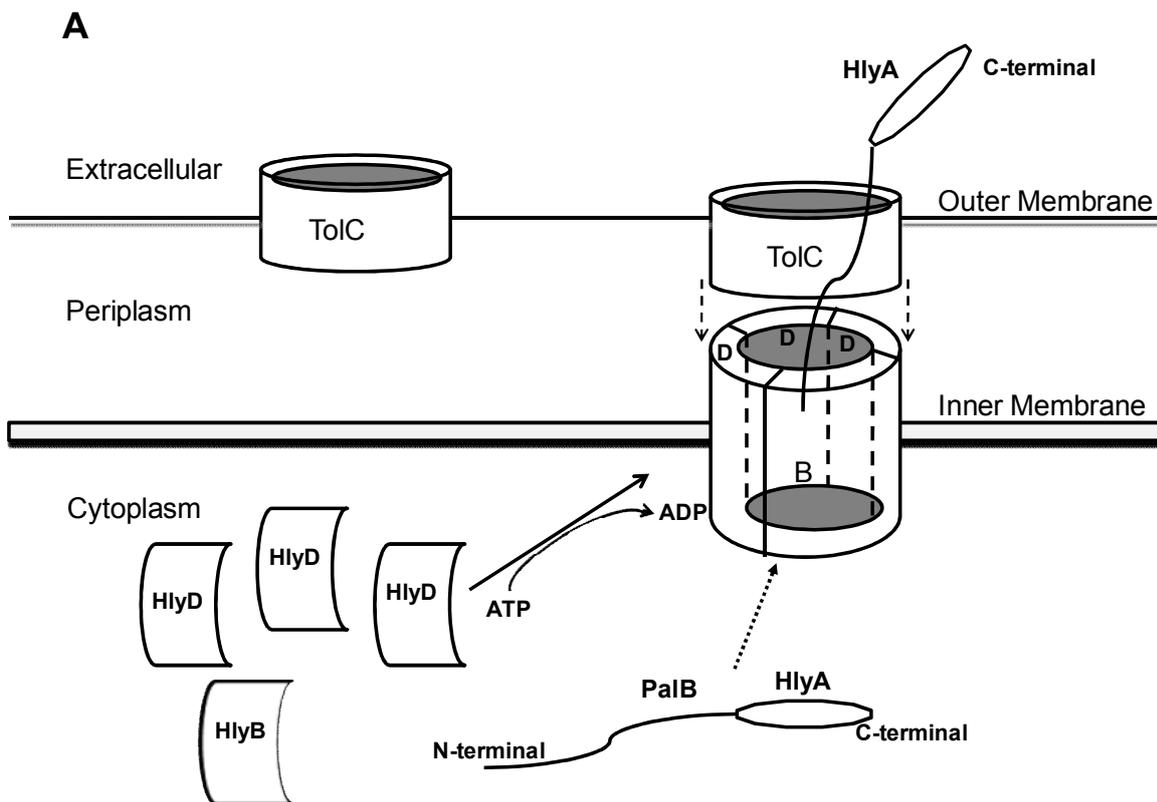
By contrast, very few *E. coli* proteins are known to be extracellularly secreted due to the presence of another secretion barrier of the outer membrane. Type I secretion system, known as the ABC transporter, secretes *E. coli* toxins and exoenzymes via a one-step process across both the cytoplasmic and outer membranes without a periplasmic intermediate (24). On the other hand, Type III secretion system represents a specific mechanism associated with the

secretion of pathogenesis factors or flagellar proteins from the cytoplasm, bypassing the periplasm, directly to the exterior of the bacteria (278).

Despite of limited natural mechanisms for protein secretion in *E. coli*, genetic strategies have been developed to release recombinant proteins extracellularly, including (1) the use of leaky mutant host strains (233) and cell-wall-less L-form *E. coli* (105), (2) chemical or biochemical permeabilization of outer membrane (52, 251), and (3) coexpression of protein release factors such as bacteriocin release protein (BRP) (288), colicin E1 lysis protein (Kil), *tolAIII* and *out* genes (294). However, these approaches on the basis of permeabilizing cells are limited to the release of periplasmic proteins (which are typically exported from the cytoplasm via Type II secretion system) and often deteriorate the physiological condition of producing cells. Consequently, the recombinant protein yield is hampered. Type I or III secretion systems, therefore, appear to be an alternative by which extracellular production of proteins can be performed with minimum physiological impact. In fact, several proteins, that are otherwise hard to be expressed intracellularly, have been functionally expressed and extracellularly secreted via the hemolysin (Type I) system (28, 79, 286) and the flagellar (Type III) system (21, 64, 220, 253).

PalB, a lipase B from *Pseudozyma antarctica* (previously known as *Candida antarctica*), is an important industrial enzyme with wide applications (9). Herein, we are exploring another strategy for functional expression of recombinant PalB, namely extracellular secretion via Type I and III systems (Figure 5.1). Previously, PalB was expressed heterologously in fungi and yeasts, such as *Aspergillus oryzae* (122), *Pichia pastoris* (237), and *Saccharomyces cerevisiae* (313). Given the popularity of *E. coli*, functional expression of PalB in this host was not demonstrated until recently (27, 167, 303, 305, 306). Since there are three intramolecular disulfide bonds in PalB and their formation can potentially affect the development of protein structure and bioactivity, functional expression of PalB in the oxidative periplasm of *E. coli* appears to be a rational approach. However, heterologous PalB targeting in the periplasm was highly unstable and subjected to proteolysis (303). Cytoplasmic expression of PalB in the reducing cytoplasm of wild-type *E. coli* suffered ineffective disulfide bond formation. Though this technical issue could be

circumvented by the expression in the oxidative cytoplasm of genetically modified *E. coli* (i.e. Origami™), protein misfolding remained problematic (305). Given the above limitations in disulfide bond formation and protein misfolding, extracellular secretion appears to be a plausible exploration. In this study, using the hemolysin secretion apparatus, PalB-HlyA protein fusion was extracellularly produced with relatively fast secretion and high PalB activities. On the other hand, though PalB secreted via the flagellar system is an intact protein, the secretion was not initiated until 24 h after cultivation. Both two protein secretion systems posed minimum physiological impact on producing cells.



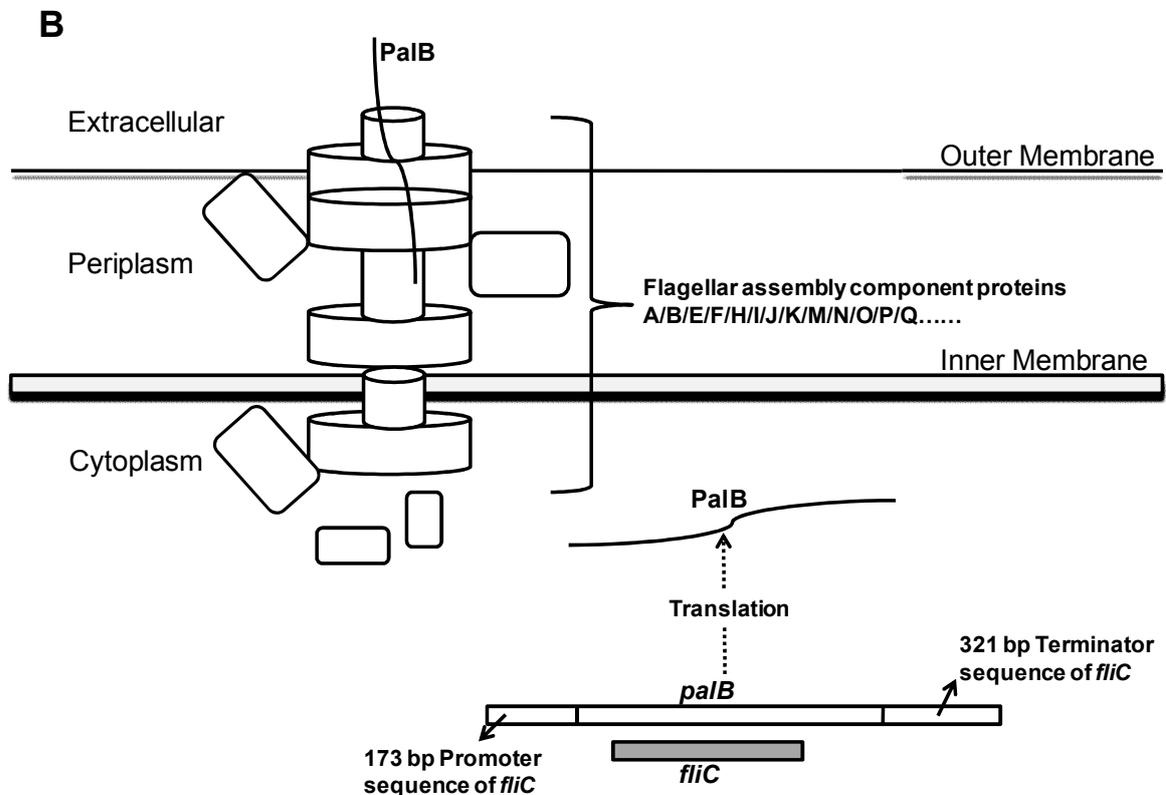


Figure 5.1 Extracellular secretion of heterologous PalB in *E. coli* via Type I and Type III secretion systems. Panel A/ The hemolysin (Type I) secretion system belongs to the ABC transporter family which recognizes the C-terminal amino acids of hemolysin toxin HlyA for protein secretion without requiring an N-terminal signal peptide (100). The inner membrane proteins, i.e. HlyB and HlyD trimer, form an assembly with concurrent ATP hydrolysis (79, 88) to interact with the outer membrane protein TolC (148). A hydrophilic channel connecting the two membranes forms as an export conduit through which the recombinant PalB fused to the 23 kDa C-terminal of HlyA is co-exported extracellularly. Panel B/ The *E. coli* flagellar (Type III) secretion system can secrete the filamentous flagellar protein (FliC) through the interaction with various

flagellar assembly component proteins (183). In order to conduct recombinant protein secretion using this system, the flagellar protein gene *fliC* is replaced by the target gene of interest (*palB* in this study) flanked by the untranslated regions (UTR) of the *fliC* gene, i.e. the 173 bp promoter sequence of *fliC*_{MG1655} at 5' end and 321 bp terminator sequence of *fliC*_{MG1655} at the 3' end (170). The exact nature of the secretion signals still remains unknown. One model suggests that the secretion could be cotranslational using the 5'UTR region of mRNA as the signal (8); whereas another model describes the secretion as posttranslational using a protein sequence as a signal (168).

5.2 Materials and Methods

5.2.1 Bacterial Strains and Plasmids

Bacterial strains, plasmids, and oligonucleotides used in this study are summarized in Table 5.1 and are briefly described below. DH5 α was used as the host strain for molecular cloning. BL21(DE3), DH5 α , HB101, JM109 and MC4100 were used as the expression hosts for PalB secretion studies using the hemolysin transporter. MKS12 was used as the host strain for PalB secretion studies using flagellar system.

The protocol for molecular cloning is described in Chapter 3. The plasmid pEHLYPalB was used for extracellular secretion of PalB-HlyA fusion via the hemolysin system. The *palB* gene was PCR-amplified with *Pfu* (Stratagene, La Jolla, CA, USA) as the PCR polymerase, pPCRscriptPalB-L as the template, and P10 and pEHREV as the primers. The amplified DNA fragment was digested with *NcoI/EagI* and ligated with similarly digested pEHLYA2-SD to form pEHLYPalB. The plasmid pVDL9.3 was used for the production of HlyB and HlyD. The plasmid p5'3'UTRPalB was used for extracellular secretion of PalB via the *E. coli* flagellar system. The *PvuI/SacI* fragment of p5'UTR was subcloned into pMCS3'UTR to form p5'3'UTR containing the 173-bp 5'-*fliC*_{MG1655} promoter sequence and the 321-bp 3'-untranslated region (UTR) of the *fliC*_{MG1655} gene. The *palB* gene was PCR-amplified with *Pfu* as the PCR polymerase, pPCRscriptPalB-L as the template, and P10 and P22 as the primers. The amplified DNA fragment was digested with *NcoI/SacI* and ligated to similarly digested p5'3'UTR to form p5'3'UTRPalB.

Table 5.1 Strains, plasmids, and oligonucleotides.

Strain	Relevant Genotype	Source & Reference
BL21(DE3)	F ⁻ <i>ompT dcm lon hsdS_B (r_B⁻, m_B⁻) gal λ(DE3[<i>lacI</i> <i>ind1 sam7 nin5 lacUV5-T7 gene 1</i>])</i>	(224)
DH5α	F ⁺ (ϕ 80 <i>dlac Δ(lacZ)M15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17 (r_K⁻, m_K⁺) phoA supE44 lambda-thi-1 gyrA96 relA1</i>)	(300)
HB101	F ⁻ <i>hsdS20 leuB6 recA13 ara-14 proA2 lacY1 thi-1 galK2 rpsL20 xyl-5 mtl-1 supE44 λ</i>	(34)
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi-1 mcrA Δlac-proAB</i> F ⁺ [<i>traD36 proAB⁺ lacI^q lacZΔM15</i>]	(307)
MC4100	<i>araD 139Δ(argF-lac)U169 rpsL150 relA1 flb3501 deoC ptsF25 rbsR</i>	(35)
MKS12	Deletion of 1407 bp <i>fliD</i> gene and 1497 bp <i>fliC</i> gene of <i>E coli</i> MG1655 Δ <i>fimA-H</i> ¹⁸	(170)
Plasmid		
p5'UTR	173 bp promoter sequence of <i>fliC</i> _{MG1655} in <i>PvuI-ScaI</i> site of pBR322, additional <i>NcoI-SacI</i> sites preceding <i>ScaI</i> , Tc ^R	(170)
pMCS3'UTR	321 bp 3'UTR DNA sequence of <i>fliC</i> _{MG1655} in <i>ScaI-EcoRI</i> site of pBR322, Tc ^R	(170)
p5'3'UTR	173 bp promoter sequence of <i>fliC</i> _{MG1655} in <i>PvuI-ScaI</i> site of pMCS3'UTR, additional <i>NcoI-SacI</i> sites	This study

	preceding <i>ScaI</i> , Tc ^R	
p5'3'UTRPalB	5'UTR:: <i>palB</i> ::3'UTR, Ori (pBR322), Tc ^R	This study
pEHLYA2-SD	P _{<i>lac</i>} ::HlyA, Ap ^R	(79)
pEHLYAPalB	P _{<i>lac</i>} :: <i>palB</i> :: <i>hlyA</i> , Ap ^R	This study
pPCRscriptPalB-L	PCR cloning of <i>palB</i> , Ap ^R	This lab
pVDL9.3	P _{<i>lac</i>} :: <i>hlyB</i> ; <i>hlyD</i> , Cm ^R	(79)

Oligonucleotides

P10	5'-GG <u>CCATG</u> GGTCTACCTTCCGGTTCGG-3'	(306)
P22	5'-CT <u>GAGCTC</u> TACAGGGGGTGACGATGCCGGAG-3'	(306)
pEHREV	5'-ACGGAGCTCGAATTC <u>GGCCG</u> GGGTGACGAT-3'	This study

* Italic: mutation nucleotides, underline: restriction site

5.2.2 Cultivation

The protocol for shaker flask experiment and bioreactor cultivation is described in Chapter 3 and Chapter 4.

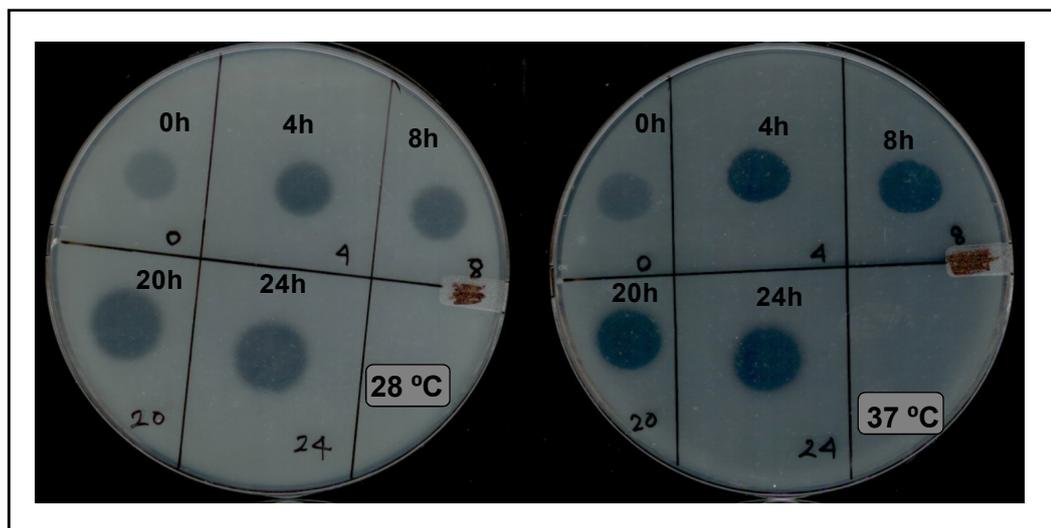
5.2.3 Analytical Methods

The protocol for various analyses is described in Chapter 3 and Chapter 4. The culture supernatant and intracellular soluble fraction were analyzed for PalB activity.

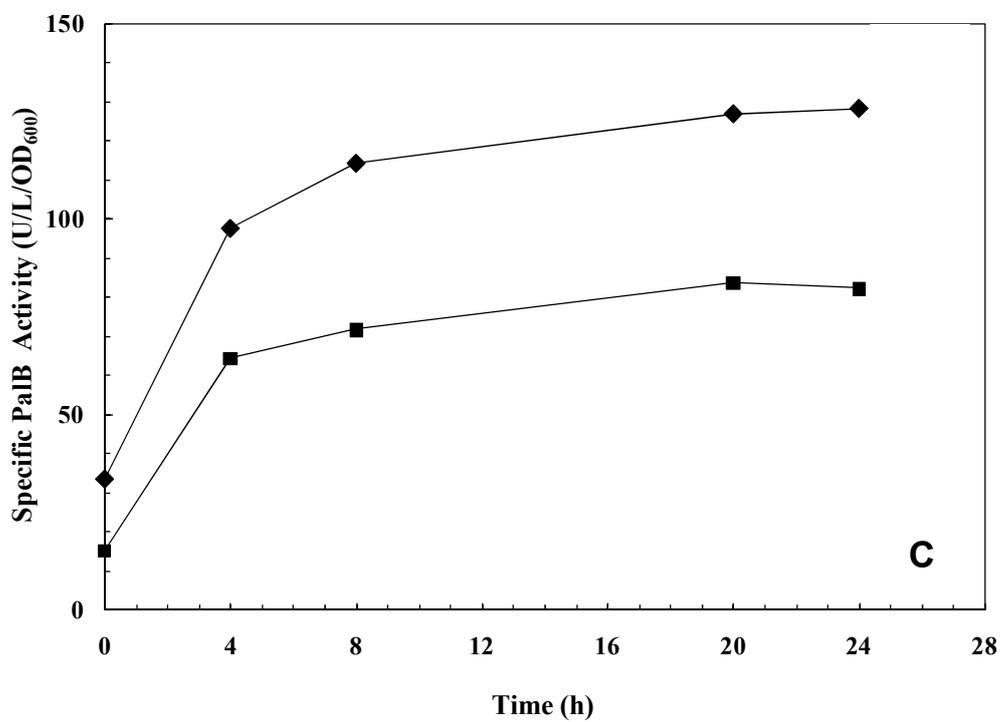
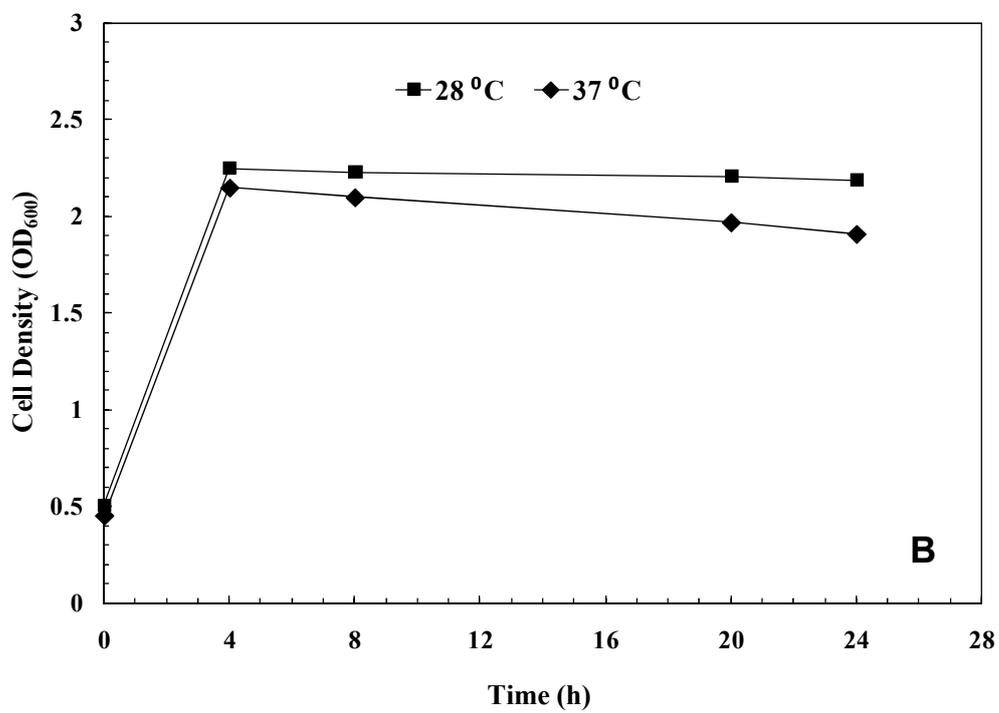
5.3 Results

For the extracellular secretion of PalB from *E. coli*, the plasmid pEHLYAPalB containing the *palB-hlyA* gene fusion (encoding the protein fusion of PalB and the 23 kDa C-terminal of HlyA whose expression was regulated by the *lac* promoter) was constructed. Using JM109

harboring pEHLYAPalB and pVDL9.3 for shaker flask cultivation, the culture performance is summarized in Figure 5.2. PalB bioactivity associated with the extracellular secretion of PalB-HlyA was visualized on tributyrin agar plates (Figure 5.2A), indicating that HlyA can functionally mediate PalB secretion without affecting its proper folding. Two cultivation temperatures, i.e. 28 °C and 37 °C, were used. While cell growth for the two cultures was similar (Figure 5.2B), the 37 °C one had a higher specific PalB activity (128 vs. 83 U/L/OD₆₀₀ in Figure 5.2C). Therefore, all subsequent cultivations were performed at 37 °C. Western blotting was also conducted to probe PalB-HlyA at approximately 58 kDa in both the extracellular and intracellular (soluble) fractions. While the PalB-HlyA band was detected in both fractions (Figure 5.2D), PalB activity was measurable only in the extracellular fraction (Figure 5.2A and 5.2C) but not the intracellular one (data not shown), implying that the secretion of PalB could assist proper protein folding to attain its bioactive form. Note that, for the later extracellular culture samples at 20 and 24 h, another PalB-related band with a molecular weight less than the expected size of 58 kDa was probed by PalB antibodies, implying that a possible proteolysis might have occurred on a specific cleavage site. Upon comparing the PalB activity with the band intensity, both extracellular bands appeared to be bioactive.



A



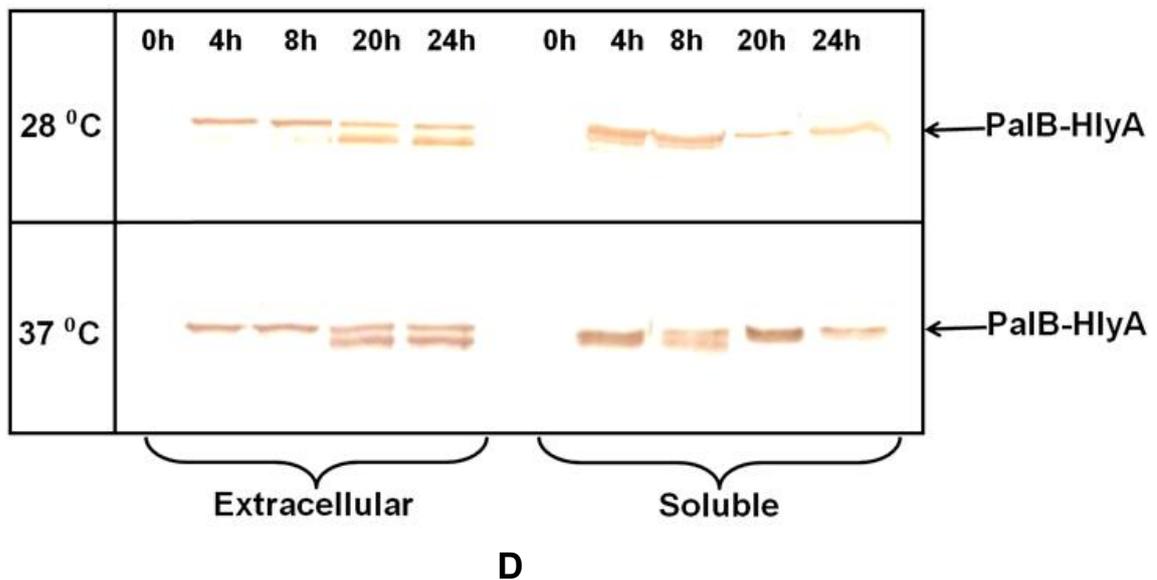
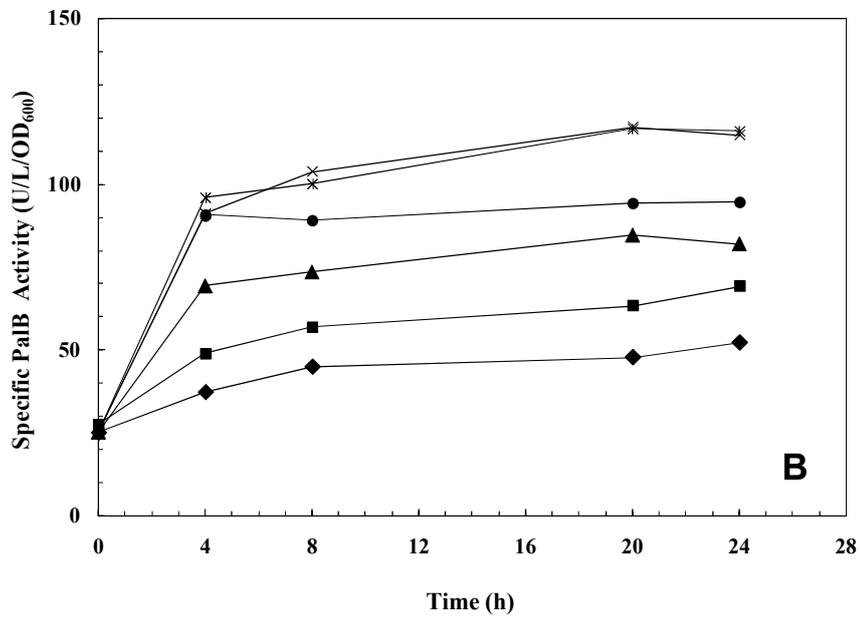
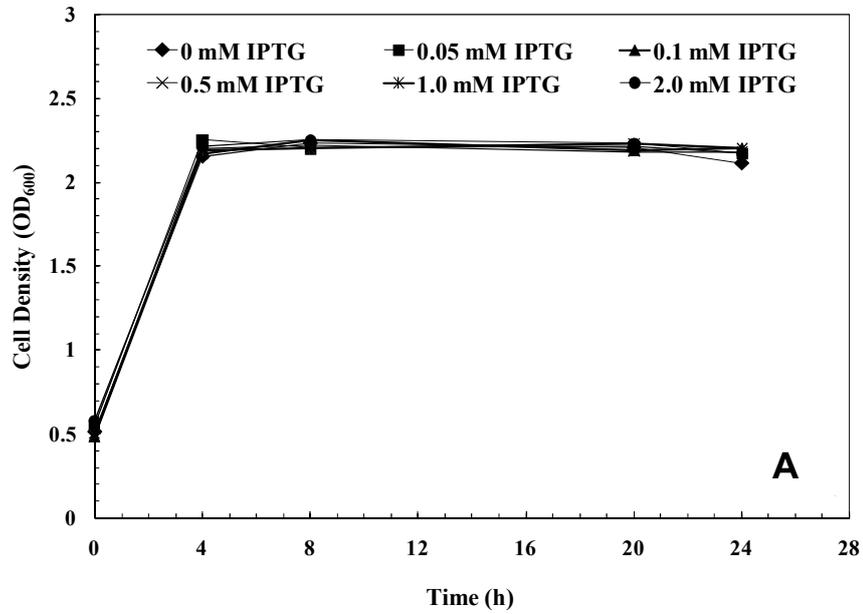


Figure 5.2 Shaker flask cultivation of JM109 (pEHLYAPalB, pVDL9.3): The cultivation was conducted at 28 °C and 37 °C. IPTG at 0.5 mM was used for induction. **Panel A/** Qualitative visualization of PalB activity using tributyrin agar plates. **Panel B/** Time profiles of cell density. **Panel C/** Time profiles of specific PalB activity. **Panel D/** Western blotting analysis of the extracellular and soluble intracellular fraction of culture samples.

