A STUDY ON INDUCTION OF MINICELL FORMATION BY FTSZ
OVERPRODUCING L-FORM <u>E. COLI</u>

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ABSTRACT

A STUDY ON INDUCTION OF MINICELL FORMATION BY FTSZ OVERPRODUCING L-FORM <u>E. COLI</u>

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L-form organisms (or L-phase, L-variants) are cell wall-deficient (CWD) bacteria consisting of protoplasts and spheroplasts that have the ability to grow and divide. The mutant strain LW1655F$^+$ (also known as L-form *E. coli*) was originally derived from the parental *E. coli* K-12 parental strain and is known to be deficient in cell wall, periplasmic space, flagella, fimbriae and outer membrane. Minicells are small cell-like structures that do not have genomic DNA and are produced during the logarithmic phase of growth during which division event does not occur. Minicell production in *E. coli* K-12 parental strain can be induced by introducing a mutation at the *minB* locus of the bacterial genome. Moreover, previous studies have shown that overproducing FtsZ to two to seven folds led to minicell production in rod-shaped *E. coli* K-12. The present study originally intended to investigate whether spherical; cell-wall deficient L-form *E. coli* is also capable of producing minicells upon overproduction of FtsZ. To achieve this, two types of model organisms were used: a) L-form *E. coli*, b) L-form *E. coli* overproducing 2.25-fold more FtsZ as constructed in the present study. Electron microscopy analyses of the organism demonstrated that L-form *E. coli* cells spontaneously produced vesicle-like structures. Such spontaneous vesicle production was previously reported for other L-form bacteria such as *Listeria monocytogenes*, yet the current study is the first to demonstrate this

event in L-form *E. coli*. Furthermore, flow cytometric analyses revealed that overproduction of FtsZ in L-form *E. coli* significantly increased the number of vesicles released into the growth medium when compared to L-form *E. coli*. Although minicell formation by both L-form E. coli and its FtsZ overproducing derivative could not be verified and remained ambiguous, the present study demonstrated two new findings: (i) L-form *E. coli* spontaneously produces vesicle-like structures without any modification, and (ii) FtsZ overproduction in such cells leads to a significantly increased vesicle secretion.

Keywords: L-form *E. coli*, FtsZ, minicell, vesicle formation, flow cytometry

ÖZ

FTSZ'NİN FAZLA ÜRETİMİ YOLUYLA L-FORM E. COLI'DE MİNİ HÜCRE OLUŞUMUNUN İNDÜKSİYONU ÜZERİNE BİR ÇALIŞMA

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Büyüme ve bölünme özelliğine sahip olan, ancak hücre duvarı olmadığı için protoplast ve sferoplast halde bulunan bakteriler için L-form, L-faz veya L-varyantları gibi terimler kullanılmaktadır. Mutasyona uğramış LW1655F$^+$ suşu *E. coli* K-12 ana suşundan türetilmiş olup hücre duvarından, periplazmik boşluktan, kamçı ve fimbriya yapılarından ve dış zardan yoksundur ve L-form *E. coli* olarak adlandırılmıştır. Mini hücreler küçük, hücre benzeri yapılardır ve hücre büyümesinin logaritmik aşamasında üretilirler. Ancak genomik DNA'ları olmadığından bölünemezler. *E. coli* K-12 ana suşundan mini hücre üretimi, bu suşun genomic DNA'sının *minB* lokusundaki bir mutasyonla indüklenebilir. Buna ilaveten, FtsZ proteininin çubuk şekilli *E. coli*'de iki-yedi misli fazla üretilmesi ile de mini hücre üretiminin sağlanabildiği daha önceki çalışmalarda gösterilmiştir. Bu çalışmanın ana amacı, FtsZ proteininin fazla üretiminin yuvarlak yapıda ve hücre duvarından yoksun olan L-form *E. coli*'de mini hücre üretimine neden olup olmayacağını incelemektir. Model organizmanın laboratuvar koşullarında sorunsuz büyütülmesi ve FtsZ proteininin bu model organizmada ifade edilip normalden 2.25 kat fazla üretilmesi gibi sorunların aşılmasının ardından yapılan elektron mikroskopik analizlerde hiçbir modifikasyon yapılmayan L-form *E. coli* hücrelerinde vesikül benzeri yapıların üretildiği saptanmıştır. Literatürde bu bakteri suşunun vesikül üretimi yaptığına dair bir bilgi bulunmamasına karşılık, *Listeria monocytogenes* türünün L-formlarında vesikül üretimi olduğu rapor edilerek bu vesikül yapılarının birer üreme birimi