The effect of the inducer concentration on the secretion of PalB-HlyA is shown in Figure 5.3. The control experiment without IPTG induction had a specific PalB activity of 25 U/L/OD₆₀₀ due to the expression leakage associated with the *lac* promoter. The PalB activity increased monotonically with the IPTG concentration up to 0.5 mM, at which the expression/secretion was optimal (Figure 5.3B). Further increase in the inducer concentration had either no or adverse effect on the culture performance. Note that the doublet bands were still observable for all the later extracellular culture samples (Figure 5.3C). Nevertheless, the inducer concentration had no effect on cell growth (Figure 5.3A), implying that the

expression and secretion of PalB-HlyA posed a minimum physiological impact on producing cells.



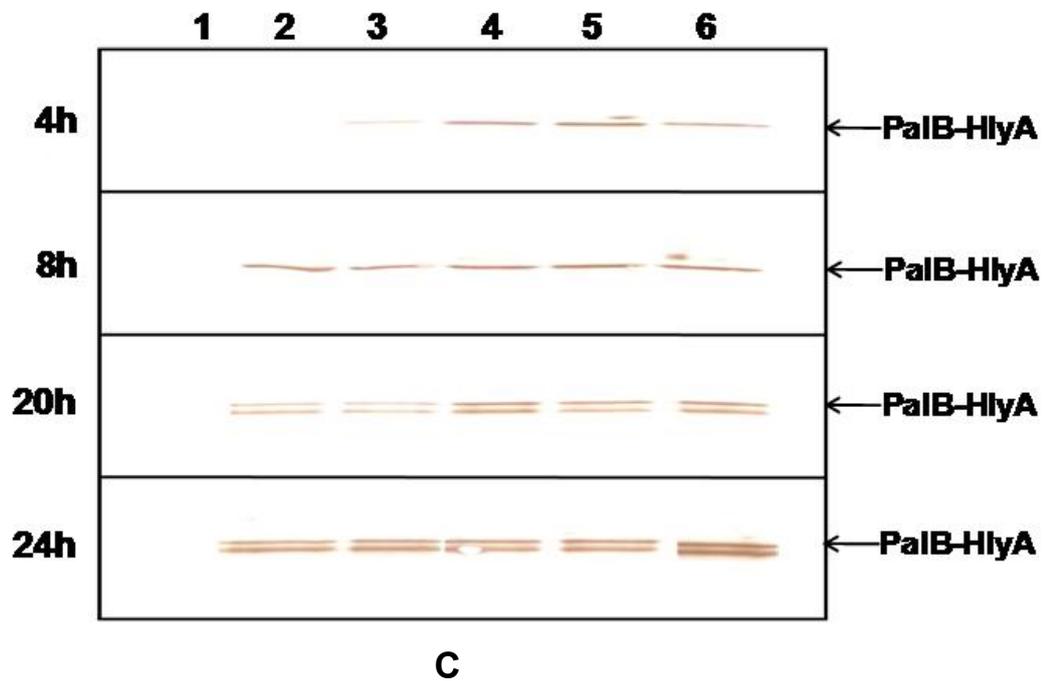
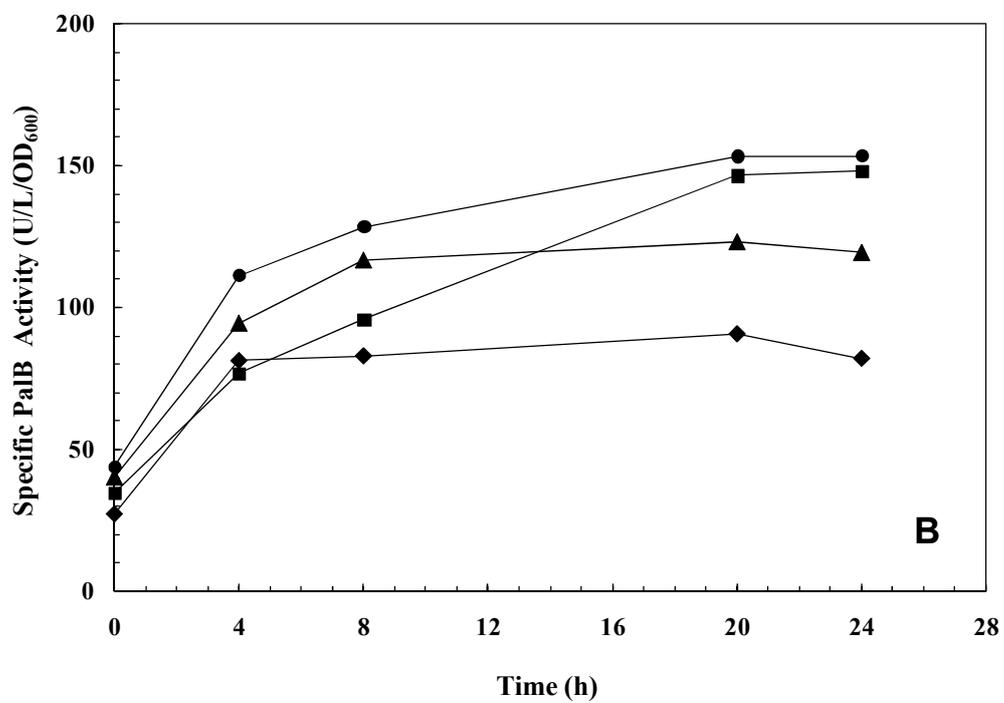
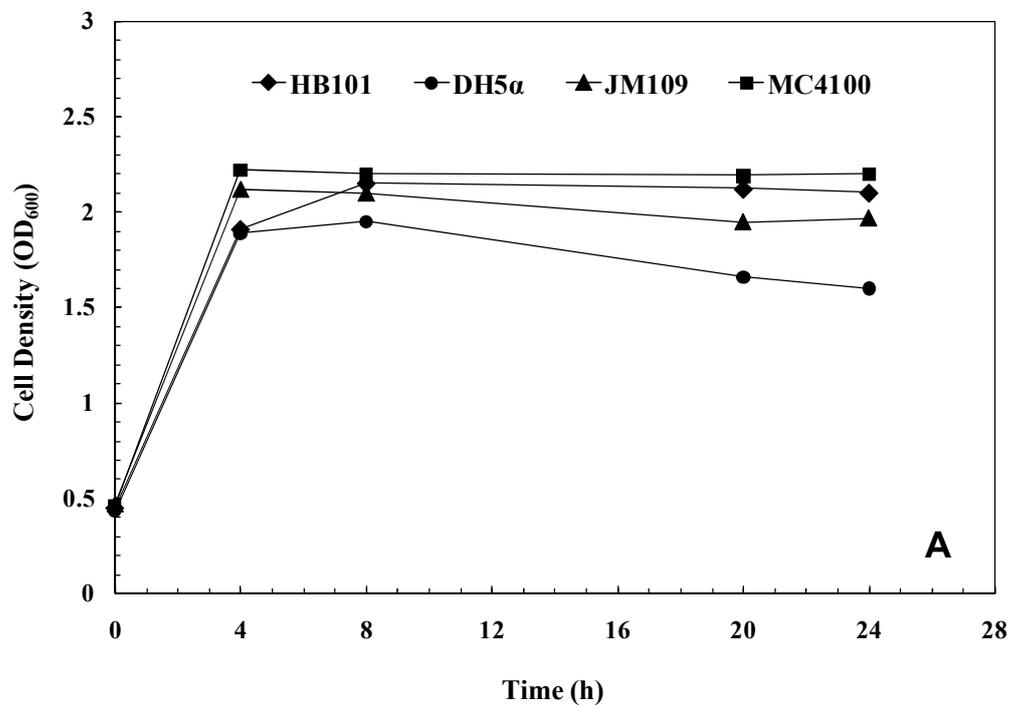
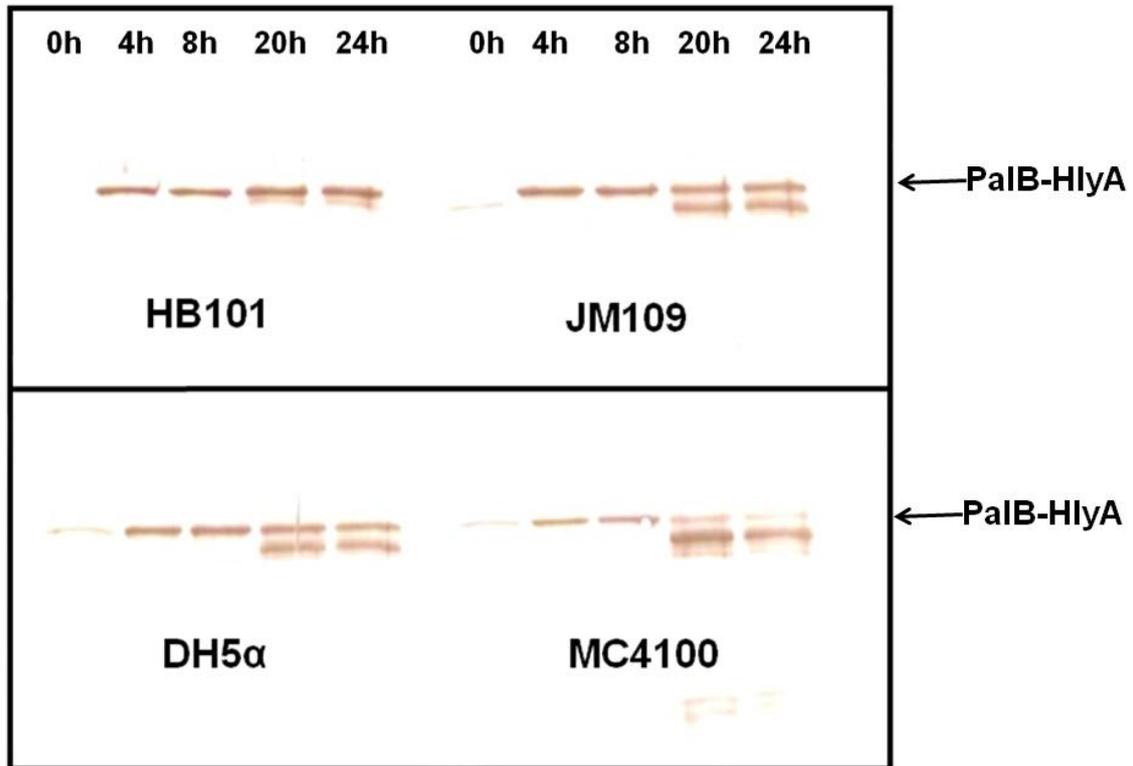


Figure 5.3 Effect of the inducer concentration on the culture performance for PalB-HlyA expression and secretion using JM109 (pEHLYPalB, pVDL9.3): The cultivation was conducted at 37 °C with various IPTG concentrations. Panel A/ Time profiles of cell density. Panel B/ Time profiles of specific PalB activity. Panel C/ Western blotting analysis of the extracellular fraction of culture samples.

Five *E. coli* hosts, i.e. BL21(DE3), DH5 α , HB101, JM109 and MC4100, were used to observe any host effect and the results are summarized in Figure 5.4. While the specific PalB activity was the highest with the use of MC4100 or DH5 α as the expression host (Figure 5.4B), the latter suffered poor growth (Figure 5.4A) which negatively impacted the overall culture performance. On the other hand, recombinant HB101 had the lowest specific PalB activity (Figure 5.4B). Note that no secretion was observed for recombinant BL21(DE3) (data not shown) though it is often considered an effective host for recombinant protein production. The results suggest the importance of selecting a proper host strain for the extracellular production of PalB using the hemolysin secretion system. Interestingly, the doublet bands on the Western blot for the late culture samples of recombinant JM109 and DH5 α were similar in pattern since the two bands had a comparable intensity (Figure 5.4C). On the other hand, the lower band of the doublet for the late recombinant HB101 culture samples was minor, whereas the upper band of the doublet for recombinant MC4100 culture samples was minor (Figure 5.4C). Note that the high PalB activities associated with the late recombinant MC4100 culture samples support the previous argument that both bands could represent a bioactive species.

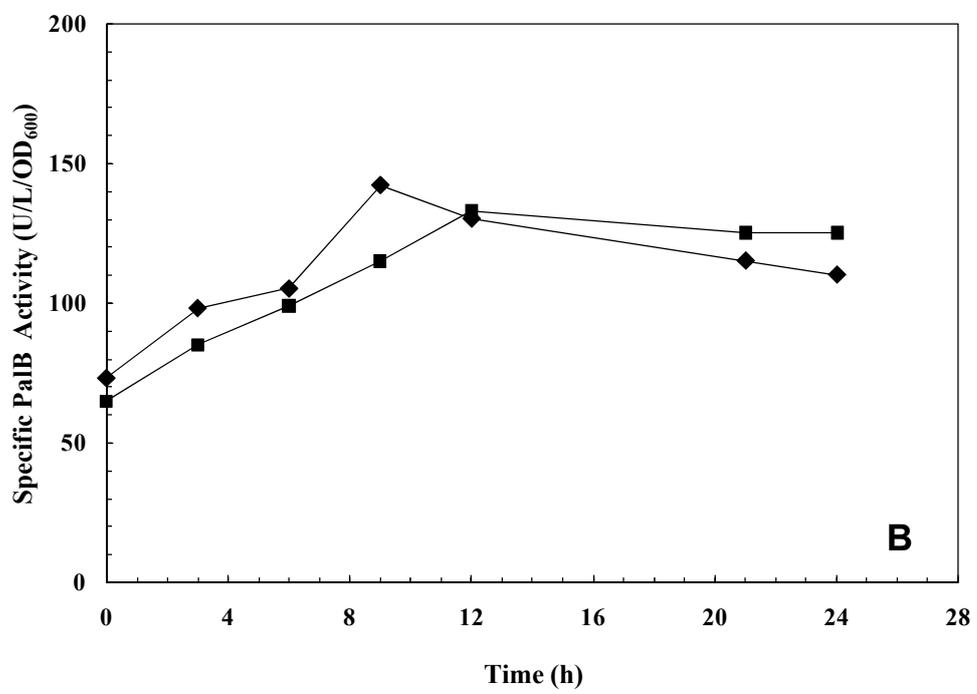
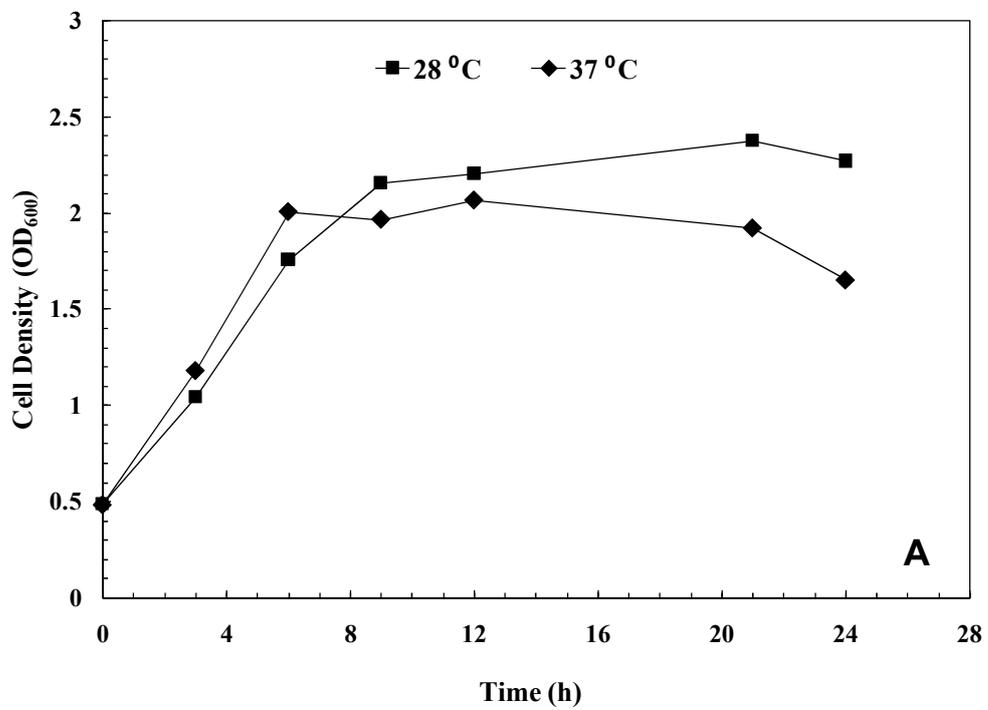




C

Figure 5.4 Effect of the host cell on the culture performance for PalB-HlyA expression and secretion: Various *E. coli* hosts, including DH5 α , HB101, JM109, and MC4100, harboring pEHLYPalB and pVDL9.3 were used. The cultivation was conducted at 37 °C with 0.5 mM IPTG. Panel A/ Time profiles of cell density. Panel B/ Time profiles of specific PalB activity. Panel C/ Western blotting analysis of the extracellular fraction of culture samples.

Extracellular secretion for functional expression of PalB was also explored in a bioreactor and the cultivation results using JM109 (pEHLYAPalB, pVDL9.3) are summarized in Figure 5.5. With the cultivation at 28 °C, the specific PalB activity was increased by 50% at 130 U/L/OD₆₀₀ as compared to the shaker flask culture in which the maximum PalB activity was 83 U/L/OD₆₀₀. On the other hand, only a slight improvement was observed in the secretion of PalB-HlyA for the bioreactor culture at 37 °C when compared to the shaker flask culture at the same temperature (i.e. 142 U/L/OD₆₀₀ vs. 128 U/L/OD₆₀₀). While PalB-HlyA was effectively produced and secreted within 9 h of bioreactor cultivation at 37 °C, the secreted protein appeared to be rather unstable and the PalB activities declined after 9 h. The results imply that a lower cultivation temperature at 28 °C might be favored for bioreactor cultivation which appears to have a better ambience for faster secretion and production of PalB-HlyA.



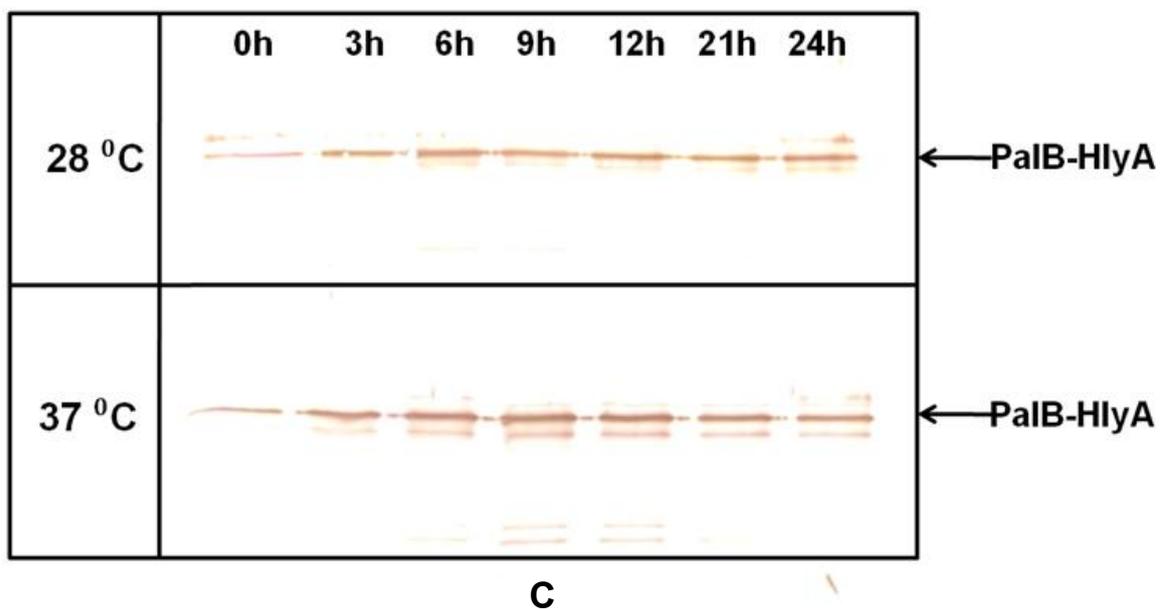
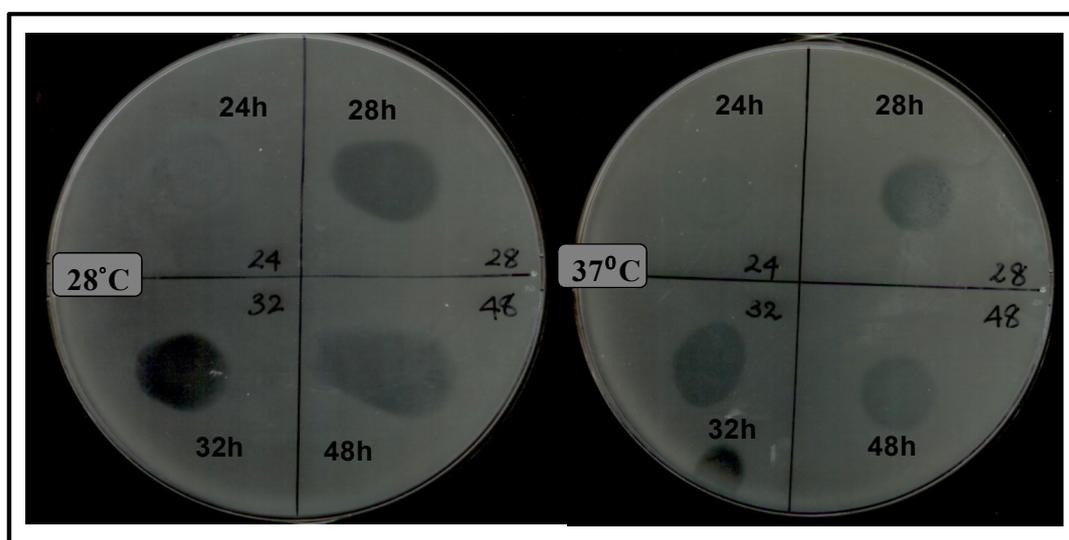
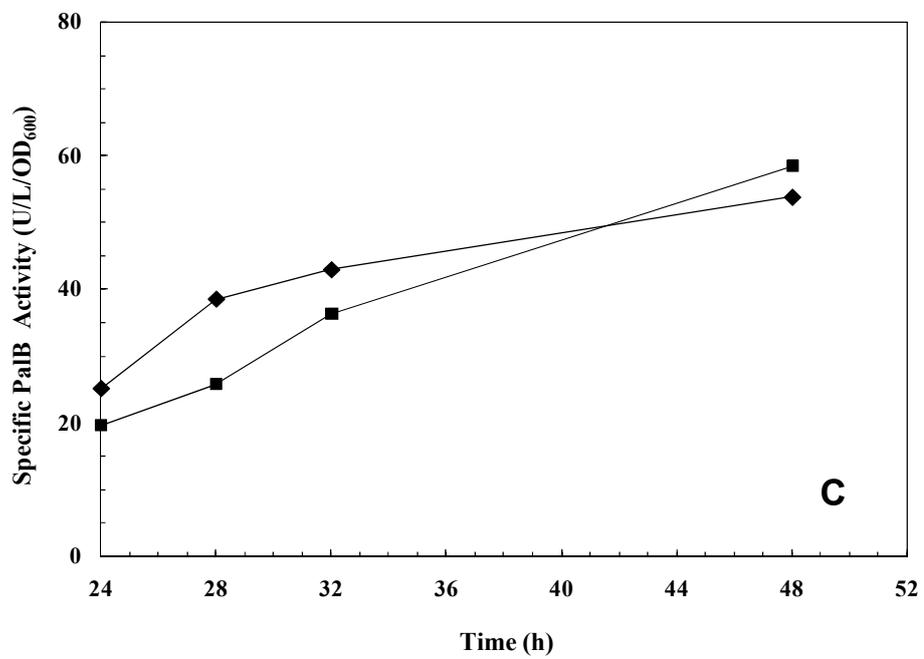
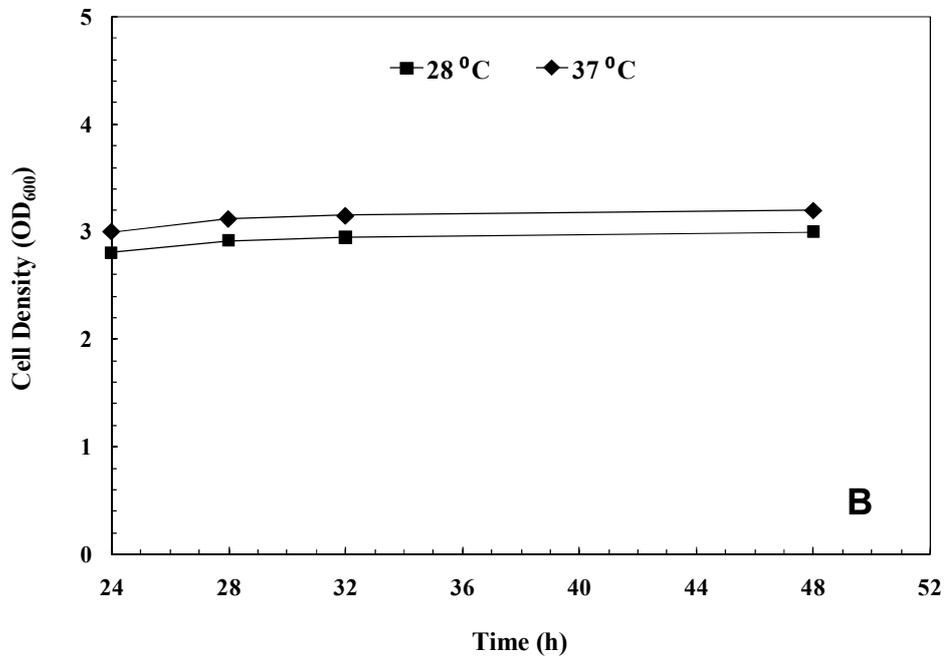


Figure 5.5 Bioreactor cultivation of JM109 (pEHLYAPalB, pVDL9.3): The cultivation was conducted at 28 °C and 37 °C. IPTG at 0.5 mM was used for induction. Panel A/ Time profiles of cell density. Panel B/ Time profiles of specific PalB activity. Panel C/ Western blotting analysis of the extracellular fraction of culture samples.

The modified flagellar secretion apparatus was also explored for the extracellular secretion of recombinant PalB using the plasmid p5'3'UTRPalB. The expression of PalB was under the regulation of the *fliC* promoter. Using *E. coli* MKS12, whose flagellin-related genes of *fliC* and *fliD* are deleted, as a host, the results of shaker flask cultivation are summarized in Figure 5.6. Consistent to the previous observation (170), protein secretion was not significantly observed until 24 h of cultivation. Hence, culture samples were collected during 24 and 48 h for analysis. Compared to the hemolysin system, the flagellar system appears to be less effective in terms of the secretion level though the intact PalB rather than a PalB protein fusion was secreted. The cultivation was conducted at both 28 °C and 37 °C with a rather similar culture performance in terms of cell growth (Figure 5.6B) and the secretion level (Figure 5.6C). Western blotting was conducted to probe the expressed PalB in both the extracellular and intracellular (soluble) fractions. While the PalB-related band at 35 kDa was observed in both fractions, PalB activity was present only in the extracellular fraction (Figure 5.6D). The results suggest that extracellular secretion could assist proper folding for functional expression of PalB.



A



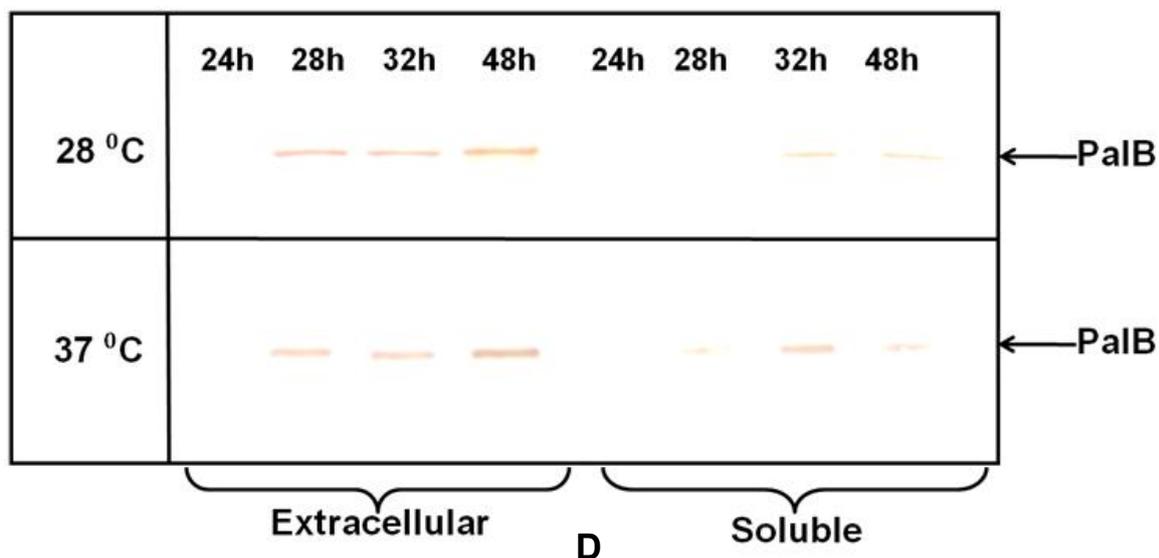
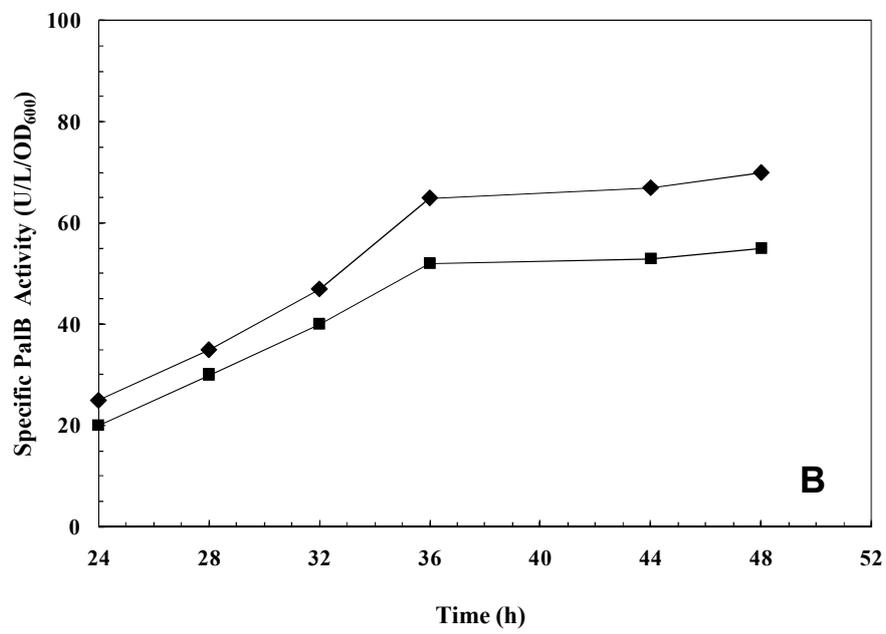
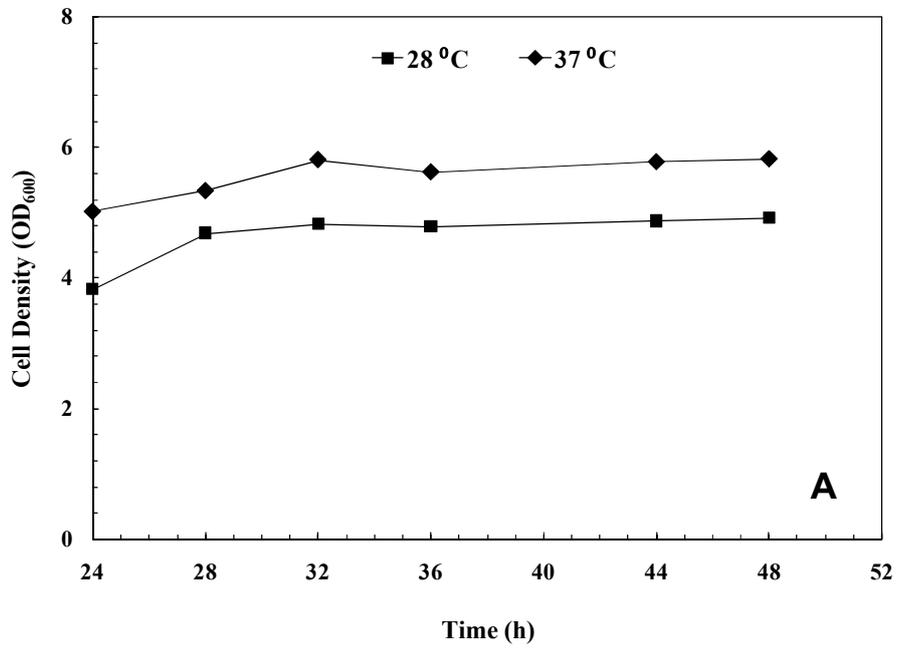
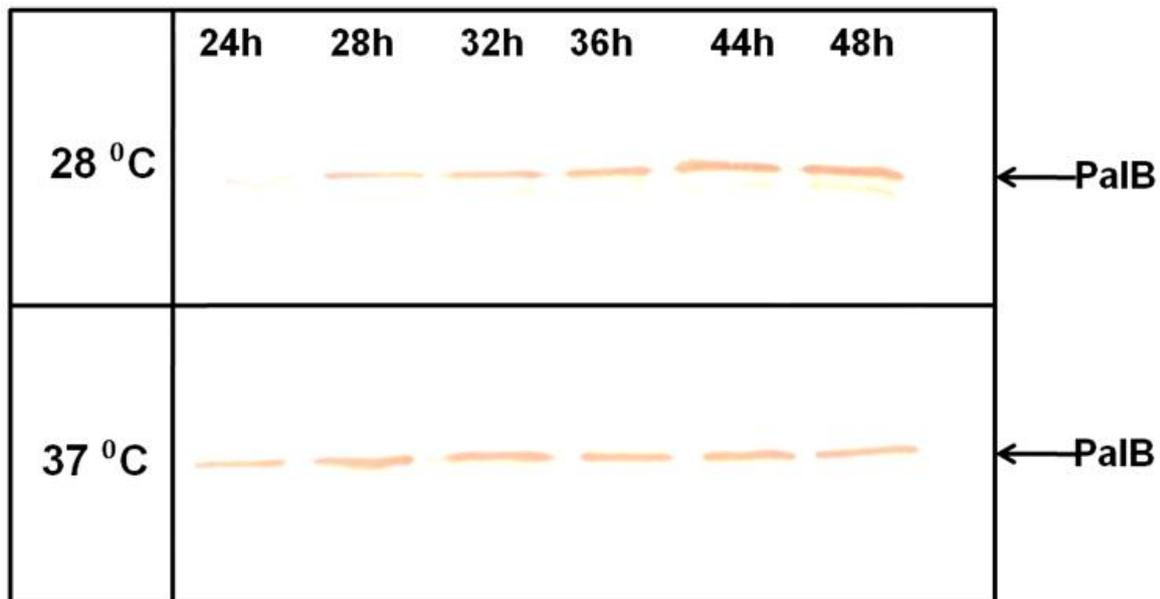


Figure 5.6 Shaker flask cultivation of MKS12 (p5'3'UTRPalB): The cultivation was conducted at 28 °C and 37 °C. Panel A/ Qualitative visualization of PalB activity using tributyrin agar plates. Panel B/ Time profiles of cell density. Panel C/ Time profiles of specific PalB activity. Panel D/ Western blotting analysis of the extracellular and soluble intracellular fraction of culture samples.

Culture performance for extracellular secretion of PalB was enhanced upon bioreactor cultivation and the results are summarized in Figure 5.7. Compared to the shaker flask cultivation at 37 °C, the corresponding bioreactor culture had not only a higher cell density (5.8 vs. 3.2 OD₆₀₀) but also a higher specific PalB activity (70 vs. 54 U/L/OD₆₀₀), resulting in a significant enhancement in the volumetric PalB activity. There was also a slight improvement for bioreactor cultivation at 28 °C due to a higher cell density compared to the shaker flask cultivation. Note that the secretion of PalB did not appear to pose any adverse effect on cell physiology based on the profile of cell growth.





C

Figure 5.7 Bioreactor cultivation of MKS12 (p5'3'UTRPalB): The cultivation was conducted at 28 °C and 37 °C. Panel A/ Time profiles of cell density. Panel B/ Time profiles of specific PalB activity. Panel C/ Western blotting analysis of the extracellular fraction of culture samples.

5.4 Discussion

Though PalB has wide industrial applications, its functional expression in *E. coli* suffered various technical limitations. In our lab, various strategies were explored to express heterologous PalB in the cytoplasm, periplasm, and cell surface of *E. coli* with protein misfolding and intracellular proteolysis as the major expression issues (303, 305, 306). Hence, extracellular secretion appears to be a plausible alternative for exploration. In this study, functional expression of heterologous PalB in *E. coli* was achieved by extracellular secretion via two Sec-independent pathways, i.e. the α -hemolysin (Type I) and the modified

flagellar (Type III) secretion systems. Bioactive and rather pure PalB was obtained in the extracellular medium with minimum contamination by the host proteins.

Since PalB has three disulfide bonds critical for developing the protein structure and biological activity, the absence of the PalB activity for the intracellular fraction of culture samples suggests that the intracellularly expressed polypeptides could be maintained in an unfolded or partially folded state presumably for preparing extracellular export. It was previously demonstrated that single chain Fv fragments secreted by the hemolysin transporter retained the antigen binding activity, which is associated with the formation of correct disulfide bridges, upon the extracellular accumulation (79). However, the *trxB* mutant of *E. coli* with an oxidizing cytoplasm had an inhibitory effect on the HlyA-dependant secretion of proteins with disulfide bonds (78). These observations suggest that proper disulfide bonds formed upon either protein translocation through the export conduit or protein exposure to the oxidative environment outside the cell.

One of the advantages of the hemolysin transporter demonstrated in this study lies in its fast and efficient secretion of PalB-HlyA with no interference in the folding and activity of PalB from the HlyA moiety. The hemolysin transporter has been competitive for the secretion of small heterologous polypeptides, such as single Ig domains (28), single chain Fv (79), and Shiga-like toxin IIeB (286). Apparently, the large size of PalB-HlyA with more than 500 amino acids did not affect its effective secretion, as demonstrated herein.

Though the hemolysin transporter is able to export the target protein extracellularly, it has at least two drawbacks. First, the secreted protein remains fused to HlyA and, depending on the application, an additional cleavage step might be required to obtain the native target protein. Second, coexpression of HlyB and HlyD secretion component proteins is required to mediate the secretion (7, 276). The simultaneous overexpression of the target protein fusion (i.e. PalB-HlyA) and transport component proteins might lead to an extra physiological burden on producing cells, resulting in relatively poor cell growth observed herein.

It is interesting to observe another protein species, with its size a bit smaller than the expected size of PalB-HlyA, in the extracellular fraction of culture samples after prolonged cultivation. Apparently, the secreted PalB-HlyA was subjected to extracellular proteolysis. In

addition to intracellular proteases, cell envelope proteases can also mediate the proteolysis of recombinant proteins heterologously expressed in *E. coli*. For example, the outer membrane protease OmpT was shown to specifically degrade several recombinant proteins incubated with intact *E. coli* K-12 (101). It was also applied for the proteolysis of secreted proteins fused with HlyA (110). OmpT acts on dibasic residues (176) and is present in all the *E. coli* hosts used herein except BL21(DE3). The PalB-related band with a lower molecular weight could possibly represent the OmpT-degradation product of PalB-HlyA. Nevertheless, it seems that the extracellular proteolysis did not affect the PalB activity, particularly for the culture of recombinant MC4100 in which most of secreted PalB-HlyA was digested but without losing the PalB activity (Figure 5.4C). Such proteolysis was almost negligible for the bioreactor cultivation at 28 °C (Figure 5.5C), implying that it could be associated with high cultivation temperature.

Type III secretion systems were originally identified when conducting studies on pathogenesis of bacteria whose invasion mechanism involves the injection of bacterial proteins into host cells (87, 126). The structural and regulatory components of the flagellum comprise the secretion apparatus, a Type III secretion system that secretes the flagellum from the cytoplasm directly to the exterior of the cell (183). Several proteins from evolutionarily distant sources, including Peb1 from *Campylobacter jejuni* (220), D1-D3 repeats from *Staphylococcus aerus* (253), α -enolase from *Streptococcus pneumoniae* (21), and the eukaryotic green fluorescent protein (64) were successfully expressed in *E. coli* and extracellularly secreted using the flagellar secretion system. However, these studies also reported that there were polypeptides highly expressed intracellularly but hardly secreted, as well as those expressed at low levels but still secreted extracellularly.

While PalB secretion was achieved by the modified flagellar secretion system herein, the secretion rate was rather slow and the expression/secretion level was relatively low possibly due to the lack of a strong promoter. Majander et al. (170) reported that different target proteins have varying levels of protein yield which could be associated with the similarity of the target protein sequence to flagellin and in turn the efficiency of transcription and translation of the target protein gene. On the other hand, the secretion rate might be

influenced by posttranslational folding since the flagellar subunits are secreted in an unfolded form (261) and fold only as they assemble (310). However, such secretion mechanism could be advantageous for functional expression of PalB which requires the formation of three disulfide bonds for proper folding. Extracellular secretion of PalB apparently made disulfide bond formation more effective and proteolysis less susceptible, unlike our previous explorations with intracellular expression (303, 305, 306). Given the slow secretion rate and relatively low PalB expression levels, the major advantage of the flagellar system is that the intact PalB, instead of PalB protein fusion as mediated by the hemolysin system, could be extracellularly secreted. This has a critical impact on biotechnological applications with respect to not only functional expression of PalB but also simpler downstream purification and recovery.

It is also noteworthy, in both cases, that the extracellular secretion of PalB protein products posed a minimum physiological impact on producing cells since the cell growth profile was hardly affected by heterologous protein expression and cell lysis was not observed during the cultivation. Also, the expression/secretion performance appears to be enhanced by bioreactor cultivation as compared to the shaker flask cultivation, presumably due to the controlled environment conducive to producing cells. The extracellularly secreted PalB products were not subjected to major proteolysis and loss of bioactivity, which was previously observed upon intracellular expression (303). While the secretion of PalB products was effective, both of the two secretion systems had a technical issue of low expression which could be improved by using a strong promoter system.

Chapter 6

Enhancing the Functional Expression of Heterologous *Burkholderia* Lipase in *Escherichia coli*

6.1 Introduction

Lipases are an important biocatalyst for industrial applications (246). Microbial cells including bacteria, fungi, and yeasts are the major sources of lipases with various enzymatic activities (e.g. hydrolysis, esterification, transesterification, alcoholysis, acidolysis, and chemo, regio, enantio-selective synthesis), enzyme properties (e.g. pH and temperature stability), and substrate specificities (131). Recent interest in lipases arises particularly from their applications as a potential biocatalyst for biodiesel production (197). Many microbial lipases have been isolated, sequenced and expressed (43, 61, 133, 285, 305). Among them, *Burkholderia* lipases have certain features of thermal stability, alkaline pH and organic solvent tolerance, transesterification activity, and high substrate selectivity, making it an attractive industrial enzyme (80, 201, 215). Hence, there is a motivation in developing an economically feasible bioprocess for their overproduction.

The Gram-negative bacterium *Escherichia coli* is still recognized as the most popular host for high-level production of recombinant proteins from both prokaryotic and eukaryotic sources (173). While the major expression steps of transcription and translation have to be effective to achieve high-level production, it is not uncommon that the recombinant protein yield is limited by posttranslational events, such as disulfide bond formation, solubility, misfolding, proteolysis, and even the toxicity to host cells (171). The expressed recombinant protein can be targeted in various intracellular compartments, including the cytoplasm and periplasm, displayed on the cell surface or secreted into the extracellular medium. The oxidative environment of the extracytoplasmic compartments is conducive for disulfide bond formation (90). Extracellular secretion often yields recombinant proteins in the culture medium with minimum contamination from host cell proteins (181). The recombinant

proteins can be displayed on the *E. coli* cell surface for specific applications, such as whole-cell biocatalysts and high throughput peptide library screening (159).

E. coli has several folding modulators to assist the folding of nascent polypeptides, particularly during stressful conditions. These folding modulators, including trigger factor, DnaK/J-GrpE, and GroEL/ES in the cytoplasm and Dsb-family chaperones, DegP, FkpA, Skp, and SurA in the periplasm, can be potentially useful for the high-level production of heterologous proteins in a bioactive conformation in the cytoplasm or periplasm (15). Alternatively, fusion tags, originally developed for protein purification with affinity chromatography, are an effective tool to enhance the functional expression, solubility, bioactivity, and stability of the partner protein (275). The N-terminal tags can potentially enhance the translational initiation and, therefore, are more popular (109). Several solubility-enhancing tags have been successfully used to produce hard-to-express heterologous proteins in *E. coli* (76).

Given the recognized industrial applicability of *Burkholderia* lipase, there are very few reports regarding its production so far. One of them is the cloning and heterologous expression of the *Burkholderia cepacia* lipase gene in *E. coli* (229). However, the recombinant lipase was predominantly expressed as insoluble inclusion bodies even with the coexpression of lipase-specific foldase (Lif) which is a natural lipase folding modulator. Another study reported the mutagenesis and screening approach to obtain soluble variants of *Burkholderia* lipase for functional expression (104). In the current study, we explored various genetic strategies for the functional expression of a lipase from *Burkholderia* sp. C20 (Lip) in *E. coli*. The *Burkholderia* strain was originally isolated from the food waste and could potentially have novel lipases with the transesterification activity for biodiesel production (166).

6.2 Materials and Methods

6.2.1 Bacterial Strains and Plasmids

Bacterial strains, plasmids, and oligonucleotides used in this study are summarized in Table 1. DH5 α was used as the host strain for molecular cloning. JM109, BL21(DE3), and Origami B(DE3) were used as the host strain for Lip expression. The protocols for molecular cloning are described in Chapter 3.

The *lip* gene was PCR-amplified using the primer pair P1 and P2, and *Burkholderia* sp. C20 chromosomal DNA as the template. The primer pair P1 and P2 was designed based on the sequence of the *lipA* gene of *Burkholderia multivorans* (NCBI Accession number NC_010805-1). The amplified *lip* gene was cloned to pPCRscriptSK(+) to obtain pPCRscriptLip and the DNA sequencing result is given in Figure 6.1A. The *lip* gene with the native signal peptide was PCR-amplified using P20/P21 primer pairs on pPCRscriptLip template, digested with *NcoI/EcoRI* and ligated to similarly digested pTrc99A to obtain pTrcLip. The leaderless(II) *lip* gene (without the native signal peptide) was PCR-amplified using the primers P58/P21 and pPCRscriptLip template, digested with *NcoI/EcoRI* and ligated to similarly digested pTrc99A to obtain the plasmid pTrcLipII. In addition, PCR was performed using pPCRscriptLip as the template and the primer pairs of P131/P27, P58/P27, and P58/P59, respectively. The PCR products were digested respectively with *SfiI*, *NcoI/SfiI* and *NcoI/SacI* and ligated to similarly digested pESTYFP, pEHLA2-SD and pETM80/pETM82 to construct pESTLipII, pEHLA2LipII and pII DsbCLipII/pDsbCLipII. The *NcoI/EcoRI* DNA fragment from pTrcLipII was subcloned into pET20b(+) and pENTR4 to obtain pETLipII and pENTRLipII, respectively. The *lip*-containing DNA in pENTRLipII was subcloned into pGGWA, pMGWA, pHNGWA, pXGWA, pDest-555, pDest-556, pETM-50, and pETM-52 using the Gateway cloning technology (Invitrogen); resulting in pGSTLipII, pMBPLipII, pHisNusALipII, pTRXLipII, pT7PKLipII, pSkpLipII, pDsbALipII, and pII DsbALipII, respectively. The plasmids pAR3GRO, pARDegP, pARDsbA pARDsbC, pARFkpA, pARIIDsbA, pARIIDsbC, pARSurA and pARSkp respectively contain the *groEL/ES*, *degP*, *dsbA*, *dsbC*, *fkpA*, *lldsbA*, *lldsbC*, *surA* and *skp* genes whose expression was under the regulation of the *araB* promoter. The plasmid pG-KJE8 contains the *dnaK/J-grpE*

and *groEL/ES* genes respectively fused with *araB* and *zt-lp* promoters. pG-TF3 contains the *tig* and *groEL/ES* genes respectively fused with *araB* and *zt-lp* promoters. All chaperone gene(s)-expression plasmids contain a chloramphenicol-resistant (Cm^R) marker and, therefore, are compatible with all Lip-expression vectors containing an Ampicillin-resistant (Ap^R) marker.

Table 6.1 Strains, plasmids, and oligonucleotides.

Strain	Relevant Genotype	Source & Reference
<i>E. coli</i>		
BL21(DE3)	F ⁻ <i>ompT dcm lon hsdS_B</i> (r _B ⁻ , m _B ⁻) <i>gal</i> λ(DE3[<i>lacI ind1 sam7 nin5 lacUV5-T7</i> gene 1])	Novagen (224)
DH5α	F'(φ80 <i>dlac</i> Δ(<i>lacZ</i>)M15) Δ(<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ , m _K ⁺) <i>phoA supE44 lambda-thi-1 gyrA96 relA1</i>)	Lab stock (300)
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi-1 mcrA</i> Δ <i>lac-proAB</i>) F'[<i>traD36 proAB</i> ⁺ <i>lacI^q lacZ</i> ΔM15]	Lab stock (307)
Origami B(DE3)	F- <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm lacY1 ahpC</i> (DE3) <i>gor522::Tn10</i> (Tc ^R) <i>trxB</i> (Km ^R)	Novagen
<i>Burkholderia</i>		
<i>Burkholderia</i> sp. C20	Isolated from the food waste of a food processing plant	(166)
Plasmid		
pAR3GRO	P _{<i>araB</i>} :: <i>groEL/ES</i> , Cm ^R	Lab stock (221)

pAR3KJ	$P_{arab}::dnaK/J::grpE$, Cm ^R	Lab stock (221)
pARDegP	$P_{arab}::degP$, Cm ^R	Lab stock (214)
pARDsbA	$P_{arab}::dsbA$, Cm ^R	Lab stock (303)
pARDsbC	$P_{arab}::dsbC$, Cm ^R	Lab stock (303)
pARFkpA	$P_{arab}::fkpA$, Cm ^R	Lab stock (301)
pARlIDsbA	$P_{arab}::lldsbA$, Cm ^R	Lab stock (305)
pARlIDsbC	$P_{arab}::lldsbC$, Cm ^R	Lab stock (305)
pARSkp	$P_{arab}::skp$, Cm ^R	Lab stock (193)
pARSurA	$P_{arab}::surA$, Cm ^R	Lab stock
pDest-555	$P_{tac}::6\times his::T7PK$, Ap ^R	Deb K. Chatterjee (47)
pDest-556	$P_{tac}::6\times his::skp$, Ap ^R	Deb K. Chatterjee (47)
pHisperiMBP	$P_{tac}::6\times his::mbp$, Ap ^R	D. Waugh (192)
pDsbALipll	$P_{T7}::dsbA::6\times his::lipll$, Ap ^R	This study
pDsbCLipll	$P_{T7}::dsbC::6\times his::lipll$, Kn ^R	This study
pETM-50	$P_{T7}::dsbA::6\times his$, Ap ^R	EMBL
pETM-52	$P_{T7}::lldsbA::6\times his$, Ap ^R	EMBL
pETM-80	$P_{T7}::dsbC::6\times his$, Ap ^R	EMBL (72)
pETM-82	$P_{T7}::lldsbC::6\times his$, Ap ^R	EMBL (72)

pENTR4	Entry vector, attL1-rrNB-attL2, Kn ^R	Invitrogen
pENTRLipII	<i>lipII</i> gene containing entry vector, Kn ^R	This study
pEHLA2-SD	P _{lac} :: <i>hlyA</i> , Ap ^R	Luis Angel (79)
pEHLALipII	P _{lac} :: <i>lip</i> :: <i>hlyA</i> , Ap ^R	This study
pESTYFP	P _{lac} :: <i>phoA</i> _{signal} :: <i>yfp</i> :: <i>estA</i> , Cm ^R	Lab stock (193)
pESTLipII	P _{lac} :: <i>phoA</i> _{signal} :: <i>lipII</i> :: <i>estA</i> , Cm ^R	This study
pET20b(+)	P _{T7} :: <i>pelB</i> , Ap ^R	Lab stock
pETLipII	P _{T7} :: <i>pelB</i> :: <i>lipII</i> , Ap ^R	This study
pG-KJE8	P _{araB} :: <i>dnaK/J</i> :: <i>grpE</i> , P _{zt-lp} :: <i>groEL/ES</i> , Cm ^R	T. Yura (198)
pG-TF3	P _{araB} :: <i>tig</i> , P _{zt-lp} :: <i>groEL/ES</i> , Cm ^R	T. Yura (198)
pGGWA	P _{T7} :: <i>gst</i> ::6× <i>his</i> , Ap ^R	D. Busso (40)
pGSTLipII	P _{T7} :: <i>gst</i> :: <i>lipII</i> , Ap ^R	This study
pHisperiMBPLipII	P _{lac} ::6× <i>his</i> :: <i>mbp</i> :: <i>lipII</i> , Ap ^R	This study
pHNGWA	P _{T7} ::6× <i>his</i> :: <i>nusA</i> , Ap ^R	D. Busso (40)
pHisNusALipII	P _{T7} ::6× <i>his</i> :: <i>nusA</i> :: <i>lipII</i> , Ap ^R	This study
pIIDsbALipII	P _{T7} :: <i>lldsbA</i> ::6× <i>his</i> :: <i>lip</i> , Ap ^R	This study
pIIDsbCLipII	P _{T7} :: <i>lldsbA</i> ::6× <i>his</i> :: <i>lip</i> , Kn ^R	This study
pMGWA	P _{T7} :: <i>mbp</i> , Ap ^R	D. Busso (40)

pMBPLipII	$P_{T7}::mbp::lipII$, Ap ^R	This study
pPCRscriptAmp SK(+)	PCR cloning vector, Ap ^R	Stratagene
pPCRscriptLip	PCR cloning of <i>lip</i> , Ap ^R	This study
pSkpLipII	$P_{T7}::skp::lip$, Ap ^R	This study
pT7PKLipII	$P_{T7}::T7PK::lipII$, Ap ^R	This study
pTF16	$P_{arab}::tig$, Cm ^R	T. Yura (198)
pTrcLip	$P_{trc}::lip$, Ap ^R	This study
pTrcLipII	$P_{trc}::lipII$, Ap ^R	This study
pTRXLipII	$P_{T7}::trx::lipII$, Ap ^R	This study
pXGWA	$P_{T7}::trx$, Ap ^R	D. Busso (40)

Oligonucleotides

P1	5'-ATGGCCAGATCGATGCGTTCCAGGGTGGTG-3'	This study
P2	5'-TCAGACGCCC GCGAGCTTCAACCGGTTTCGC-3'	This study
P20	5'-CCGCCCATGGCCAGATCGATGCGTTCCAGG-3'	This study
P21	5'-GCGGCCG <u>G A A T T C</u> A C C C G C C C G C G A G C T T C-3'	This study
P25	5'-CTGGAGCTCCACCGCG <u>G C C C C C G A G G C C C T A G C</u> CCCGCCGCGAGCTT-3'	This study
P27	5'-CACCGCGGTGGCGGG <u>A G C T C A</u> A G C C C C G C C-3'	This study

P58	5'-GCGGCAACCG <u>CCATGG</u> CCGACGACTATGCG-3'	This study
P59	5'-GCGGCAACCC <u>CTCGAGG</u> CCGACGACTATGCG-3'	This study
P131	5'-ACGCACGCGGCGATGG <u>CCCAGCCGGCCATGGCC</u> GACGACTATGCGACG-3'	This study

* Italic: mutation nucleotides, underline: restriction site

6.2.2 Cultivation

The protocol for cultivation is described in Chapter 3. After induction, the Erlenmeyer flasks were further shaken at the same conditions for another 4h at 28 °C, or 24h at 15 °C. All the shaker-flask cultivations were conducted at least in duplicate.

6.2.3 Analytical Methods

The protocols for various analyses are described in Chapter 3 and Chapter 4. Lip activity was qualitatively evaluated by the area and transparency of the halo formed on 1% tributyrin agar plates. To conduct this, soluble fractions and extracellular fractions of 50- μ L were loaded on the plate and incubated at 37 °C overnight. On the other hand, Lip enzyme assay was conducted using a pH-stat (Brinkman Metrohm 842 Titrando, FL, USA). An appropriate volume of the soluble fraction of cell lysates was added to 5 mL of 2% tributyrin emulsion in water at 55 °C and the reaction solution was maintained at pH 9.0 by controlled addition of 0.02 N NaOH (279). One unit of enzyme activity is defined as the amount of enzyme required to liberate one μ mole of fatty acid per min.

1-6_complete NC_010805-1 (1) ATGGCCAGATCGATGCGTTCCAGGGTAA TGGCAGGGGCAGTGGGA TGCGC
ATGGCCAGATCGATGCGTTCCAGGGTGG TGGCAGCAGCAGTGGGGTGGCGC

1-6_complete NC_010805-1 (51) GATGAGCGTCGCGCCGTTTCGCGGGGACGACCCGCGC FGA C GACGCTCGCGA
GATGAGCGCCGCGCCGTTTCGCGGGGACGACCCGCGCTFGA T GACGCTCGCGA

1-6_complete NC_010805-1 (101) CGACGCATGCGGCGA TGGG GCA ACCGCGCC SCCGACGACTAT SCCGACG
CGACGCATGCGGCGCTCGC CCGATTGCGCCGCCGACGATTACGCGGGC

1-6_complete NC_010805-1 (151) ACGCGCTATCCGATCAT TCTCGTGACGGGT T GACC GGCACCCACAAGTA
ACGCGCTATCCGATGCTCTCGTGACGGGCTCACG GGCACCCACAAGTA

1-6_complete NC_010805-1 (201) CGCGGGCGTACTCGA T TACTGGTACGGCATCCAGGAAGACCTGCAACAGC
CGCGGGCGTACTCGA T TACTGGTACGGCATCCAGGAAGACCTGCAACAGC

1-6_complete NC_010805-1 (251) ATGGCGGACCTCTC TACTCGCGAACCTGTCGGGC TTCCAGAGCGACGAC
ACGGTGGGACCTGTG TATGTCGCGAACCTGTCGGGAT TCCAGAGCGACGAC

1-6_complete NC_010805-1 (301) GGC CCGAACGGG CCGCGGCGAACAGCTGCTGGG T TACGTGAAGACGGTGT
GGT CCGAACGGA CCGCGGCGAACAGCTGCTCGA TACGTGAAGCAGGTGT

1-6_complete NC_010805-1 (351) CGCCGCGACCGGCGCGACCAAGGTCAATCTCG TCGGTCA CAGCCAGGGCG
GGCCGCGACCGGCGCGACCAAGGTCAATCTGATCGGTCA T AGCCAGGGCG

1-6_complete NC_010805-1 (401) GGCTGACGTTCGCGCTATGT TCGCGCCGTCGCGCC C GATCTCGTCGCGTGG
GTCTGACGTTCGCGCTACTGTGGCGCCGTCGCGCC GATCTCGTCGCGTGG

1-6_complete NC_010805-1 (451) GTGACGACGATCGGCACG CCGCATCGCGGCTC GAGTTCGCGGACTTCGT
GTGACGACGATCGGCAC TCCGATCGCGGCTCGGAGTTCGCGGACTTCGT

1-6_complete NC_010805-1 (501) GCAGGACGTGCTCGGTACGATCCGACCGGACTGTGCTCG CAGGTGATCG
GCAGGACGTGCTCGGTACGACCCGACCGGCTGTGCTCGCTCGGTGATCG

1-6_complete NC_010805-1 (551) CCGCGTTCGTCAATGTA T TCGGATCCTGACGAGCAGCAGCCACAACAG
CCGCGTTCGTCAATGTG T TCGGATCCTGACGAGCAGCAGCCACAACAG

1-6_complete NC_010805-1 (601) AACCAGGACGCACTCGCGTGGCTGAGACGCTGACGACCGCGCAGGCCCG
AACCAGGACGCGCTCGCGCACTGAGACGCTGACGACCGCGCAGGCCCG

1-6_complete NC_010805-1 (651) CACCTACAACCAAGAACTATCCGAGCGCGGGCTTCGGCGCTCCCGGCACTT
GACCTACAACCAAGAACTATCCGAGCGCGGGTCTCGGTGCAACCGGCACTT

1-6_complete NC_010805-1 (701) GCCAGACCGGC GCGC CGACGGAAACCGTCGGCGGCAACACGCATCTGCTG
GCAGACCGGTGCGT CGACGGAAACCGTCGGCGGCAATACGCATCTGCTG

1-6_complete NC_010805-1 (751) TATTCGTGGGCGGCGACGGCGATCCAGCCGACGCTGTCGCTGTTTCGGCGT
TATTCGTGGGCGGCGACGGCGATCCAGCCGACGAA TCGGCSTTCGGCGT

1-6_complete NC_010805-1 (801) GACGGGGCGGACCGATACGAGCACCGTTCCGGTCTCGATCCGGGGAACG
CACGGGGCGGACCGATACGAGCACGATTCGGGTCATCGATCCGGGCAATG

1-6_complete NC_010805-1 (851) TGCTCGA CCTGTGACGCTCGCGCTGTTCGGCACGGGCACGGTGTATGATC
CACTCGATGCTGTCGACGCTCGCGCTGTTCGGCACGGGCACCGTGTATGATC

1-6_complete NC_010805-1 (901) AACCGCGGTTCGGGCGAGAACGACGGCTCTGTGTCGAAGTGCAGCGCGCT
AATCGCGGTTCGGGCGAGAACGACGGACTCTGTGTCGAAGTGCAGCGCGCT

1-6_complete NC_010805-1 (951) GTACGGCAAGGTGCTGAGCACGCGCTACAA GTGGAACCATATCGACGAGA
GTACGGCAAGGTGCTCGGCACGCTTCAAATGGAA TCA TCGACGAGA

1-6_complete NC_010805-1 (1001) TCAA CAGCTGCTCGGCGTGC CGCGGTATGCGGAAGACCCGGTTCGG
TCAA T CAGTGTCTCGGCGTGC CGGTATGCGGAAGATCCGGTTCGG

1-6_complete NC_010805-1 (1051) GTGATCCGCACGCA TCGAACC GGTTGAAGCTCGCGGGCGGTGA A
ATGATCCGCACGCA TCGAACC GGTTGAAGCTCGCGGGCGTCTGA A

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1                                                    50
Translation of 1-6_complete (1)
MARSMSRVMAAGAVACAMSVAPFAGTTALTLATTHAAMAA↑TAPADDYAT
Translation of NC_010805-1 (1)
MARSMSRVVAAAVACAMSAAPFAGTTALMTLATTRTALAAIAPADDYAA
51                                                    100
Translation of 1-6_complete (51)
TRYPIILVHGLTGTDKYAGVLDYWYGIQEDLQHGATVYVANLSGFQSDD
Translation of NC_010805-1 (51)
TRYPIVLVHGLTGTDKYAGVLEWYWYGIQEDLQRHGATVYVANLSGFQSDD
101                                                    150
Translation of 1-6_complete (101)
GPNGRGEQLLAYVKTVLAATGATKVNLVGHSQGGLTSRYVAAVAPDLVAS
Translation of NC_010805-1 (101)
GPNGRGEQLLAYVKQVLAATGATKVNLIHGSQGGLTSRYVAAVAPDLVAS
151                                                    200
Translation of 1-6_complete (151)
VTTIGTPHRGSEFADFVQDVLAYDPTGLSSIVIAAFVNVFGILTSSSHNT
Translation of NC_010805-1 (151)
VTTIGTPHRGSEFADFVQS↓VLAYDPTGLSSSVIAAFVNVFGILTSSSHNT

201                                                    250
Translation of 1-6_complete (201)
NQDALASLKTTLTQAATYNQNYPSAGLGAPGSCQTGA↑P↑TETVGGNTHLL
Translation of NC_010805-1 (201)
NQDALAA↑LTTLTQAATYNRNFPSAGLGAPGSC↑TGAS↑TETVGGNTHLL
251                                                    300
Translation of 1-6_complete (251)
YSWAGTAIQPTLSLFGVTGATDTSTVPVVDPANVLDLSTLALFGTGTVMI
Translation of NC_010805-1 (251)
YSWAGTAIQPTNSA↑FGVTGASDTSTIPVIDPANALDASTLALFGTGTVMI
301                                                    350
Translation of 1-6_complete (301)
NRGSGE↑NDGLVSKCSALYGVLS↑TRYKWNHIDEINQLLGVRGAYAEDPVA
Translation of NC_010805-1 (301)
NRGSGQ↑NDGLVSKCSALYGV↑LGTH↑FKWNH↑LDEINQLLGVRGAYAEDPVA
351            363
Translation of 1-6_complete (351)
VIRTHANRLKLAGG
Translation of NC_010805-1 (351)
MIRTHANRLKLAGV-----

```

B

Figure 6.1 A) Nucleotide sequence analysis of the open reading frame (ORF) of the *lip* gene amplified by PCR using the genomic DNA of *Burkholderia* sp. C20 as the template and the primer pair of P1 and P2 (denoted as '1-6'). The sequences of the primers P1 and P2 were determined based on terminal sequences of the 1095-bp lipase gene (*lipA*) on the chromosome 2 of *Burkholderia multivorans* ATCC 17616 (NCBI accession number NC_010805, region 699394~700488). The sequences of *lip* (1-6) and *lipA* (denoted as 'NC_010805') have been aligned to share a sequence similarity of 87.9%. The stop codon 'TGA' at the 3' end of 1-6 in the figure was originally 'GCT' in the amplified DNA, which was replaced with a 'STOP' codon for further cloning experiments. B) The translated amino acid sequences of the *lip* gene (denoted as 'translation of 1-6 complete') and *lipA* gene (depicted as 'translation of NC-010805_1') have been aligned to share a sequence identity of 85.6% and a sequence consensus of 89.5%. The 1-364 amino acid sequence of *lip* gene (1-6) was subjected to the BLAST query (www.ncbi.nlm.nih.gov). A sequence identity of 99% (362/364) and 100% positive (364/364) was observed with a potential lipase gene (NCBI accession number YP_002233567) identified from the genomic sequence of *Burkholderia cenocepacia* J2315. Further DNA sequence analysis of the two ORFs showed 100% similarity (4 nucleotides being different and no gap), confirming the missing of a 'STOP' codon in the original PCR product of *lip* (1-6). Lipase genes from other *Burkholderia* sp. shared 70% to 96% sequence identity with *lip* (1-6). It may be noted that *lip* (1-6) shared only 92% and 93% sequence similarity to two other species of *Burkholderia cenocepacia*. The arrow represents the predicted cleavage site associated with the signal peptide (40 amino acids) using the SignalP 3.0 server (www.cbs.dtu.dk/services/SignalP/). However, the extracellular lipase purified from *Burkholderia* sp. HY-10 (215) was determined to have the N-terminal amino acid sequence of 'ADTYAATRYPIILVHGLTGTDKYAG' which is similar to the underlined amino acid sequence of 'ADDYATTRYPIILVHGLTGTDKYAG'. This was determined as the N-terminal sequence of mature Lip for the subsequent cloning of the *lip* gene without its native signal peptide coding sequence (*ll-lip*).

6.3 Results

Seven scenarios (i.e. A1, A2, B, C, D, E, and F summarized in Figure 6.2) for the functional expression and targeting of a lipase from *Burkholderia* sp. C20 (Lip) in *E. coli* are compared.

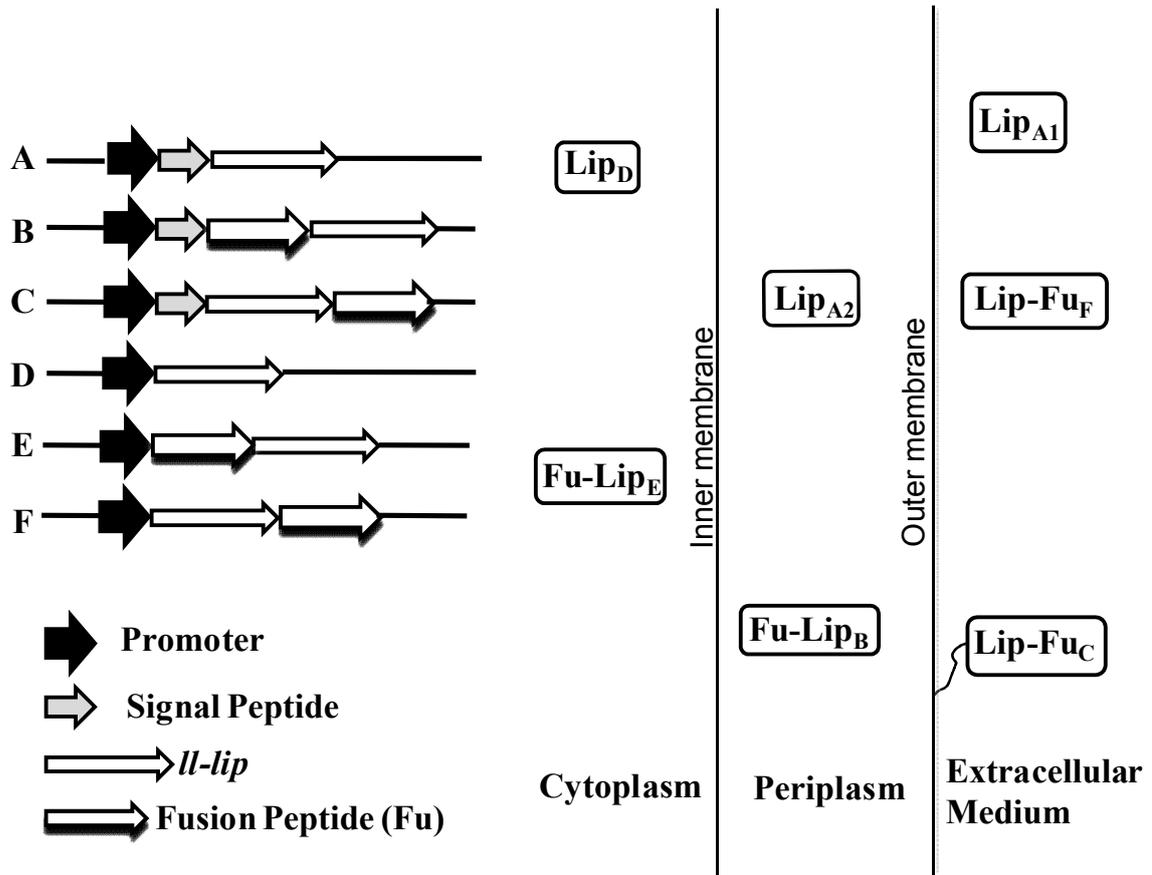
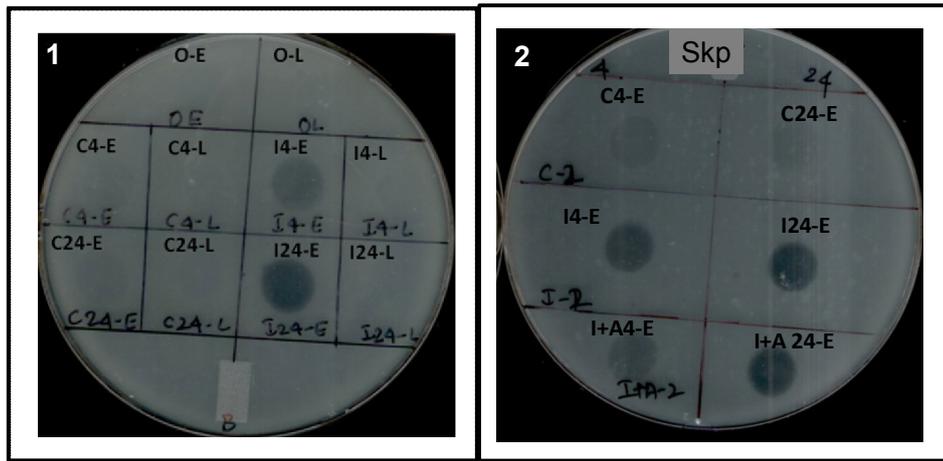


Figure 6.2 Various expression strategies adopted in this study for the production and targeting of Lip in *E. coli*. A1/ Secretion of Lip into the extracellular medium: The *lip* gene containing the native signal peptide is expressed under the regulation of the *trc* promoter. The native signal peptide is responsible for the extracellular secretion of Lip (denoted as Lip_{A1}). A2/ Secretion of Lip into the periplasm: The *lip* gene without its native signal peptide coding sequence (*ll-lip*) is fused with the *pelB* signal sequence and the *pelB::ll-lip* fusion is expressed under the regulation of the T7 promoter. The PelB

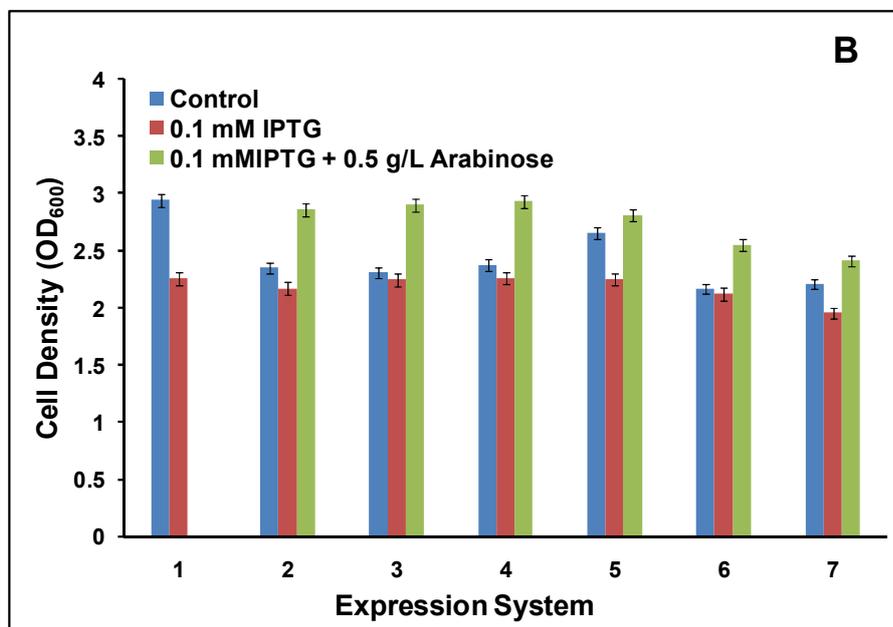
signal peptide is responsible for the translocation of Lip to the periplasm (denoted as Lip_{A2}). B/ Periplasmic expression of Lip fusions: Lip is N-terminally fused with various expression enhancing tags (i.e. DsbA, DsbC, and HisperiMBP) and the *Fu::ll-lip* gene fusions are expressed under the regulation of the T7 or *tac* promoter. The signal peptides of DsbA, DsbC, and MBP are respectively responsible for the translocation of Fu-Lip to the periplasm (denoted as Fu-Lip_B). C/ Surface display of Lip: Lip is C-terminally fused with an inactive variant of EstA from *Pseudomonas aeruginosa* and the *ll-lip::estA* fusion is expressed under the regulation of the *lac* promoter. The signal peptide of PhoA is responsible for exporting Lip-Fu (denoted as Fu-Lip_C). D/ Cytoplasmic expression of Lip: The *ll-lip* gene is expressed in the cytoplasm under the regulation of *trc* promoter (denoted as Lip_D). E/ Cytoplasmic expression of Lip with an N-terminal fusion tag: Lip is N-terminally fused with various expression enhancing tags (i.e. DsbA, DsbC, GST, MBP, NusA, Skp, T7PK, and TRX) and the *Fu::ll-lip* gene fusions are expressed in the cytoplasm under the regulation of the T7/*tac* promoter (denoted as Fu-Lip_E). F/ Secretion of a Lip fusion into the extracellular medium: Lip is C-terminally fused with HlyA and the *ll-lip::hlyA* gene fusion is expressed under the regulation of the *lac* promoter. Lip-HlyA is extracellularly secreted via the α -hemolysin transporter (denoted as Lip-Fu_F).

The culture performance of Lip expression using JM109 (pTrcLip) (i.e. scenario A1) is summarized in Figure 6.3. Visible halos were observed on tributyrin plates (Figure 6.3A) for the extracellular medium samples of the IPTG-induced culture with the lipase activity up to 55 U/L (expression system 1 in Figure 6.3C), but not for the soluble intracellular fraction. The results imply that bioactive Lip was expressed and secreted into the extracellular medium under the direction of the native signal peptide which is recognized by *E. coli*. The extracellular expression posed no adverse physiological impact on the bacterial cell growth (Figure 6.3B). Since *Burkholderia* lipase is translocated across the inner membrane while being unfolded via the Sec secretion system prior to the secretion into the extracellular medium (86), folding factors in the cell envelope were coexpressed to investigate their effect

on Lip expression and the results are summarized in Figure 3 as well. Among the folding factors investigated, Skp significantly increased the lipase activity by 36% to 75 U/L (#7 in Figure 6.3C); whereas DegP had an adverse effect with neither halos being present on tributyrin plates nor a measurable lipase activity (#6 in Figure 6.3C).



A



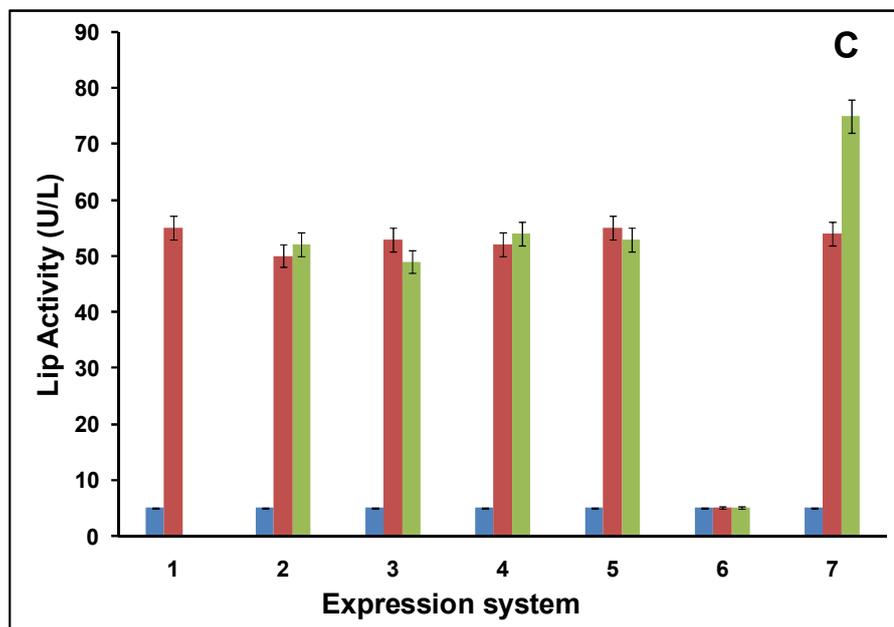
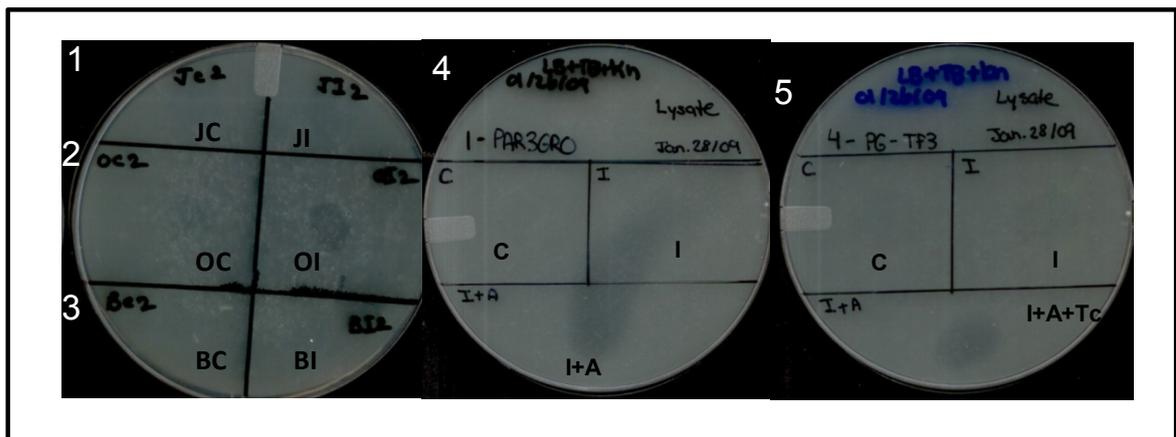


Figure 6.3 Culture performance of Lip expression (scenario A1) using (1) JM109 (pTrcLip), (2) JM109 (pTrcLip, pARFkpA), (3) JM109 (pTrcLip, pARDsbA), (4) JM109 (pTrcLip, pARDsbC), (5) JM109 (pTrcLip, pARSurA), (6) JM109 (pTrcLip, pARDegP), (7) JM109 (pTrcLip, pARSkp). Panel A/ Qualitative visualization of lipase activity on tributyrin agar plates. Panel B/ Cell density. Panel C/ Lipase activity. ‘C’, ‘I’, and ‘A’ represent the cultures ‘without induction’, ‘with IPTG induction’, and ‘with arabinose induction’, respectively. ‘E’ and ‘L’ represent the ‘extracellular fraction’ and ‘soluble lysate fraction’, respectively.

The culture performance for Lip expression in the cytoplasm of *E. coli* (i.e. scenario D) is described in Figure 6.4. Among the three hosts investigated, i.e. JM109, BL21(DE3), and Origami B(DE3), the halo associated with tributyrin hydrolysis was visualized only for the culture sample of recombinant Origami B(DE3) and the lipase activity appeared to be extremely low (#2 in Figure 6.4A). However, neither lipase activity was measurable by pH stat nor the protein band corresponding to Lip overexpression was identifiable by SDS-PAGE (data not shown) for all the culture samples grown at 15 or 28 °C. The results suggest

two possible expression issues, i.e. ineffective translation or protein degradation. Several cytoplasmic chaperones including GroEL/ES, Trigger factor, DnaK/J-GrpE, and the Dsb-chaperones were coexpressed to investigate their possible effect on the cytoplasmic expression of Lip. Visible halos developed on tributyrin agar plates were slightly visible only for BL21(DE3) (pTrcLipII, pAR3GRO) and BL21(DE3) (pTrcLipII, pG-KJE8) (#4 and #5 in Figure 6.4A), but however not for recombinant Origami B(DE3) (data not shown). Again, the lipase activity was not measurable by pH stat and the Lip band was not identifiable by SDS-PAGE (data not shown).



A

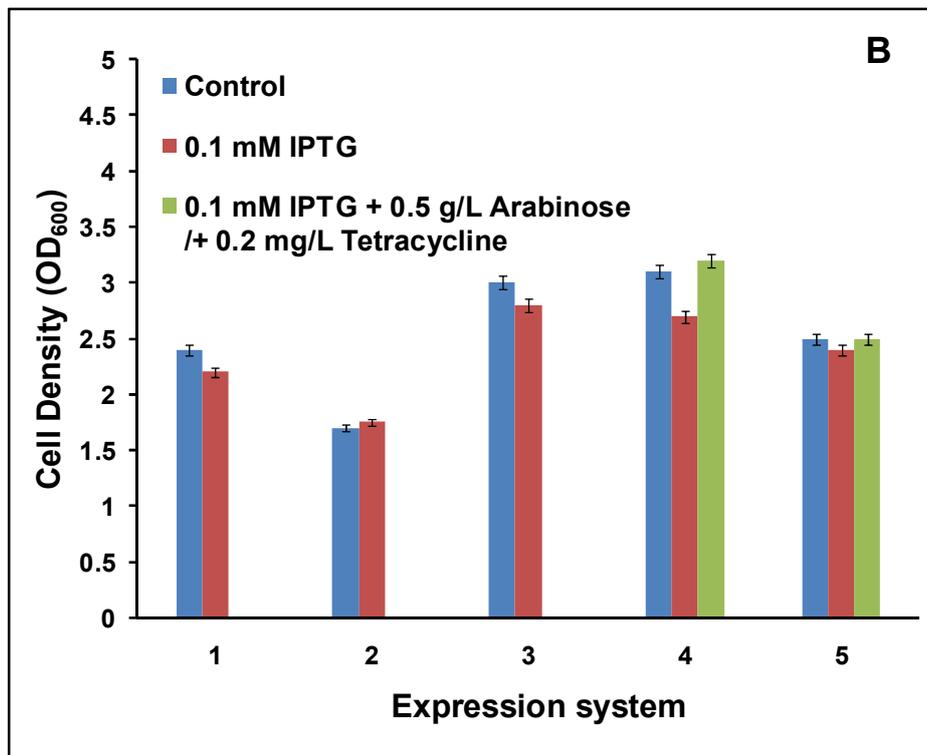
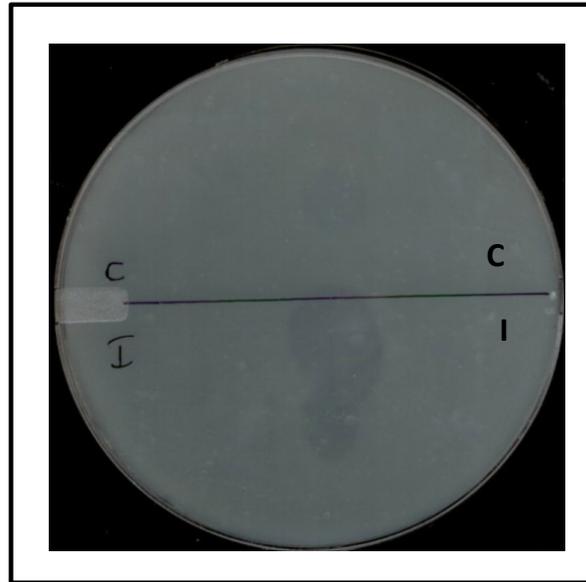


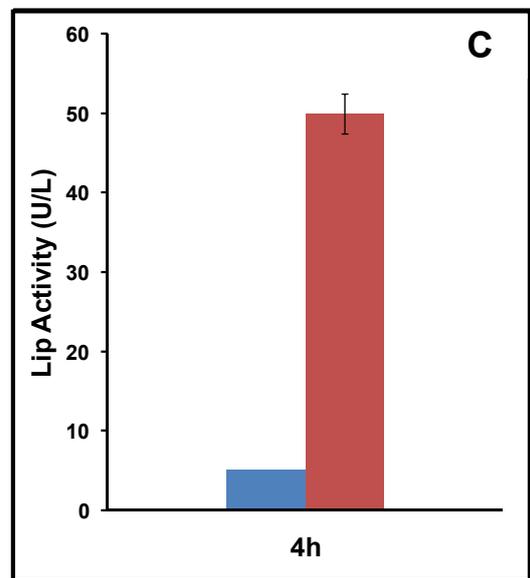
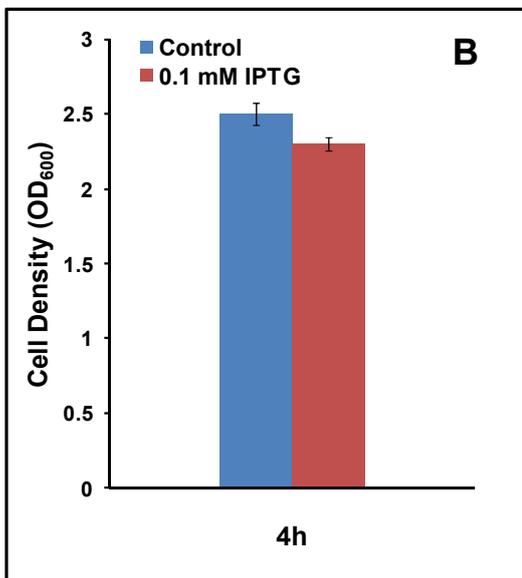
Figure 6.4 Culture performance of Lip expression (scenario D) using (1) JM109 (pTrcLipII), (2) Origami B(DE3) (pTrcLipII), (3) BL21(DE3) (pTrcLipII), (4) BL21(DE3) (pTrcLipII, pAR3GRO), (5) BL21(DE3) (pTrcLipII, pG-KJE8). Panel A/ Qualitative visualization of lipase activity on tributyrin agar plates. Panel B/ Cell density. ‘C’ ‘I’ ‘A’ and ‘Tc’ represent the cultures ‘without induction’, ‘with IPTG induction’, ‘with arabinose induction’, and ‘with tetracycline induction’, respectively.

The culture performance for Lip expression in the periplasm of *E. coli* (i.e. scenario A2) is summarized in Figure 6.5. Using the cell lysate of the BL21(DE3) (pETLipII) culture sample with IPTG induction, a halo was visualized on tributyrin agar plates (Figure 5A) and a lipase activity up to 50 U/L was measured (Figure 6.5C). A protein band corresponding to Lip at 35 kDa were visualized upon the SDS-PAGE analysis, but most of the expressed Lip existed in insoluble fraction (Figure 6.5D), implying a potential expression issue of protein

misfolding. Neither lowering the cultivation temperature to 15 °C nor the coexpression of periplasmic chaperones improved the solubility or lipase activity (data not shown).



A



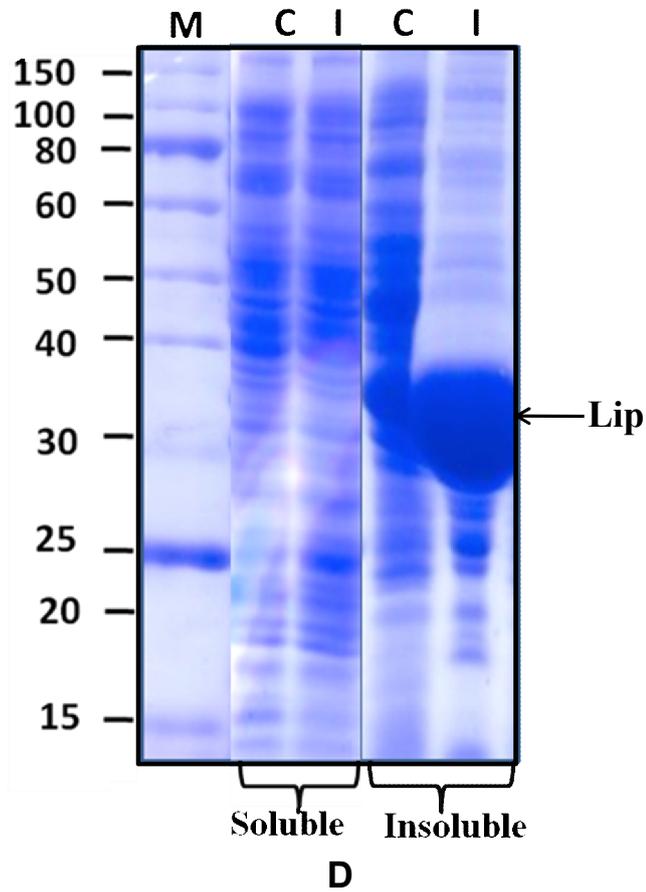
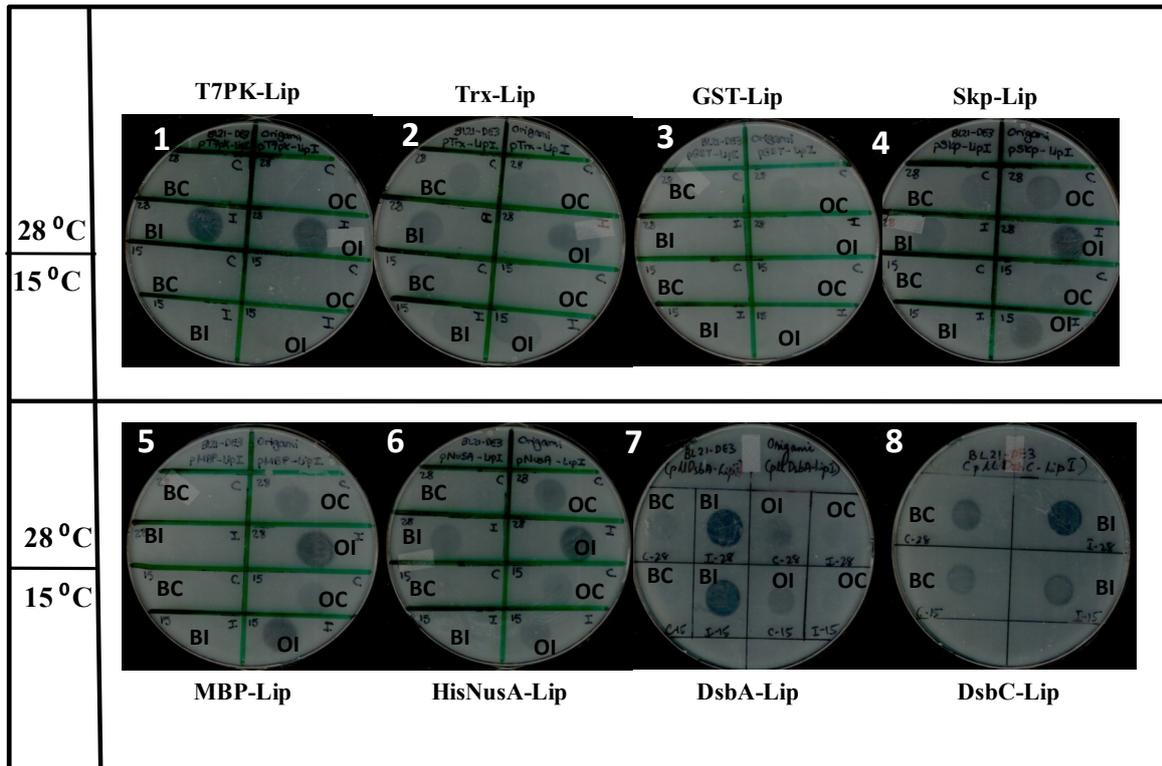


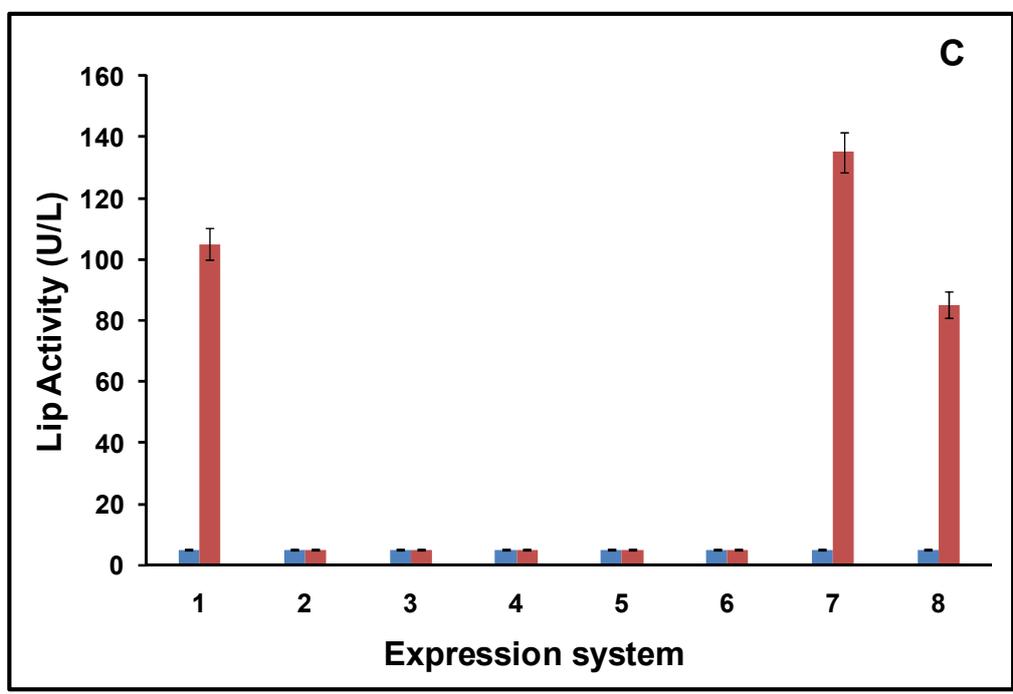
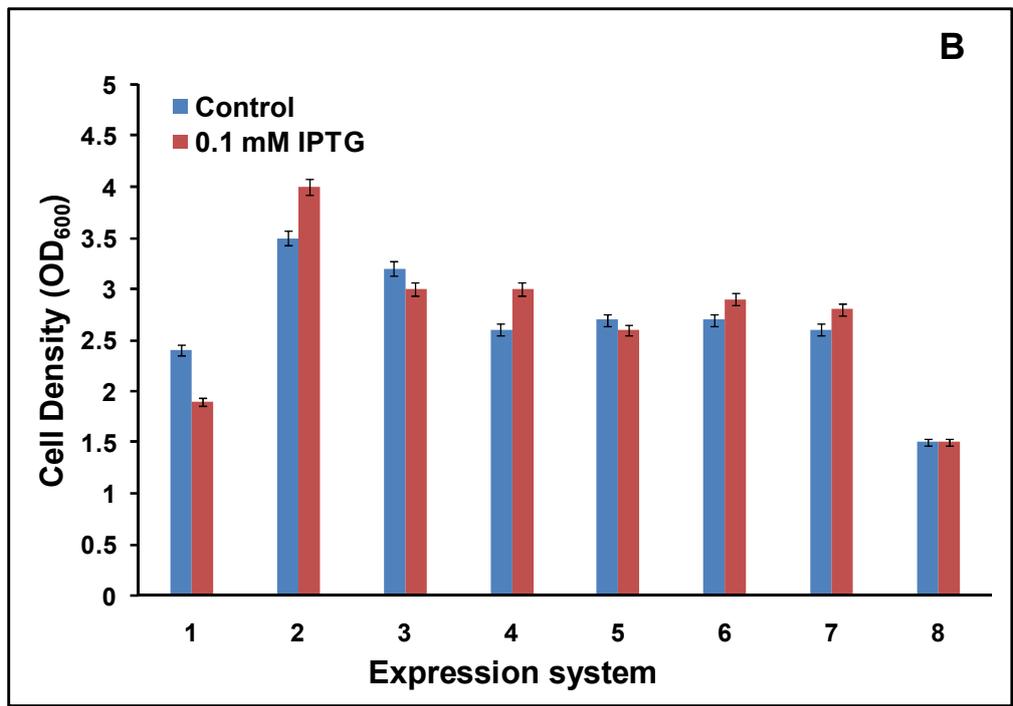
Figure 6.5 Culture performance of Lip expression (scenario A2) using BL21(DE3) (pETLipII). Panel A/ Qualitative visualization of lipase activity on tributyrin agar plates. Panel B/ Cell density. Panel C/ Lipase activity. Panel D/ SDS PAGE analysis of the soluble and insoluble fractions of culture samples. ‘C’ and ‘I’ represent the cultures ‘without induction’ and ‘with IPTG induction’, respectively.

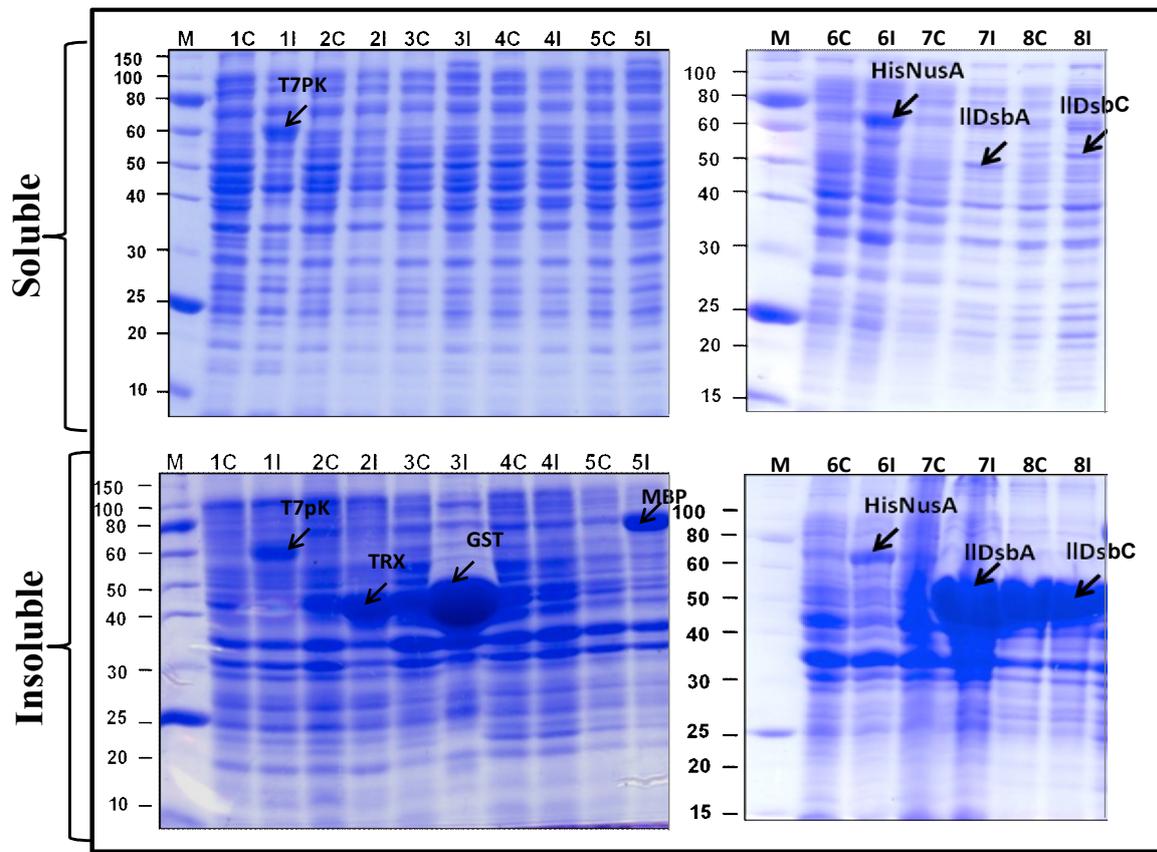
Given the poor culture performance for the cytoplasmic expression of Lip, eight N-terminal fusion tags, i.e. DsbA, DsbC, GST, MBP, HisNusA, Skp, T7PK, and TRX, were used to construct various translational fusions with Lip for their respective expression in the cytoplasm (i.e. scenario E). The culture performance for the expression of these Lip fusions in BL21(DE3) and Origami B(DE3) is summarized in Figure 6.6. In general, 28 °C appeared to be a better cultivation temperature than 15 °C (Figure 6.6A). Halos with a higher intensity were observed for T7PK-Lip, DsbA-Lip and DsbC-Lip fusions (#1, #7 and #8 respectively in Figure 6.6A) using BL21(DE3) as the expression host and for T7PK-Lip, Skp-Lip, MBP-Lip, and HisNusA-Lip (#1, #4, #5, and #6 respectively in Figure 6.6A) using Origami (DE3) as the expression host. GST-Lip fusion hardly had any lipase activity (#3 in Figure 6.6A) no matter which host was selected. The majority of the overexpressed Lip fusions aggregated as insoluble inclusion bodies (insoluble fraction in Figures 6.6D and 6.6G). In general, the lipase activity assayed by pH stat in general correlated with the solubility of Lip fusion proteins. Among the eight fusion tags investigated in recombinant BL21(DE3), T7PK, DsbA, and DsbC were effective in solubilizing Lip (Lanes 1, 7, and 8 in the soluble fraction of Figure 6.6D) and the Lip activities of T7PK-Lip, DsbA-Lip, and DsbC-Lip fusions were assayed to be 105 U/L, 135 U/L and 85 U/L, respectively (Figure 6.6C). On the other hand, both lipase activity (Figure 6.6C) and protein bands corresponding to TRX-Lip, GST-Lip, Skp-Lip, and MBP-Lip (Lanes 2, 3, 4, and 5 in Figure 6.6D) were minimally detected in the soluble fraction even though halos can be slightly visualized on tributyrin plates for TRX-Lip, and Skp-Lip (Figure 6.6A). Though HisNusA-Lip fusion had a visible protein band in the soluble fraction (Lane 6 in Figure 6.6D) and showed a faint halo on the tributyrin plate (Figure 6.6A), its lipase activity was hardly detected by pH stat (Figure 6.6C). Among the seven fusion tags investigated in recombinant Origami B(DE3), the lipase activities of T7PK-Lip, Skp-Lip, MBP-Lip, HisNusA-Lip fusion proteins were assayed to be 92 U/L, 55 U/L, 25 U/L, and 35 U/L (Figure 6.6F), respectively, whereas the other fusions had no detectable lipase activities with faint halos on tributyrin plates. Note that the lipase activity of Skp-Lip was detected by both pH stat and a visible halo on the tributyrin plate though the protein band corresponding to Skp-Lip was hardly detectable (Lane 4 in Figure 6.6G). In general, the

expressed fusions had a higher protein solubility in Origami B(DE3) than BL21(DE3) (Figures 6.6D vs. 6.6G), implying that the enhancement in the protein solubility could be associated with disulfide bond formation.

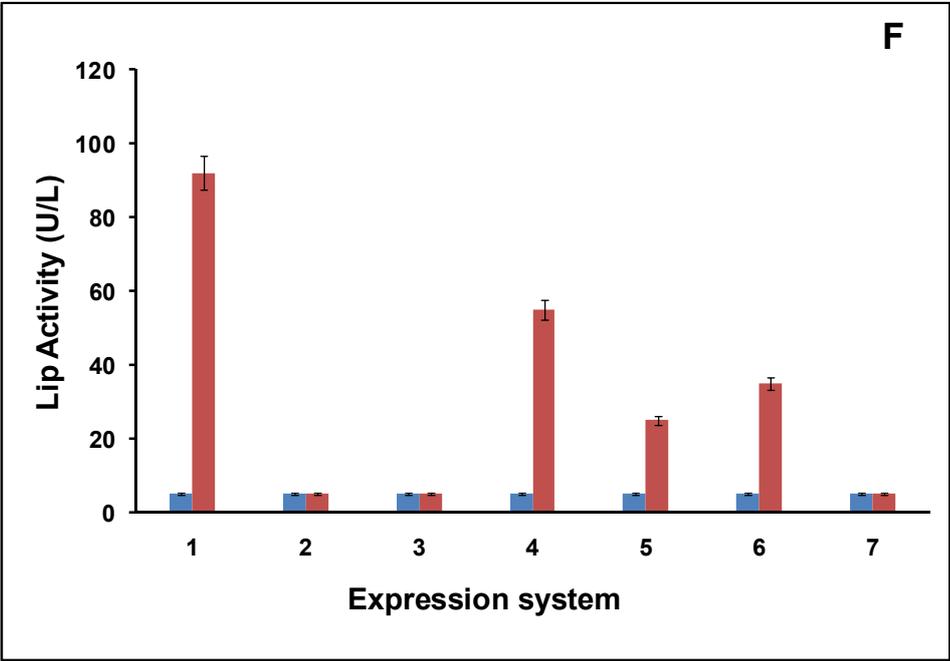
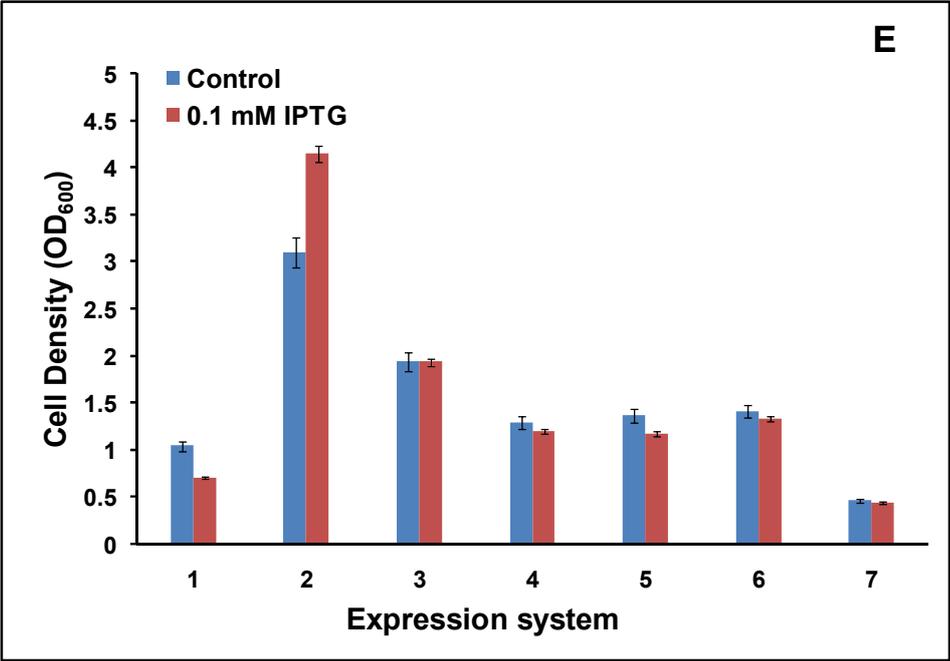


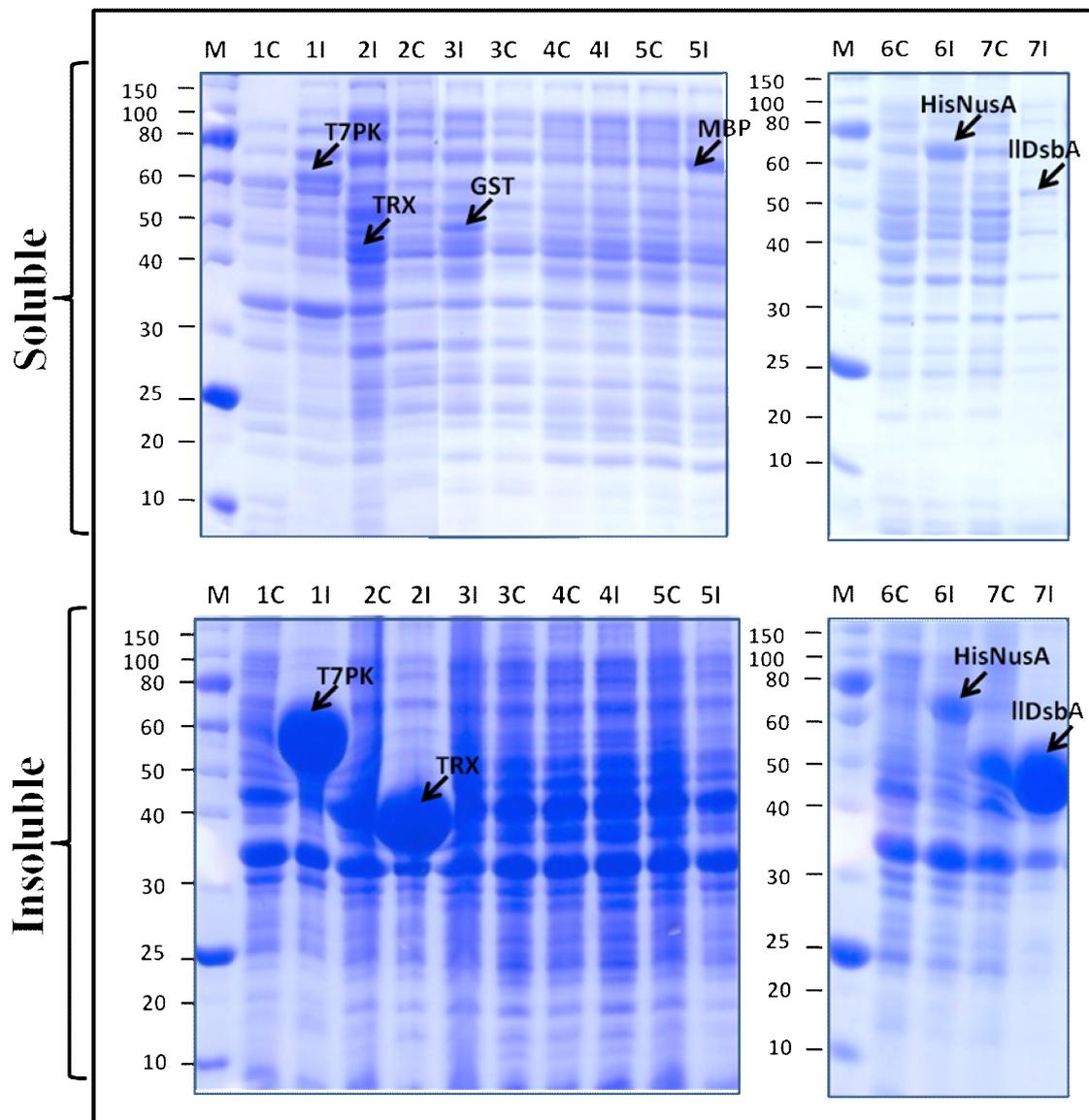
A





D



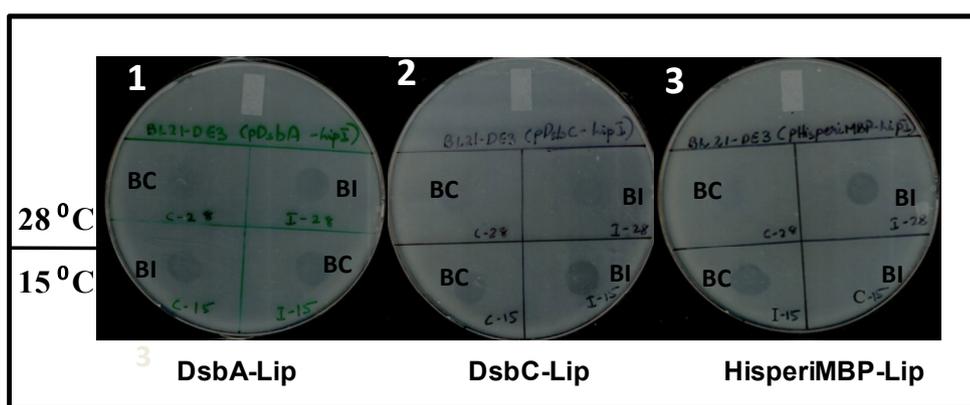


G

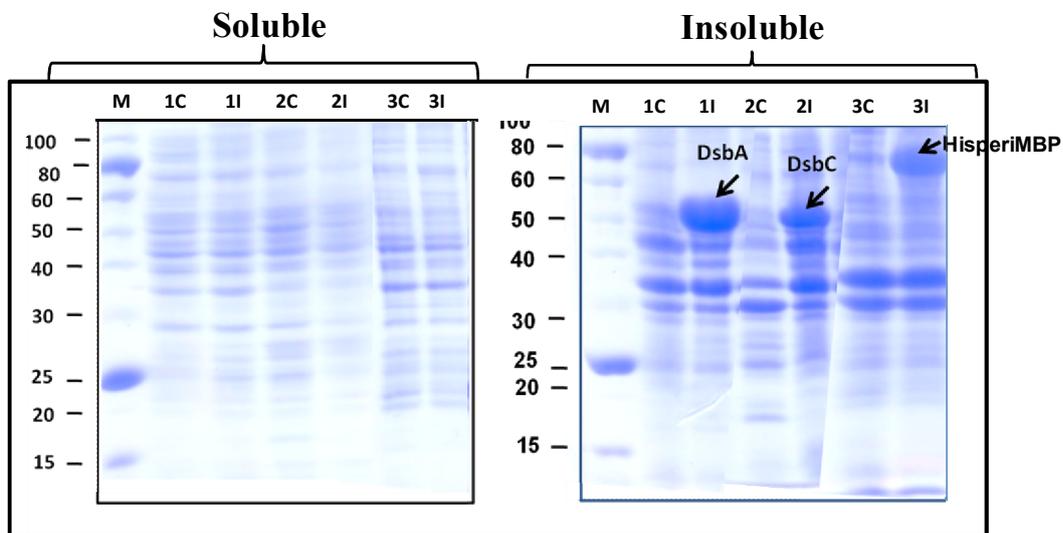
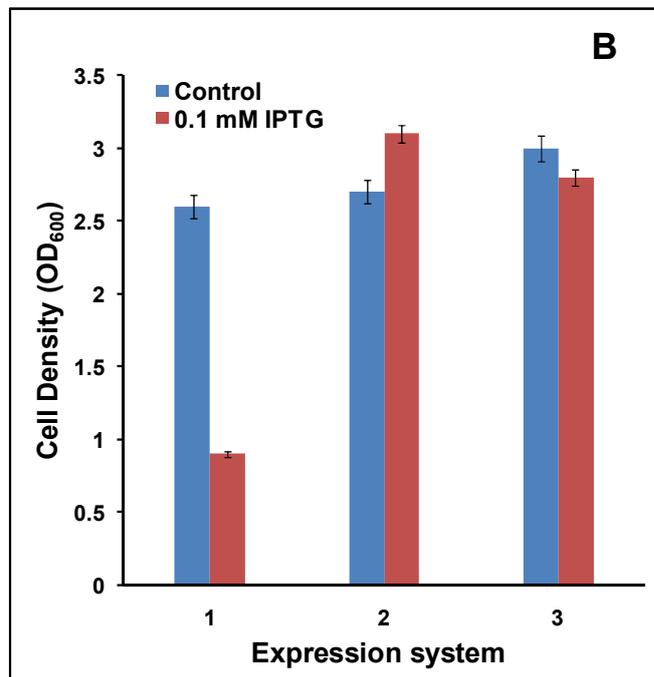
Figure 6.6 Culture performance of Lip expression (scenario E) using an *E. coli* host harboring (1) pT7PKLipII, (2) pTRXLipII, (3) pGSTLipII, (4) pSkpLipII, (5) pMBPLipII, (6) pHisNusALipII, (7) pIIDsbALipII, (8) pIIDsbCLipII. Panel A/ Qualitative visualization of lipase activity on tributyrin agar plates. The right and left halves of each plate represent the culture samples respectively using BL21(DE3) (denoted as ‘B’)

and Origami B(DE3) (denoted as ‘O’) as the expression host. The upper half and lower halves of each plate represent the cultivation temperatures of 28 °C and 15 °C, respectively. Plate 7 corresponds to recombinant BL21(DE3) only since Origami B(DE3) is not compatible with the kanamycin-resistant plasmid pIIDsbCLipII. Panel B & E/ Cell density. Panel C & F/ Lipase activity. Panel D & G/ SDS PAGE analysis of the soluble and insoluble fractions of culture samples. Various Lip protein fusions have been indicated. Note that Panels B, C, and D represent the cultures using BL21(DE3) as the expression host, and Panels E, F, and G represent the cultures using Origami B(DE3) as the expression host. ‘C’ and ‘I’ represent the cultures ‘without induction’ and ‘with IPTG induction’, respectively.

Similarly, three N-terminal fusion tags, i.e., HisperiMBP, DsbA, and DsbC, were used to construct various translational fusions with Lip for their respective expression in the periplasm (i.e. scenario B). The culture performance for the expression of these Lip fusions in BL21(DE3) is summarized in Figure 6.7. Though visible halos developed on tributyrin plates for these Lip fusions (Figure 6.7A), their lipase activities were undeterminable by pH stat. Apparently, these fusion tags were incompetent in solubilizing the expressed Lip since almost all the Lip fusions were detected in the insoluble fraction as inclusion bodies (Figure 6.7C).



A

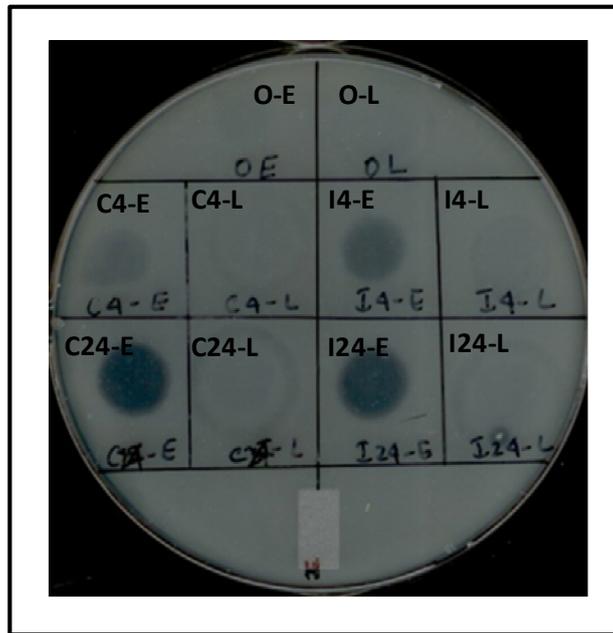


C

Figure 6.7 Culture performance of Lip expression (scenario B) using (1) BL21(DE3) (pDsbA-LipII), (2) BL21(DE3) (pDsbC-LipII), (3) BL21(DE3) (pHisperiMBP-LipII). Panel A/ Qualitative visualization of lipase activity on tributyrin agar plates. The upper

half and lower halves of each plate represent the cultivation temperatures of 28 °C and 15 °C, respectively. Panel B/ Cell density. Panel C/ SDS-PAGE analysis of the soluble and insoluble fractions of culture samples. Various Lip protein fusions have been indicated. ‘C’ and ‘I’ represent the cultures ‘without induction’ and ‘with IPTG induction’, respectively.

Given the above technical limitations associated with intracellular expression in *E. coli* and the fact that Lip is an extracellular lipase in the original Gram-negative bacterium *Burkholderia*, extracellular secretion of Lip appears to be a plausible approach. To do this, a translational fusion of Lip with the 23 kDa C-terminal of HlyA was constructed for extracellular secretion of Lip-HlyA via the Hly-transporter (i.e. scenario F). The secretion was assisted by HlyB and HlyD which were coexpressed from the plasmid pVDL9.3. Using JM109 harboring pEHLYALipII and pVDL9.3, the culture performance for the expression and secretion of Lip-HlyA is summarized in Figure 6.8. Visible halos were developed on tributyrin agar plates for the extracellular medium samples (Figure 6.8A), but not the intracellular fractions; indicating that HlyA can mediate the secretion of bioactive Lip. Lipase activity up to 95 U/L was obtained in the 24-h IPTG-induced culture sample without affecting cell growth (Figure 6.8C). Note that the samples of the control cultures without IPTG induction also developed a visible halo owing to the leaky expression associated with the *lac* promoter, but its lipase activity was very low compared to that of the IPTG-induced culture (Figure 6.8C). The Hly-transporter appeared to be more effective than using the native signal peptide of Lip for achieving functional Lip secretion in the growth medium of *E. coli* (95 U/L in Figure 6.8C vs. 55 U/L in Figure 6.3C). However, Lip secreted with the use of the native signal peptide is an intact Lip as opposed to the Lip-HlyA fusion secreted via the Hly-transporter.



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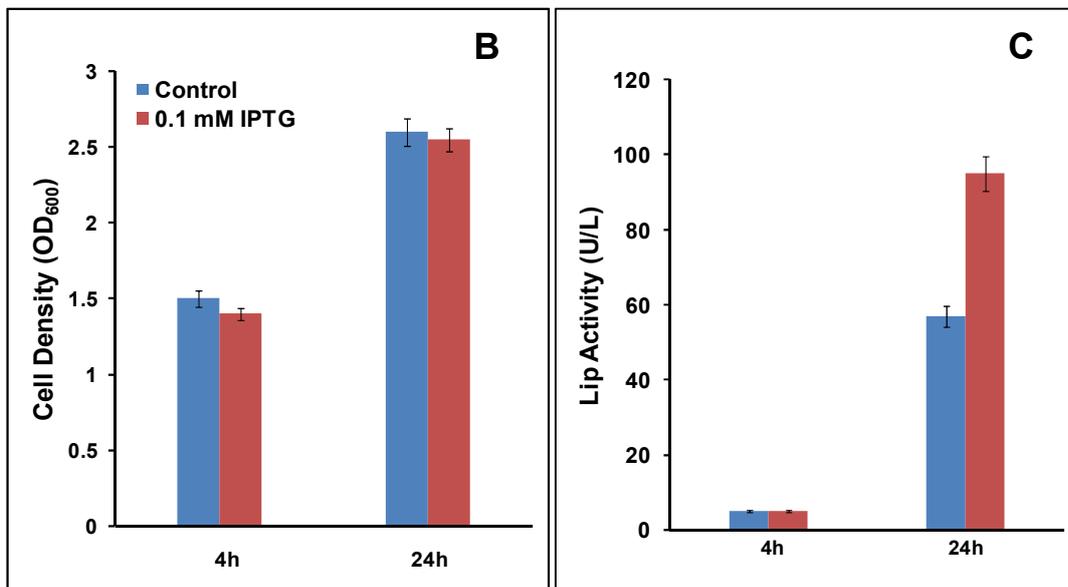
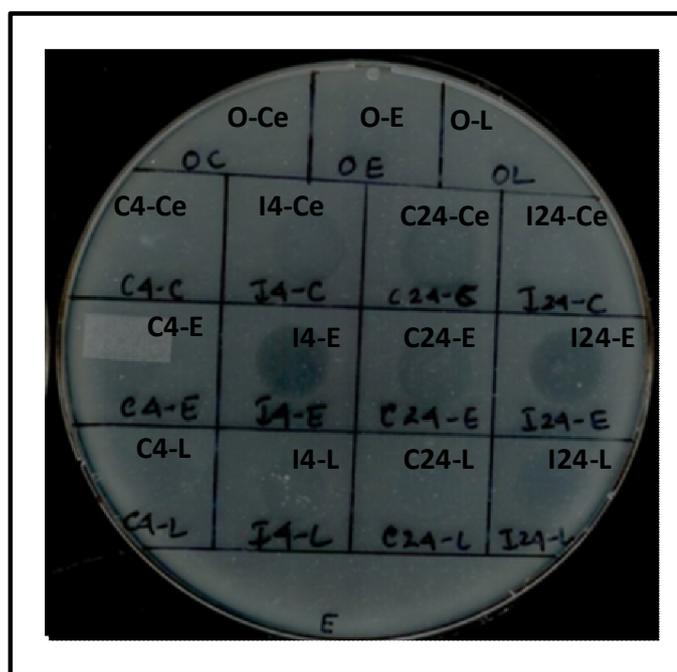


Figure 6.8 Culture performance of Lip expression (scenario F) using JM109 (pEHLYA-LipII) Panel A/ Qualitative visualization of lipase activity on tributyrin agar plates. Panel B/ Cell density. Panel C/ Lipase activity. ‘C’ and ‘I’ represent the cultures

‘without induction’ and ‘with IPTG induction’, respectively. ‘E’ and ‘L’ represent the ‘extracellular fraction’ and ‘soluble lysate fraction’, respectively.

Finally, displaying Lip on the *E. coli* cell surface using the carrier protein of EstA*, which is an inactive variant of esterase from *Pseudomonas aeruginosa* (18), was explored (scenario C) and the culture performance of JM109 harboring pESTLipII is summarized in Figure 6.9. Upon the qualitative analysis of the lipase activity on tributyrin agar plates, halos were observed for both the whole cell and the extracellular fraction, whereas no halo was visible for the soluble intracellular lysate (Figure 6.9A). The presence of the lipase activity in the extracellular fraction suggests Lip detachment from the cell membrane and/or a potential cell lysis. The whole cells harvested from the IPTG-induced culture yielded a lipase activity up to 25 U/L upon conducting the lipase assay by pH stat (Figure 6.9C), confirming the functional display of Lip on the *E. coli* cell surface.



A

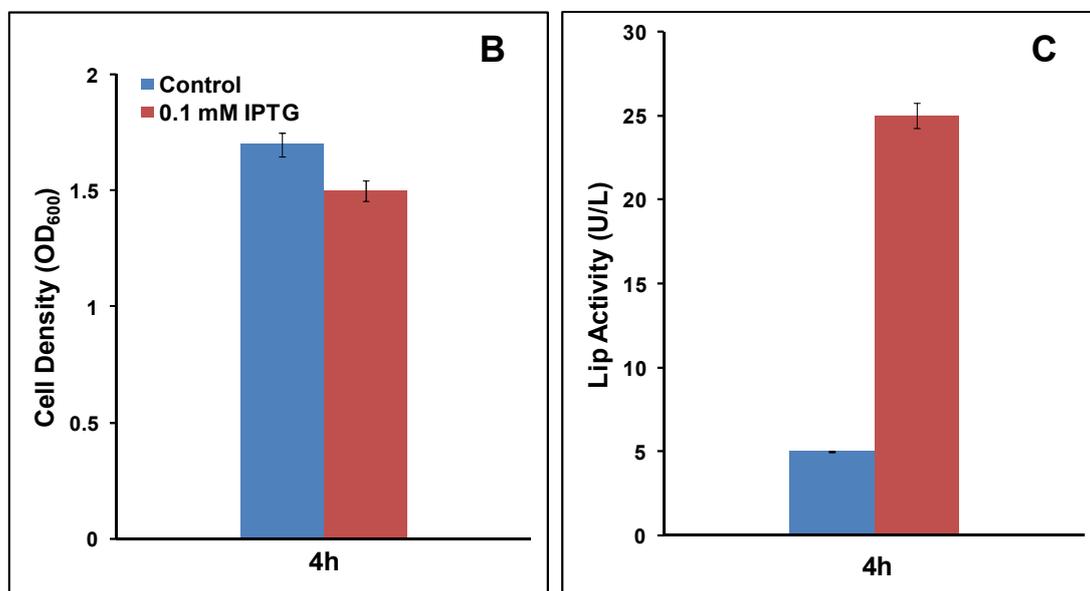


Figure 6.9 Culture performance of Lip expression (scenario C) using JM109 (pEST-LipII) Panel A/ Qualitative visualization of lipase activity on tributyrin agar plates. Panel B/ Cell density. Panel C/ Lipase activity. ‘C’ and ‘I’ represent the cultures ‘without induction’ and ‘with IPTG induction’, respectively. “Ce”, “E”, and “L” represent the ‘cell’, ‘extracellular fraction’, and ‘soluble lysate fraction’, respectively, of culture samples.

6.4 Discussion

Functional expression of recombinant proteins in *E. coli* can be potentially limited by disulfide bond formation. In this study, it appears that Lip expression in the oxidative cytoplasm of Origami B(DE3) slightly developed the lipase activity, as opposed to the cytoplasmic expression of Lip in the reductive cytoplasm of BL21(DE3) which has no lipase activity (Figure 6.4). The results suggest certain disulfide bond(s) could be critical for functional expression of Lip. It was reported that the Cys190-Cys269/Cys270 disulfide

bridge, which is conserved in lipases from Gram-negative bacteria, is required for protein stabilization against heat denaturation and proteolysis (144, 152, 199). Such conserved cysteine residues of Cys190 and Cys270 exist in the mature Lip. In addition to disulfide bond formation, ineffective translation or proteolysis could also contribute to the poor Lip yield associated with the cytoplasmic expression. Coexpression of GroEL/ES, which assists the folding of de-novo proteins and the refolding of misfolded proteins (77, 112), had a slightly positive effect in developing the lipase activity in BL21(DE3). The low expression yield could be partially alleviated with the use of fusion tags and high-level expression was achieved for all the Lip fusions except Skp-Lip. Though lipase activity was detected for several Lip fusions, the majority of the expressed Lip fusions aggregated as insoluble inclusion bodies in the cell, implying another expression issue of protein misfolding. It is interesting to observe that the functional expression of the Lip fusion with DsbA was achieved in BL21(DE3), but not in Origami B(DE3); whereas the functional expression of Lip fusions with MBP, Skp, and HisNusA was achieved in Origami B(DE3), but not in BL21(DE3). T7PK was reported to be effective in producing hard-to-express proteins in *E. coli* (47) and it was the only fusion tag achieving the functional expression in both BL21(DE3) and Origami B(DE3).

Similarly, the majority of the heterologously expressed Lip, either non-fused Lip or Lip fusions, in the periplasm of *E. coli* formed inclusion bodies though the periplasm is an oxidative compartment suitable for disulfide bond formation. The first step for the secretion of extracellular lipase in *Burkholderia* is protein translocation across the cytoplasmic membrane to the periplasm via the Sec secretion system and the export occurs concurrently with the removal of the N-terminal signal sequence while maintaining a partially folded and near native conformation for the secreted lipase (86). Further transport of proteins across the outer membrane into the extracellular medium can be mediated by the Xcp secretion system, which is a complex machinery comprising of at least 12 different proteins and well conserved in the Gram-negative bacteria species such as *Pseudomonas* and *Burkholderia* (280). Though the expressed Lip was targeted to the periplasm of *E. coli* via the Sec secretion system, the presence of insoluble Lip aggregates, even under the situation with the coexpression of

periplasmic folding factors, suggests that proper folding of Lip was prevented in the periplasm. Interestingly, the expression of Lip with its native signal peptide produced active enzyme in the extracellular medium of *E. coli*, implying that extracellular secretion assists the proper folding of Lip. Apparently, the signal peptide of *Burkholderia* Lip is recognized by *E. coli* for the extracellular secretion of Lip even though no Xcp-secretion-like system has been reported for *E. coli*. Technically, it will be interesting to explore the use of the Lip signal peptide for the extracellular secretion of recombinant proteins since most of the signal peptides adopted in *E. coli* are primarily for the secretion of recombinant proteins into the periplasm (143, 169, 189, 245). The extracellular expression of Lip was further improved by Skp coexpression. Skp is a cell envelope chaperone that helps maintaining an intermediate folded conformation for outer membrane proteins to be competent for export and insertion onto the outer membrane (39). It was demonstrated to be effective in improving the folding of recombinant antibody fragments in the periplasm (31) and the display of recombinant proteins on the cell surface (193). The improved extracellular expression of Lip associated with Skp coexpression might be due to the prevention of premature Lip folding in the periplasm so that the subsequent export across the outer membrane was facilitated.

Since extracellular secretion appears to be an effective approach for the functional expression of Lip, another strategy of using the α -hemolysin transporter for extracellular secretion was successfully explored in this study. The extracellular secretion was conducted through the construction of a Lip-HlyA fusion whose translocation across the inner and outer membranes in *E. coli* is mediated by an export conduit which is assembled by the Hly-transport component proteins of HlyB, HlyD (88) and the outer membrane protein TolC (7). No signal peptide is required for such direct secretion of Lip-HlyA from the cytoplasm to the cell exterior without a periplasmic intermediate (276). It was reported that functionally active single chain Fv antibodies with correct disulfide bonds were secreted via the Hly-transporter (79), whereas the activity was demolished when an *E. coli* mutant defective in thioredoxin reductase was used as the host for the secretion (78); suggesting that proper disulfide bonds could be formed during the protein transport through the export conduit or in the oxidative culture medium. Such a feature might be helpful in terms of forming the disulfide bridge of

Cys190-Cys270 in Lip-HlyA which is required for stabilizing the active protein (144). Comparing the two secretion strategies, both of them posed a minimum physiological impact on cell growth, but the one via the Hly-transporter appeared to produce a higher lipase activity. However, a Lip-HlyA fusion was produced and, as a result, an additional protein cleavage step would be required to obtain the native Lip. On the other hand, the native Lip was produced through the secretion based on the native signal peptide.

Finally, Lip was functionally expressed on the *E. coli* cell surface using EstA^{*}, an inactive variant of EstA from *Pseudomonas aeruginosa*, as a carrier protein. EstA has been shown to be properly localized on the outer membrane upon heterologous expression in *E. coli* and, as a result, it can serve as a carrier for the functional display of several lipolytic enzymes from *Fusarium solani*, *Bacillus subtilis*, and *Serratia marcescens* on the *E. coli* cell surface (18). Since the N-terminal extracellular domain of EstA is intact in this design, the passenger protein fused to the carrier can be relatively large, fully exposed on top of the carrier protein, and placed far from the lipopolysaccharide layer as compared to other displaying systems. In this study, whole cells with the expressed Lip-EstA^{*} were found to have the lipase activity, implying the functional expression of Lip on the cell surface. However, the lipase activity was also detected in the extracellular medium, suggesting some of the displayed Lip was released. The release of the displayed Lip could be possibly due to the proteolytic attack by certain outer membrane protease, such as OmpT (101), or the release of the whole Lip-EstA^{*} fusion into the medium since EstA is not a native *E. coli* protein and the integration of EstA with the outer membrane might not be firm enough. The *E. coli* cells with bioactive Lip being expressed on the outer membrane can be used as a whole-cell biocatalyst though the displaying performance could be further improved.

Chapter 7

Original Contributions and Recommendations

7.1 Original Contributions

7.1.1 Periplasmic Chaperone Skp Reduces Extracytoplasmic Stress and Improves Cell Surface Display in *E. coli*

Cell physiology seriously deteriorated when yellow fluorescence protein (YFP) was displayed on *E. coli* cell surface, resulting in growth arrest, cell lysis, and poor display performance. Coexpression of Skp, a periplasmic chaperone known to be associated with several outer membrane proteins for their targeting onto the outer membrane, was demonstrated to be effective to restore the deteriorated cell physiology. Upon Skp coexpression, cells with YFP display became less sensitive to EDTA and SDS, implying cell physiology was improved. Display performance was highly enhanced due to the increased specific fluorescence intensity without growth arrest and cell lysis. Results of transmission electron microscopy (TEM) indicated that the density of surface-displayed YFP was highly increased upon Skp coexpression. Cells with YFP display experienced a high level of extracytoplasmic stress, as reflected by the induced promoter activities of three stress-responsive genes, i.e. *degP* and *rpoH*. The extracytoplasmic stress reflected by the *degP* promoter activity appeared to correlate well with the phenotypical cell physiology observed under various culture conditions for cell-surface display. The gene fusion of P_{*degP*}::*lacZ* was therefore proposed to be a suitable “sensor” for monitoring extracytoplasmic stress and cell physiology during the course of *E. coli* cell-surface display.

7.1.2 Stabilization of Proteolytically Sensitive Periplasmic Recombinant PalB Expression in *E. coli*

Using a eukaryotic protein of lipase B (PalB) from yeast *Pseudozyma antarctica* as the target protein for heterologous expression in *E. coli*, we experimentally demonstrated that the

proteolytic specificity can be intrinsically determined by the sequence of protein substrate and can be drastically altered by simple amino acid substitutions. Intracellular proteolysis has been recognized as one of the key factors limiting recombinant protein production, particularly for eukaryotic proteins heterologously expressed in the prokaryotic expression systems of *E. coli*. However, both the rationale as why these eukaryotic proteins often fall into the proteolytic category and the molecular mechanism associated with such proteolysis remain incompletely understood. Two proteolytically sensitive amino acid residues, i.e. Leu149 and Val223, were identified to critically affect PalB's susceptibility to proteolysis in *E. coli*. Functional expression was significantly enhanced with the use of PalB mutant derivatives that can dodge the proteolysis. While the majority of expressed PalB double mutant was localized in the periplasm, the functional expression appeared to be limited by protein misfolding. Coexpression of a periplasmic folding factor of DsbA could alleviate the limitation and, as a result, functional expression of the PalB double mutant was significantly enhanced. The study offers an alternative genetic strategy in molecular manipulation to enhance recombinant protein production in *E. coli*.

7.1.3 Efficient Secretion of Active PalB in the Extracellular Medium of *E. coli*

To overcome the technical problems related to insolubility in the cytoplasm and protein instability associated with proteolysis in the periplasm in *Escherichia coli*, PalB secretion from the cytoplasm to the bacterial culture medium without a periplasmic intermediate was investigated in this study. The methods are based on the *E. coli* α -hemolysin transport system (Hly) and the modified flagellar Type III secretion apparatus (TTSS) that can export proteins of interest from the cytoplasm directly to the exterior of the cell. Cultivation conditions including inducer strength, temperature and suitable host strain for optimal PalB-HlyA secretion were identified using shaker flask experiments for hemolysin transporter. PalB accumulated in the extracellular medium was functionally active with appropriate disulfide bridges formed and the secretion was non toxic to the growing cells. However, the secretion intermediates in the intracellular fraction of culture samples were non-bioactive even though they were soluble, suggesting that the extracellular secretion did mediate the development of

PalB activity. Proteolysis of the recombinant PalB-HlyA did not affect PalB activity as PalB was likely intact. Pure PalB secretion, unlike PalB-HlyA fusion protein; was achieved using the modified flagellar secretion system; however the secretion was slow with lower specific PalB activities but effective cell growth.

7.1.4 Cloning and Functional Expression of *Burkholderia* Lip in *E. coli*

Functional expression of lipase (Lip) from *Burkholderia* sp. C20 in *E. coli* was explored. Active Lip was expressed in the *trxB* and *gor* mutant Origami B(DE3) cytoplasm, suggesting the importance of disulfide bond formation for Lip expression. While overexpression limitation related to low protein limits was observed, coexpression of GroEL/ES folding chaperone was found to be enhancing cytoplasmic Lip expression. Though high yields of overexpressed Lip were attained during periplasmic expression, most of the polypeptides formed insoluble inclusion bodies. Fusion tag technique was also explored by constructing several Lip fusions for the evaluation of their expression performance. While cytoplasmic overexpression limitation was overcome by using Lip fusions except with Skp, the fusion tags T7PK, DsbA, and DsbC were the most effective in boosting the solubility and biological activity of the expressed Lip. On the other hand, Lip fusions expressed in the periplasm aggregated to form non-native protein. To overcome the technical limitations related to intracellular Lip expression, extracellular secretion was explored. Biologically active Lip was secreted to the *E. coli* culture medium using its native signal peptide; coexpression of the periplasmic chaperone Skp that assists the folding of periplasmic and outer membrane proteins translocated using the Sec-pathway, improved the secretion performance of Lip. Enhanced secretion of bioactive Lip fused to HlyA; from the cytoplasm to the culture supernatant without a periplasmic intermediate was conducted by α -hemolysin transporter. Lip fused to the carrier EstA was functionally displayed on the outer surface of *E. coli* and the whole cells were verified to possess enzymatic Lip activity.

7.2 Recommendations

1. The extracellular lipase (Lip) in *Burkholderia* requires the help of a specific lipase-folding-chaperone (Lif) that is encoded in its operon for appropriate folding and secretion of the mature protein. Lif maintains Lip in a partially folded conformation competent required for secretion of *Burkholderia* lipase using the Xcp secreton. The *lif* gene may be isolated by PCR-amplification from the genomic DNA of *Burkholderia* sp. C20. It will be interesting to investigate the coexpression of *lif* with the various constructs of Lip described in Chapter 6, to improve the solubility and minimize the inclusion bodies of Lip in *E. coli*. The coexpression of Lif can be targeted to cytoplasm or periplasm depending on the cytoplasmic or extracytoplasmic expression of Lip in *E. coli*.
2. The extracellular proteins including lipase in gram negative bacteria such as *Burkholderia* and the related species *Pseudomonas* are secreted by the Type II secretion pathway, also called the main terminal branch (MTB) of the general secretory pathway (GSP), more specifically the Xcp-secreton (226). These proteins are translocated in two steps across the bacterial cell envelope. After translocation of the signal peptide-bearing exoproteins across the cytoplasmic membrane, a step similar to the Sec-mediated transport of proteins in *Escherichia coli* (227), the exoproteins are transported from the periplasm across the outer membrane. The secretion by Xcp secreton requires the products of at least 12 *xcp* genes namely *xcpAPQRSTUVWXYZ* located in the cell envelope (82). XcpP and/or XcpQ determine this secretion specificity, based on their results from *Erwinia* (163). It is worth investigation whether the coexpression of the XcpP and XcpQ can improve the secretion of *Burkholderia* Lip in *E. coli*.
3. Based on the analysis by SignalP software, the cleavage site of the signal peptide from *Burkholderia* Lip was found to be located between the 40th and 41st amino acid residue. However the N-terminal sequencing analysis of the extracellular lipase from *Burkholderia* sp HY-10 starts at the 45th amino acid (described in Chapter 6). Therefore the Lip polypeptide might be present as a propeptide after translocation

across the cytoplasmic membrane of gram negative bacteria and might possibly contain recognition features for folding and secretion. The majority of the periplasmically expressed Lip in *E. coli* was found to form insoluble aggregates. It would be worth exploration to express the propeptide rather than the mature peptide of Lip in *E. coli* periplasm to investigate the effect on Lip folding and solubility.

4. Lipase expression is an excellent system for high throughput screening due to the possibility of identification of clones expressing active enzyme on tributyrin hydrolysis agar plates. Soluble versions of Lip in *E. coli* may be generated by random mutagenesis. Promising clones might be identified; the plasmid DNA isolated from the clones can be sequenced to identify the 'hot spot' amino acid residues in the sequence of Lip. They can be manipulated further by site directed mutagenesis to investigate the importance of the recognized amino acids in the structure and functional expression in *E. coli*. Similar methodology may be applied to the cytoplasmic expression of Lip in *E. coli* to identify mutants that have enhanced expression with respect to efficient translation and/or resistance to proteolysis.
5. Several other genes (six) named as putative lipase or esterase were isolated from the genomic DNA of *Burkholderia* sp. C20 based on the chromosome sequence of other *Burkholderia* species (NCBI) and cloned in various vectors for cytoplasmic and extracytoplasmic expression in *E. coli*. It would be exciting to identify any novel lipolytic enzymes present in *Burkholderia* sp. C20 and overexpress them in *E. coli*.
6. Apart from the many interesting applications of PalB, recent interests have arisen due to its potential for biotransformation for biodiesel production as opposed to the chemical conversion of oils. However this process requires the overproduction of the enzyme in a cost effective manner. The improvement in the bioprocess of functional overexpression of PalB mutant in *E. coli* might provide an effective alternative for large-scale production of PalB as opposed to the rather expensive preparations of PalB from Novazym (Novozymes A/S) and Chirazyme (Roche Molecular Biochemicals) (145). The pure protein preparation of PalB mutant can be compared with the standard PalB (from Novazym).

7. A few other clones, with halos of intensities relatively lower than the double mutant of PalB (Leu149, Val223) were identified. It is worthwhile to investigate the relevant mutations present in those clones responsible for the enhanced expression in *E. coli*. Although it has been known widely that the protein sequence determines their susceptibility to proteolysis and a number of proteases in *E. coli* have been identified, their substrate specificity or recognition sequences have not been explored properly. Identification of the proteolytically sensitive sequences of PalB described in Chapter 4 should be used to identify any novel protease or the substrate specificity of the existing proteases. Such information can be used for the construction of potential mutant strains in *E. coli* deficient in proteases, which would be useful for expression of eukaryotic proteins.
8. PalB expressed in the extracellular medium which has minimum contamination from host proteins, could be concentrated and/or purified to be used in the preparation of immobilized enzymes.
9. In our previous studies (193), the *degP* promoter activity was found to be a suitable marker for probing the level of extracytoplasmic stress that cells encountered under various culture conditions for surface display and periplasmic expression of recombinant proteins. However, the *rpoH* promoter activity failed to show such correlation, particularly for the culture conditions in which extracytoplasmic stresses appeared to be partially relieved when DegP was coexpressed (194). Since *rpoH* is a member of the σ^E pathway whose expression is associated with RseA degradation, the degradation could possibly be mediated by periplasmic proteases other than DegS. It has been reported that DegP, DegQ, and DegS are homologous periplasmic serine proteases with a conserved catalytic triad of Ser-His-Asp (146, 292). Hence, DegP and DegQ could be alternative proteases for RseA degradation. It would be very interesting to investigate if these periplasmic proteases could share an overlapping biological function of RseA degradation to induce the *rpoH* promoter activity under stressful conditions associated with the display of a recombinant protein on the cell surface of *E. coli*.

Appendix A

Protocols for Molecular Cloning

A1. Genomic DNA Isolation from *Burkholderia* sp. C20

(DNeasy® Tissue Kit, Qiagen)

1. Harvest *Burkholderia* sp. C20 from 1 ml of overnight culture (at 30 °C, 220 rpm) by centrifuging for 10 min at 5000 × g, at room temperature. Discard the supernatant.
2. Resuspend the cells in 600 µl of 1 M sorbitol buffer. Add 200 units lyticase and incubate at 30 °C for 30 min.
3. Spin down spheroplasts at 300 × g for 10 min.
4. Resuspend the spheroplasts in 180 µl of Buffer ATL. Add 20 µl of proteinase K, vortex, and incubate at 55 °C in a shaking water-bath until the cells are completely lysed.
5. Vortex for 15 s. Add 200 µl of Buffer AL to the sample, vortex, and incubate at 70 °C for 10 min.
6. Add 200 µl of ethanol (96~100%) to the sample, and vortex.
7. Pipette the mixture from Step 6 into the DNeasy Mini-spin column placed in a 2 ml collection tube. Centrifuge at 6000 × g for 1 min. Discard the flow-through with the collection tube.
8. Place the DNeasy column in a new 2 ml collection tube, add 500 µl of Buffer AW1, and centrifuge at 6000 × g for 1 min. Discard the flow-through with the collection tube.
9. Place the DNeasy in a new 2 ml collection tube, add 500 µl of Buffer AW2, and centrifuge at 15000 × g for 5 min to dry the DNeasy membrane. Discard the flow-through with the collection tube.
10. Place the DNeasy column in a clean 1.5 ml microcentrifuge tube, and pipette 200 µl of Buffer AE directly onto the membrane. Incubate at room temperature for 1 min, and then centrifuge at 6000 × g for 1 min to elution of the genomic DNA.

A2. Plasmid DNA Isolation from *E. coli* (Qiaprep Spin Miniprep Kit, Qiagen)

1. Resuspend pelleted bacterial cells in 250 μ l Buffer P1 (containing RNase) and transfer to a micro-centrifuge tube.
2. Add 250 μ l Buffer P2 (Lysis buffer) and mix thoroughly by inverting the tube gently 4–6 times.
3. Add 350 μ l Buffer N3 (Neutralization buffer) and mix immediately and thoroughly by inverting the tube 4–6 times.
4. Centrifuge for 10 min at 13,000 rpm (\sim 17,900 x g) in a table-top microcentrifuge. A compact white pellet will form.
5. Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.
6. Centrifuge for 30–60 s. Discard the flow-through.
7. Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through. This step is necessary to remove trace nuclease activity when using endA⁺ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content.
8. Wash QIAprep spin column by adding 0.75 ml Buffer PE (containing ethanol) and centrifuging for 30–60 s.
9. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.
10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 μ l Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

A3. DNA Restriction Digestion

1. For analytical purposes, set up the reaction mixture in microfuge tube as follows:

Component	Stock Concentration	Required Concentration	Volume/Reaction
DI Water	x μL		
Reaction Buffer	10X	1X	1 μL
DNA	50-200ng/ μL	10~30ng/ μL	y μL
Restriction Enzyme	1U/ μL #	~1 U *	~1 μL *
Total			10 μL

The restriction enzymes have to be diluted with the appropriate Diluent buffer to a final concentration of 1 U/ μl . * For most of the commercial enzymes, 1 Unit of enzyme is defined as the amount required to digest 1 μg of DNA (e.g. λ DNA, check with source) in one hour at 37 °C.

- Mix the ingredients by pulse-spinning the microfuge tubes.
- Incubate the reaction solution at 37°C (or other recommended temperature for specific restriction enzymes) for at least 1 hr.
- Inactivate restriction enzyme by heating at 65°C (or other recommended temperature for specific restriction enzymes) for 15 min (optional).

A4. DNA Ligation Reaction

- Digest the plasmid DNA and the insert DNA with the appropriate restriction enzymes. If necessary, isolate the desired fragments by gel electrophoresis, gel extraction and purify by ethanol precipitation.
- Set up the ligation reaction as follows:

Component	Amount	Volume used (μL)
Insert DNA	0.1-1 μg *	x
Vector DNA	0.05-1 μg *	y
10X Ligation Buffer	-	1.0

T4 DNA Ligase Enzyme	0.1 Weiss Units @	0.2 μ L	*
Deionised Water	To make up the reaction volume to 10 μ L.		It is
Total		10	reco

mm

ended to use equimolar amount of vector and insert DNA for cohesive end ligation. (Molecular cloning-Maniatis).

@ One Weiss Unit is defined as the amount of enzyme that catalyzes the exchange of 1 nmole of P^{32} from pyrophosphate into ATP in 20 minutes at 37 $^{\circ}$ C. One Weiss Unit corresponds to 60 cohesive end units as defined by New England Biolabs. (Molecular cloning-Maniatis)

- Mix the components in a pulse spinner.
- Incubate the reaction mixture 1-4 hours at 16 $^{\circ}$ C or overnight at room temperature.

A5. DNA Transformation using Chemical Method (54)

TSB (Transformation and Storage Buffer): Stored at 4 $^{\circ}$ C

LB broth containing 10% PEG, 5% DMSO and 20 mM Mg⁺² (10 mM MgCl₂ and 10 mM MgSO₄)

TSBG: TSB + 20 mM Glucose, stored at 4 $^{\circ}$ C

Stock solution

Preparation of 1 M MgCl₂

Dissolve 1.904 g MgCl₂ (MW = 95.2) into 20 mL DI water and autoclave for 15 min.

Preparation of 1 M MgSO₄

Dissolve 2.408 g MgSO₄ (MW = 120.4) into 20 mL DI water and autoclave for 15 min.

Preparation of 2 M glucose

- Dissolve 36 g glucose into 100 mL DI water and autoclave for 15 min.
- Store it at 4 $^{\circ}$ C.

Preparation of TSB (or TSBG)

1. Mix 93 mL LB broth, 10 g PEG, and 5 mL DMSO.
2. Autoclave the mixture for 15 min.
3. Aseptically add 1 mL of 1 M MgCl₂ and 1 mL of 1M MgSO₄.
4. *For TSBG, aseptically add 1 mL of 2 M glucose.
5. Store both buffers at 4°C.

Procedures

Preparation of competent cells

1. Inoculate 5 mL of LB with a colony. Grow the culture at 37°C.
2. Inoculate 100-1000 µL of the LB culture to another new LB (say, 25 mL). Grow the culture to the early log phase (OD₆₀₀ = 0.3-0.6).
3. Collect 1 mL of culture in each microfuge tube and centrifuge at 0 4°C, 4000×g for 5 min.
4. Discard the supernatant and resuspend the pellet in 1/10 volume of TSB (100µL). Sit on ice for about 10 min and ready to be used.
5. *The competent cells can be stored at -70°C.

Transformation

1. For 0.1 mL of the competent cells in TSB in a microfuge tube, add 0.1 µg of plasmid DNA.
2. Sit on ice for 5-30 min (Heat shock is not necessary).
3. Add 0.9 mL of TSBG.
4. Incubate at 37°C with shaking (200 rpm) for 60-90 min to allow the expression of the antibiotic resistance gene.
5. Plate 200 µL on the appropriate plates for selection of transformants.

A6. DNA Transformation using Electroporation (Modified from Electroporation Manual, BioRad)

Preparation of Electrocompetent Cells

1. Inoculate 100 ml of L-broth with 1/100 volume of a fresh overnight *E. coli* culture.
2. Grow the cells at 37 °C shaking at 200 rpm to an OD₆₀₀ of approximately 0.5–0.7 .

3. Chill cells on ice for ~20 min. For all subsequent steps, keep the cells as close to 0 °C as possible (in an ice/water bath) and chill all containers in ice before adding cells. To harvest, transfer the cells to two cold 60-ml centrifuge bottles and spin at 6000 x g for 15 minutes at 4 °C.
4. Carefully pour off and discard the supernatant. It is better to sacrifice the yield by pouring off a few cells than to leave any supernatant behind.
5. Gently resuspend the pellet in 50 ml of ice-cold 10% glycerol each. Centrifuge at 6000 x g for 15 minutes at 4 °C; carefully pour off and discard the supernatant.
6. Resuspend the pellet in 50 ml of ice-cold 10% glycerol, transfer to one 60-ml bottle. Centrifuge at 6000 x g for 15 minutes at 4 °C; carefully pour off and discard the supernatant.
7. Resuspend the pellet in ~4 ml of ice-cold 10% glycerol. Transfer to two 2-ml sterile microfuge tubes. Centrifuge at 6000 x g for 15 minutes at 4 °C; carefully pour off and discard the supernatant.
8. Resuspend the cell pellet in a final volume of 400µl of ice-cold 10% glycerol. The cell concentration should be about $1-3 \times 10^{10}$ cells/ml.

This suspension may be frozen in aliquots (40µl each in a sterilized 1.5ml microfuge tube) on dry ice and stored at -80 °C. The cells are stable for at least 6 months under these conditions.

Electroporation

1. Thaw the cells on ice. For each sample to be electroporated, place a 1.5 ml microfuge tube and either a 0.1 or 0.2 cm electroporation cuvette on ice.
2. In a cold, 1.5 ml polypropylene microfuge tube, mix 40 µl of the cell suspension with 1 to 2 µl of DNA (DNA should be in deionized water). Mix well and incubate on ice for ~1 minute. (Note: it is best to mix the plasmids and cells in a microfuge tube since the narrow gap of the cuvettes prevents uniform mixing.)
3. Set the MicroPulser to "Ec1" when using the 0.1 cm cuvettes. Set it to "Ec2" or "Ec3" when using the 0.2 cm cuvettes. See Section 4 for operating instructions.

4. Transfer the mixture of cells and DNA to a cold electroporation cuvette and tap the suspension to the bottom. Place the cuvette in the chamber slide. Push the slide into the chamber until the cuvette is seated between the contacts in the base of the chamber. Pulse once.
5. Remove the cuvette from the chamber and immediately add 1 ml of SOC medium to the cuvette. Quickly but gently resuspend the cells with a Pasteur pipette. (The period between applying the pulse and transferring the cells to outgrowth medium is crucial for recovering *E. coli* transformants (Dower *et al.*, 1988). Delaying this transfer by even 1 minute causes a 3-fold drop in transformation. This decline continues to a 20-fold drop by 10 minutes.
6. Transfer the cell suspension to a 17 x 100 mm polypropylene tube and incubate at 37 °C for 1 hour, shaking at 225 rpm.
7. Check and record the pulse parameters. The time constant should be close to 5 milliseconds. The field strength can be calculated as actual volts (kV) / cuvette gap (cm).
8. Plate on selective medium.

Solutions and Reagents for Electroporation

1. L-Broth: 10 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl; dissolve in 1.0 L water. Autoclave. 100ml
2. 10% (v/v) Glycerol: 12.6 g glycerol (density = 1.26 g/cc) in 90 ml of water. Autoclave or filter sterilize. 200ml
3. SOC: separately autoclave,
 - (1) 2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl,
 - (2) 10 mM, MgCl₂,
 - (3) 10 mM MgSO₄,
 - (4) 20 mM glucose.

A7. Gel-Extraction of DNA (Qiaquick Gel Extraction Kit, Qiagen)

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 μ l).
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation. Solubilize agarose completely. For >2% gels, increase incubation time.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). Add 1 gel volume of isopropanol to the sample and mix.
5. Place a QIAquick spin column in a provided 2 ml collection tube. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.
6. Discard flow-through and place QIAquick column back in the same collection tube.
7. Recommended: Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.
8. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.
9. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x g (13,000 rpm).
10. Place QIAquick column into a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 μ l of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min.

A8. PCR Purification of DNA (Qiaquick Gel Extraction Kit, Qiagen)

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix.
2. Place a QIAquick spin column in a provided 2 ml collection tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.
4. Discard flow-through. Place the QIAquick column back into the same tube.
5. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.

6. Discard flow-through and place the QIAquick column back in the same tube.
7. Centrifuge the column for an additional 1 min.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 μ l Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 μ l elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

A9. PCR Cloning (pPCR-ScriptAmp Cloning Kit, Stratagene)

1. To prepare the ligation reaction, add the following components in order in a 0.5-ml microcentrifuge tube:
 - 1 μ l of the pPCR-Script Amp SK(+) cloning vector (10 ng/ μ l)
 - 1 μ l of PCR-Script 10 \times reaction buffer
 - 0.5 μ l of 10 mM rATP
 - 2–4 μ l of the blunt-ended PCR product or 4 μ l of the control PCR insert
 - 1 μ l of Srf I restriction enzyme (5 U/ μ l)
 - 1 μ l of T4 DNA ligase (4U/ μ l)
 - Distilled water (dH₂O) to a final volume of 10 μ l
2. Mix the ligation reaction gently and incubate this reaction for 1 hour at room temperature.
3. Transform the ligation mixture to competent *E. coli* cells and select on agar plate with ampicillin and 80 μ g/ml X-gal.

A10. Error-Prone PCR (GeneMorph EZClone Domain Mutagenesis Kit, Stratagene)

Mutant Megaprimer Synthesis

1. Prepare the sample reaction(s) as indicated below:
 - 5 μ l of 10 \times Mutazyme II reaction buffer

- x μ l template
 x μ l of sample primers (125 ng/ μ l of each primer)
 1 μ l of 40 mM dNTP mix (200 μ M each final)
 x μ l of ddH₂O for a final reaction volume of 50 μ l
 1 μ l of Mutazyme II DNA polymerase (2.5 U/ μ l)
2. Centrifuge each reaction briefly.
 3. Place the reaction in a temperature cycler.

Segment	Number of cycles	Temperature	Duration
1	1	95 °C	2 minutes
		95 °C	1 minute
		Primer Tm-5 °C	1 minute
2	30		1 minute (\leq 1kb targets) or 1minute/kb (\geq 1kb targets)
		72 °C	
3		72 °C	10 minutes

EZClone Reaction

1. Prepare the sample reaction(s) as indicated below and place in a thermal cycler:
 25 μ l of the 2 \times EZClone enzyme mix
 50 ng of template plasmid
 250 ng megaprimer (<1kb), or 500 ng for \geq 1 kb megaprimer
 3 μ l of EZClone solution
 ddH₂O to a final volume of 50 μ l

Segment	Number of cycles	Temperature	Duration
1	1	95 °C	1 minute
2	25	95 °C	50 seconds

	60 °C	50 seconds
	68 °C	1 minute/kb of plasmid

2. Add 1 μl of Dpn I restriction enzyme (10 U/ μl) directly to each amplification reaction below the mineral oil overlay using a small, pointed pipet tip.
3. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate each reaction at 37°C for 2 hours to digest the parental (i.e., the nonmutated) supercoiled dsDNA.
4. Transform the reaction into *E. coli* and select on screening agar plate with appropriate antibiotic for the plasmid vector.

A11. Site-Directed Mutagenesis (Quikchange Multisite Directed Mutagenesis Kit, Stratagene)

1. Prepare the mutant strand synthesis reactions for thermal cycling as indicated below. Add the components in the order listed then mix gently by pipetting or tapping the reaction tube.

Experimental Reaction

Templates ≤ 5 kb

2.5 μl 10 \times QuikChange® Multi reaction buffer

X μl double-distilled H₂O to a final volume of 25 μl

X μl ds-DNA template (50 ng)

X μl mutagenic primers (If using 1–3 primers, add 100 ng of each primer. If using 4–5 primers, add 50 ng of each primer.)

1 μl dNTP mix

1 μl QuikChange® Multi enzyme blend

Templates > 5 kb

2. Cycle the reactions using the cycling parameters outlined in the table below.

Segment	Number of cycles	Temperature	Duration
1	1	95 °C	1 minute
2	30	95 °C	1 minute
		55 °C	1 minute
		65 °C	2 minute/kb of plasmid

3. Add 1 μ l of Dpn I restriction enzyme (10 U/ μ l) directly to each amplification reaction below the mineral oil overlay using a small, pointed pipet tip.
4. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 minute, then immediately incubate each reaction at 37°C for 1 hour to digest the parental (nonmutated) ds-DNA.
5. Transform the reaction into *E. coli* and select on screening agar plate with appropriate antibiotic for the plasmid vector.

A12. Gateway Cloning (GatewayLR_clonase Enzyme Mix, Invitrogen)

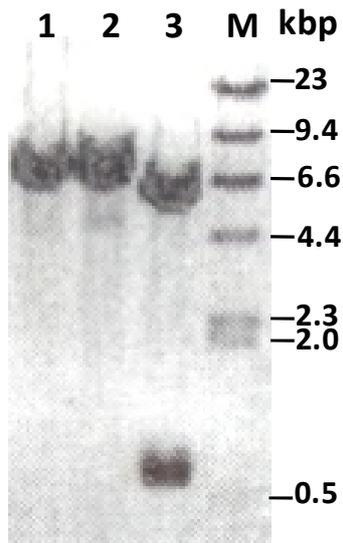
1. Add the following components to a 1.5 ml microcentrifuge tube at room temperature and mix:
 - Entry clone (50-150 ng) 1-7 μ l
 - Destination vector (150 ng/ μ l) 1 μ l
 - TE buffer, pH 8.0 to 8 μ l
2. Thaw on ice the LR Clonase™ II enzyme mix for about 2 minutes. Vortex the LR Clonase™ II enzyme mix briefly twice (2 seconds each time).
3. To each sample (Step 1, above), add 2 μ l of LR Clonase™ II enzyme mix to the reaction and mix well by vortexing briefly twice. Microcentrifuge briefly.
4. Return LR Clonase™ II enzyme mix to -20°C or -80°C storage.
5. Incubate reactions at 25°C for 1 hour.

6. Add 1 μl of the Proteinase K solution to each sample to terminate the reaction.
Vortex briefly. Incubate samples at 37°C for 10 minutes.
7. Transform into *E. coli* and plate on selective plates.

Appendix B

Restriction/Digestion Analysis of the Plasmids

The following plasmid maps were constructed using Vector NTI software, Invitrogen. The DNA gels were obtained by DNA-gel electrophoresis. Please note that the plasmids generated by Gateway Cloning have not been listed here.



Lane 1: NcoI
 Lane 2: PstI
 Lane 3: SfiI

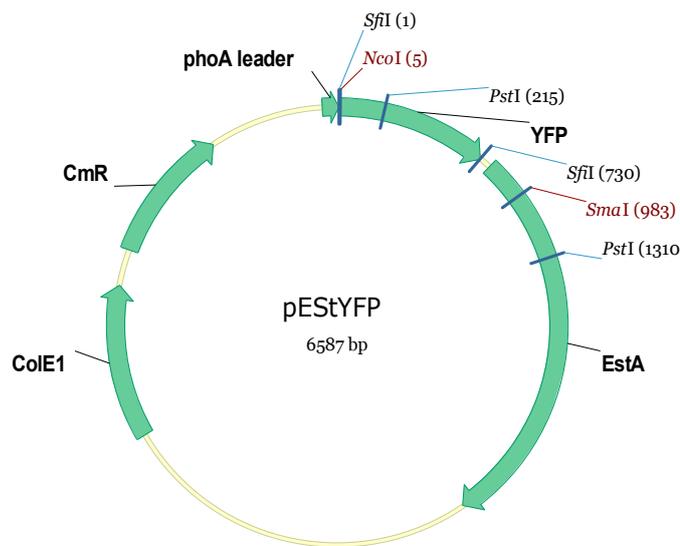


Figure B1 Restriction analysis of pESTYFP

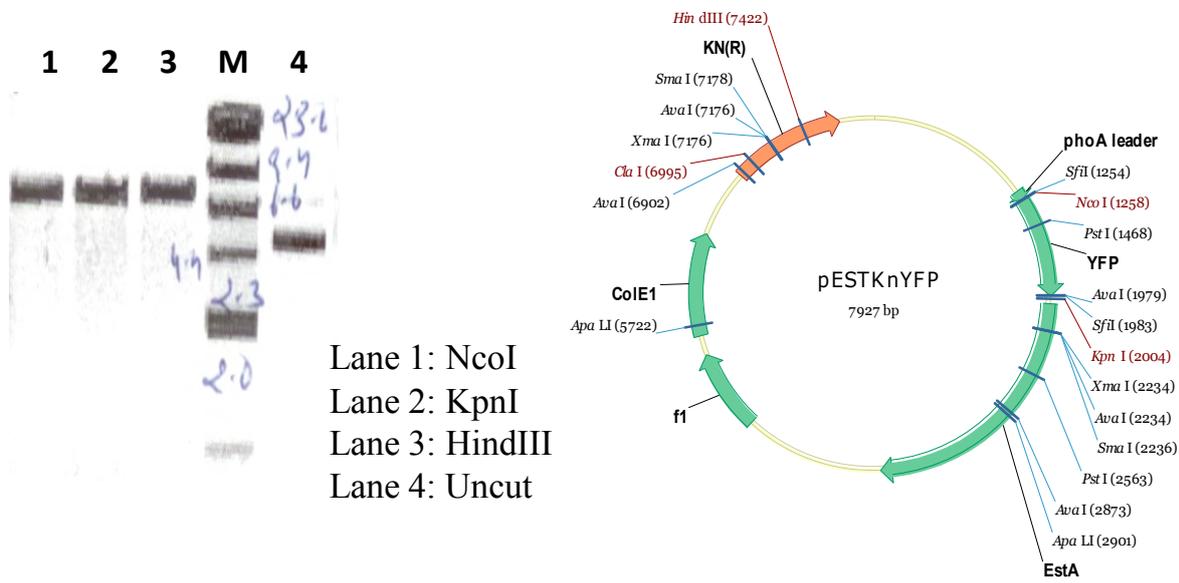


Figure B2 Restriction analysis of pESTKnYFP

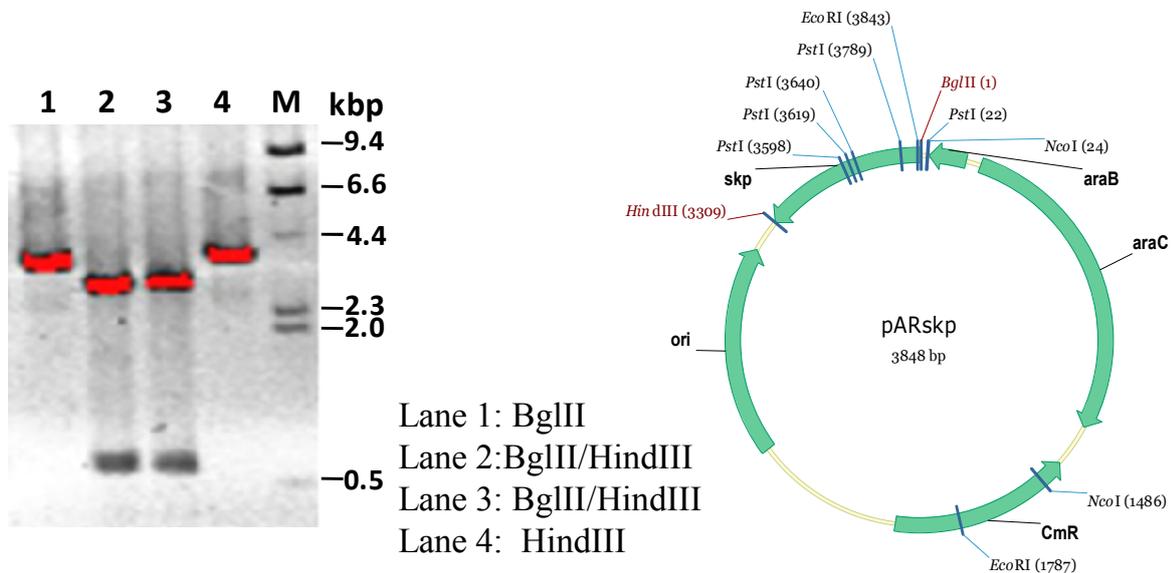


Figure B3 Restriction analysis of pARskp

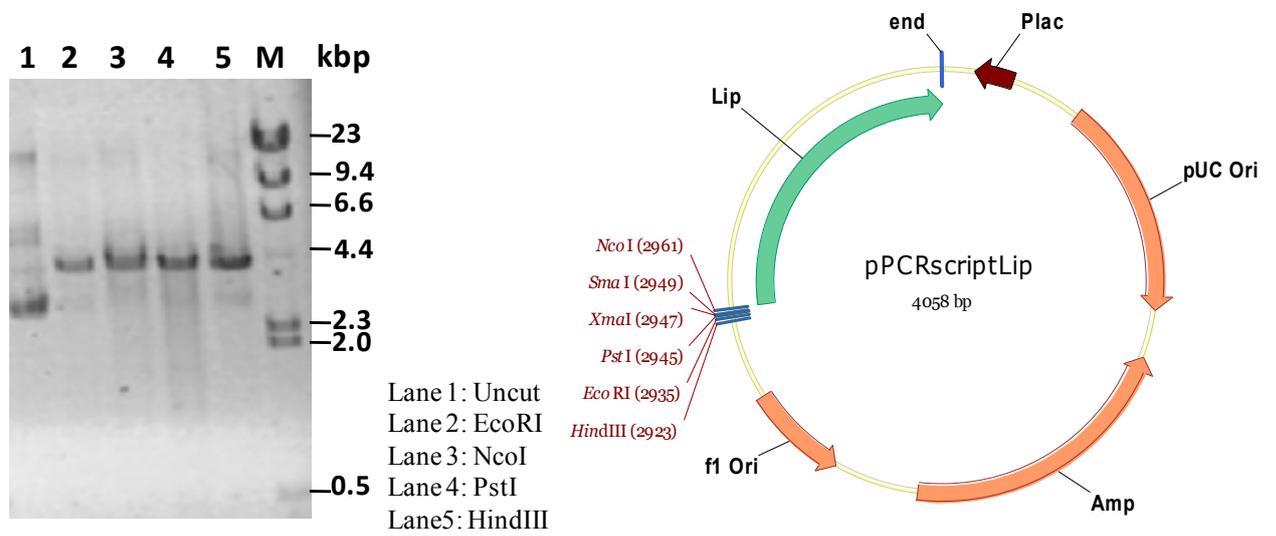


Figure B4 Restriction analysis of pPCRscriptLip

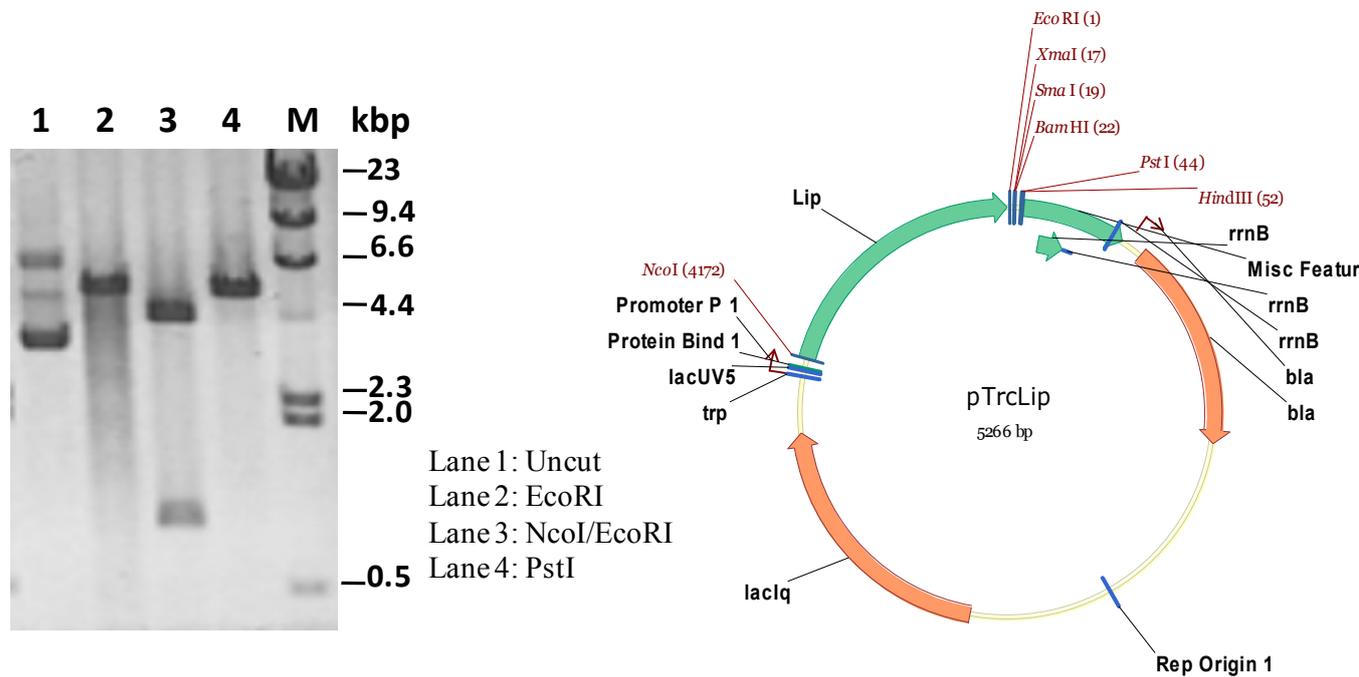


Figure B5 Restriction analysis of pTrcLip

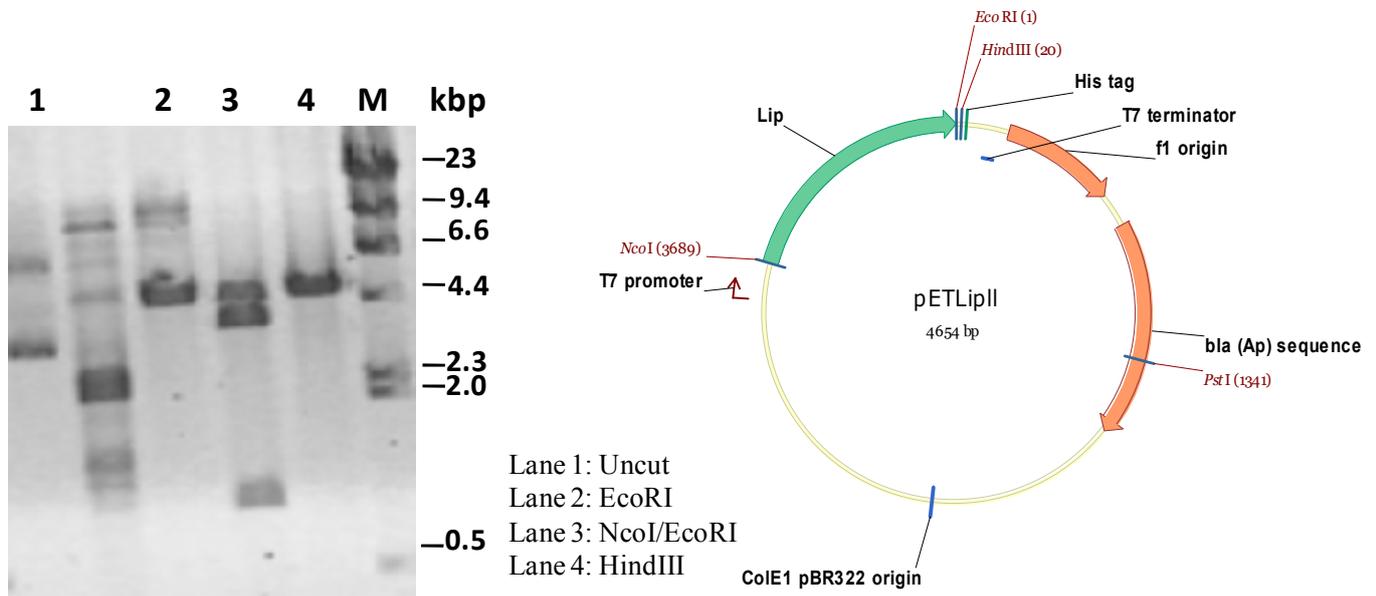


Figure B6 Restriction analysis of pETLipII

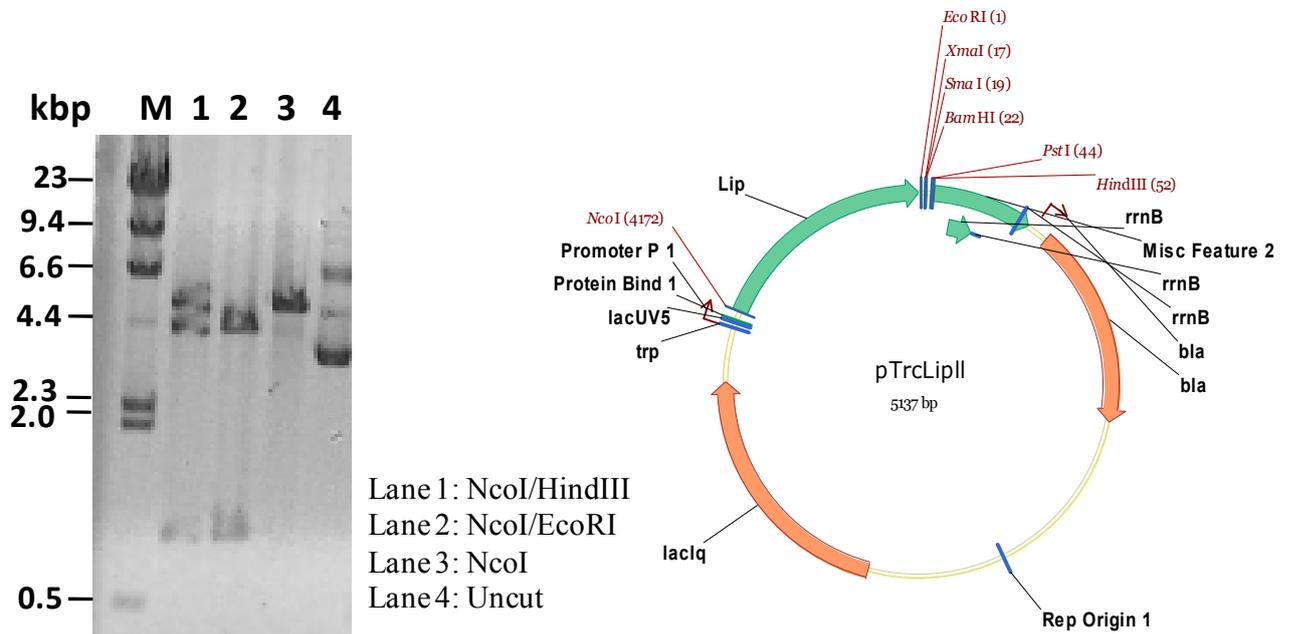


Figure B7 Restriction analysis of pTrcLipII

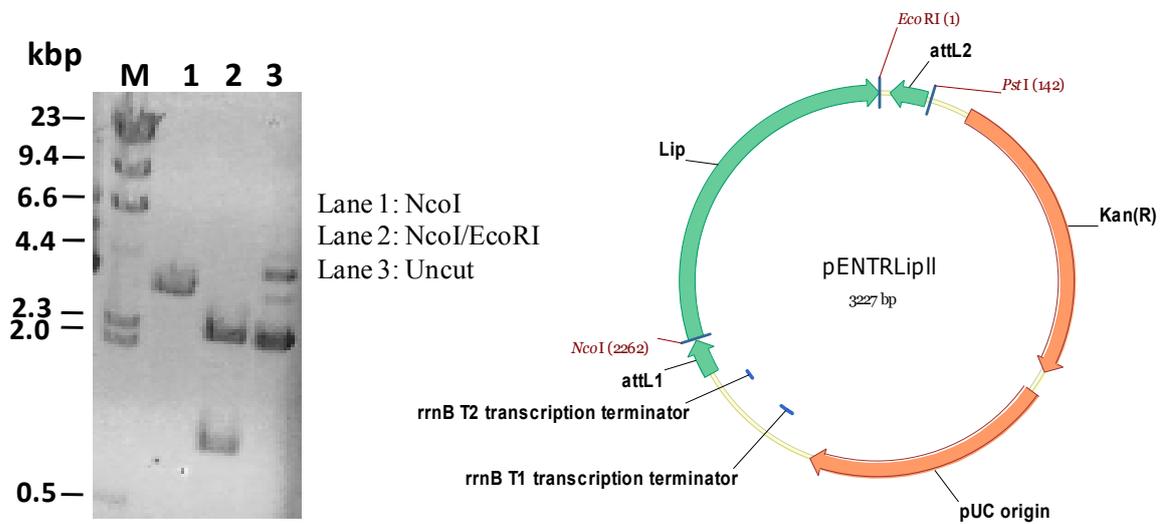


Figure B8 Restriction analysis of pENTRLipII

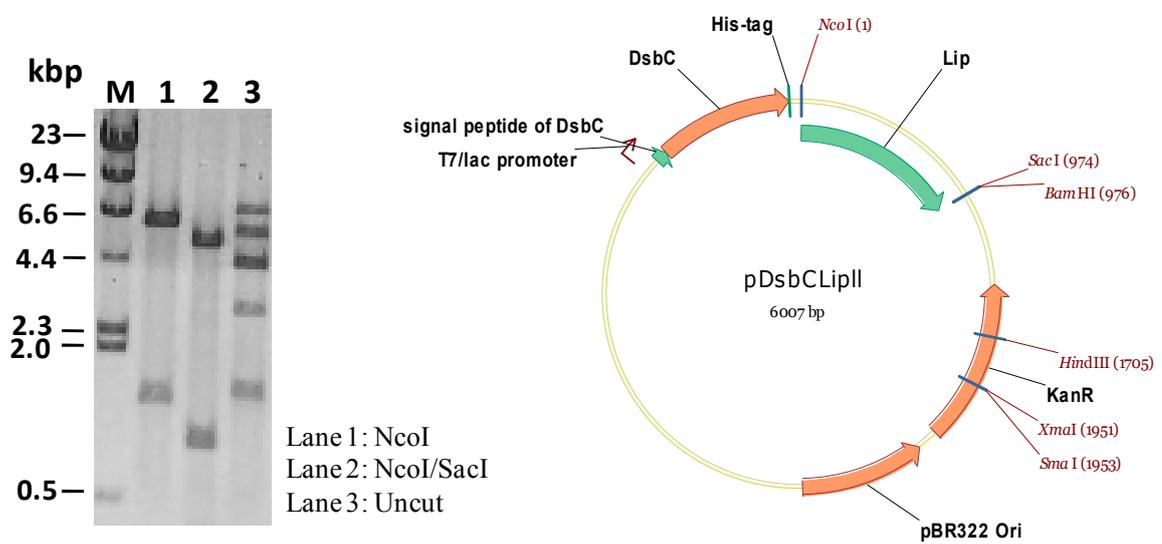


Figure B9 Restriction analysis of pDsbCLipII

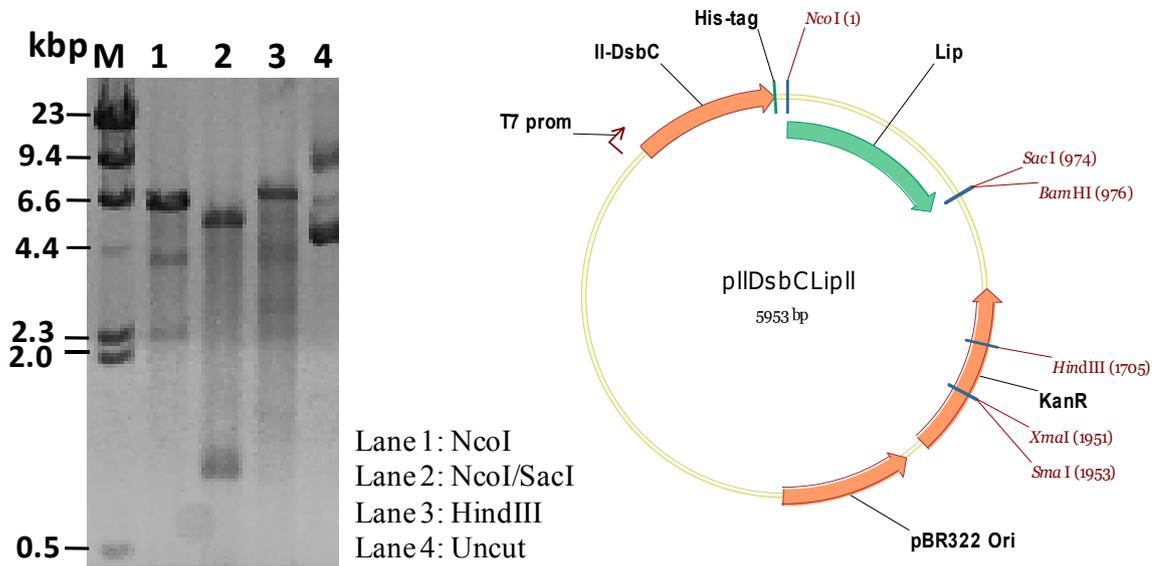


Figure B10 Restriction analysis of pIIDsbCLipII

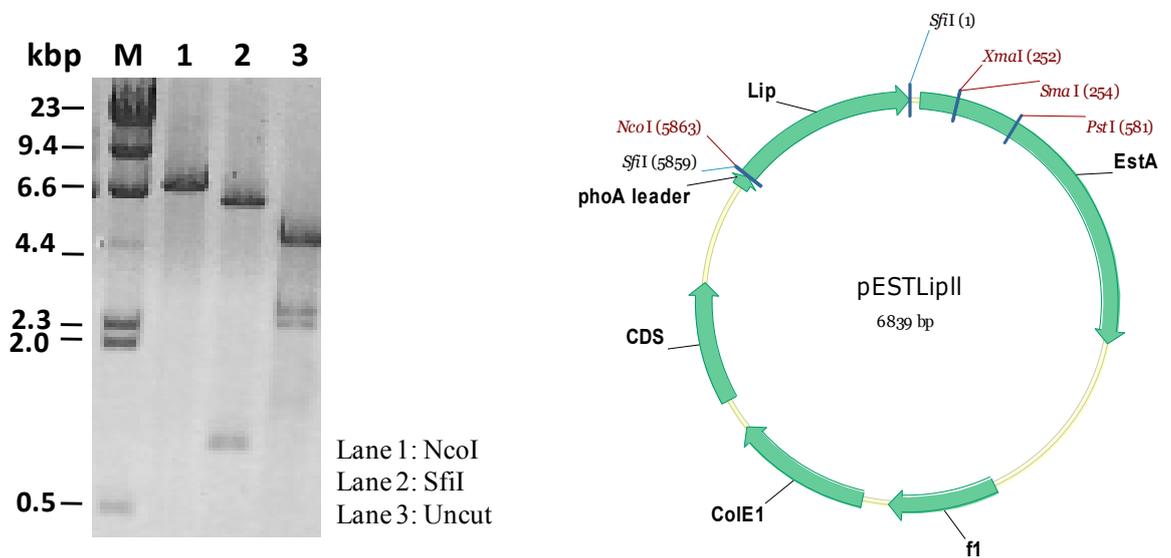
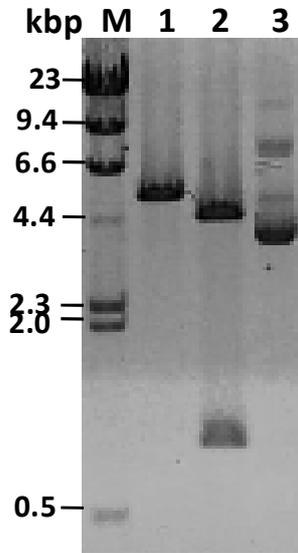
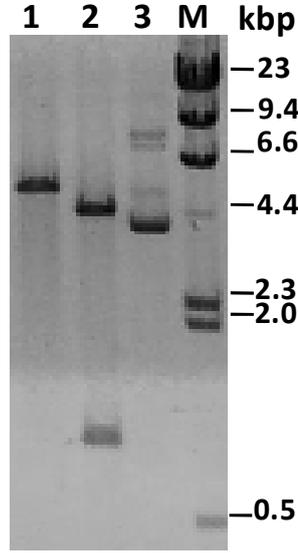


Figure B11 Restriction analysis of pESTLipII



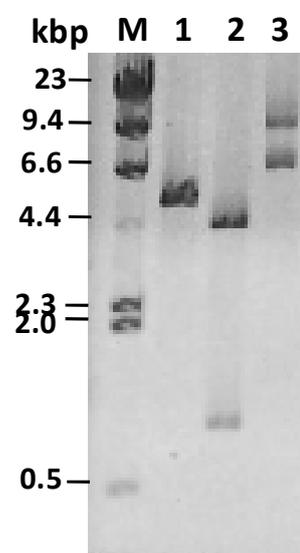
pEHLYPalB
 Lane 1: NcoI
 Lane 2: NcoI/EagI
 Lane 3: Uncut

A



pEHLYPalB
 Lane 1: NcoI
 Lane 2: NcoI/SfiI
 Lane 3: Uncut

B



p5'3'UTRCALB
 Lane 1: PvuI
 Lane 2: NcoI/SacI
 Lane 3: Uncut

C

Figure B12 Restriction analysis of (A) pEHLYPalB, (B) pEHLYPalB, (C) p5'3'UTRPalB. The DNA sequences of these plasmids were not available for sonstruction of plasmid map on Vector NTI.

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