oldukları öne sürülmüştür. Aynı oluşumların L-form *E. coli*'de de üretildikleri ilk kez bu çalışmada gösterilmiştir. Akış sitometri çalışmaları, L-form *E. coli*'de FtsZ'nin fazla üretiminin L-form *E. coli*'ye kıyasla ortama daha dazla sayıda vesikül salgılanmasına neden olduğunu göstermiştir. Gerek L-form *E. coli*, gerekse de onun FtsZ proteinini fazla üreten türevi tarafından mini hücre üretilip üretilmediği belirsiz kalmışsa da, bu araştırma iki yeni bulguyu ortaya koymuştur: (i) L-form *E. coli*, hiç bir değişikliğe uğratılmaksızın spontane biçimde vesikül-benzeri yapılar üretmektedir, ve (ii) bu organizmanın FtsZ proteinini fazla üreten türevinde vesikül sekresyonu önemli ölçüde artmıştır.

Anahtar kelimeler: L-form *E. coli*, FtsZ, mini hücre, vesikül sekresyonu, akış sitometrisi

To My Family

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bp(s)   : Base pair(s)

E. coli   : *Escherichia coli*

IPTG   : Isopropyl-b D-thiogalactopyranoside

kDa   : Kilodalton

LPS   : Lipopolysaccharide

NCBI   : National Center for Biotechnology Information

OD   : Optical Density

SDS-PAGE   : Sodium Dodecyl Sulphate-Polyacrylamide Gel
                         Electrophoresis

dH$_2$O   : Distilled water

dNTP   : Deoxynucleotide Triphosphate

ORF   : Open Reading Frame

Rpm   : Revolution per minute

Kb   : Kilo base

RT   : Room temperature

CHAPTER 1

INTRODUCTION

1.1 Model organism L-form *Escherichia coli*

L-form bacteria were first observed by Emmy Klieneberger in 1935 and "L" was used in honor of Lister Institute. The terms such as L-phase, L-variants, L-organisms and cell wall deficient (CWD)-forms in the literature can be used for L-form bacteria. The nomenclature "L-form" is specifically used for protoplast and spheroplast that have the ability to grow and divide. There are four types of L-form to include stable and unstable spheroplasts as well as stable and unstable protoplasts. Stable and unstable spheroplasts may still have some cell wall fractures, however stable and unstable protoplasts do not have any fracture of cell wall which are called cell wall-less (Allan *et al*., 2009). L-form *E. coli* represents a stable protoplast which is constructed from *E. coli* K-12 strain (Figure 1.1) and its capacity of cell wall formation has been irreversibly lost (Siddiqui*et al*., 2006). Wild type *E. coli* is a rod shaped gram-negative bacterium while L-form *E .coli* has a spherical shape and gram staining is not appropriate for this organism due to the absence of cell wall structure (Nanninga, 1998; Allan *et al*., 2009).

Stable protoplast L-form *E. coli* cells are cultivated more than 30 years and changed characteristics have been preserved during this time. Shifted cell morphology and colony morphology, loss of pili and flagella, essence of complex media, loss of cell wall, biophysical and biochemical changes in the cell membrane are the main differences between parent strain of *E. coli* and L-form *E. coli* (Gumpert and Hoischen, 1998). Induction, selection, stabilization and adaptation are the four main steps that have to be applied to isolate stable L-form cells after selective cultivation. Induction step is conducted by growing the parental strain on selective media which have essential agents that interact with cell wall synthesis such as antibiotics, lytic enzymes or some amino acids. Selection step includes replacement of a single colony

onto two fresh media, one being supplemented with cell wall inhibitors and the other is not supplemented. Stabilization of selected L-form colonies from the cell wall inhibitors deficient medium is followed by adaptation of L-form colonies by using different growth parameters in order to increase stability (Allan *et al*., 2009). Although early experiments performed with X-rays and some mutagenic substances had failed to create stable L form cells, one exception carried out with a mutagenic substance called N-methyl- N'-nitrosoguanidine led to the production of stable L form *E. coli* (Onoda *et al.,* 1987).

Strain designation for L-form *E. coli* derived from *E. coli* K-12 parental strain is LW1655F$^+$. This mutant strain is known to be deficient in cell wall, periplasmic space, flagella and fimbriae, the outer membrane as shown by electron microscope experiments and absence of them leads to some advantages and disadvantages for L-form cells. LW1655F$^+$ cells have resistance to cell-wall reactive antibiotics; however they are very sensitive to osmotic pressure (Siddiqui*et al*., 2006). Therefore, the use of osmotic protectors such as sucrose and/or salt is necessary to grow L-form cells. Moreover L-form *E. coli* has to be grown in complex media like BHIB (Brain Heart Infusion broth), and/or TSB (Tryptic Soy Broth) supplemented with yeast extract (Allan *et al*., 2009).
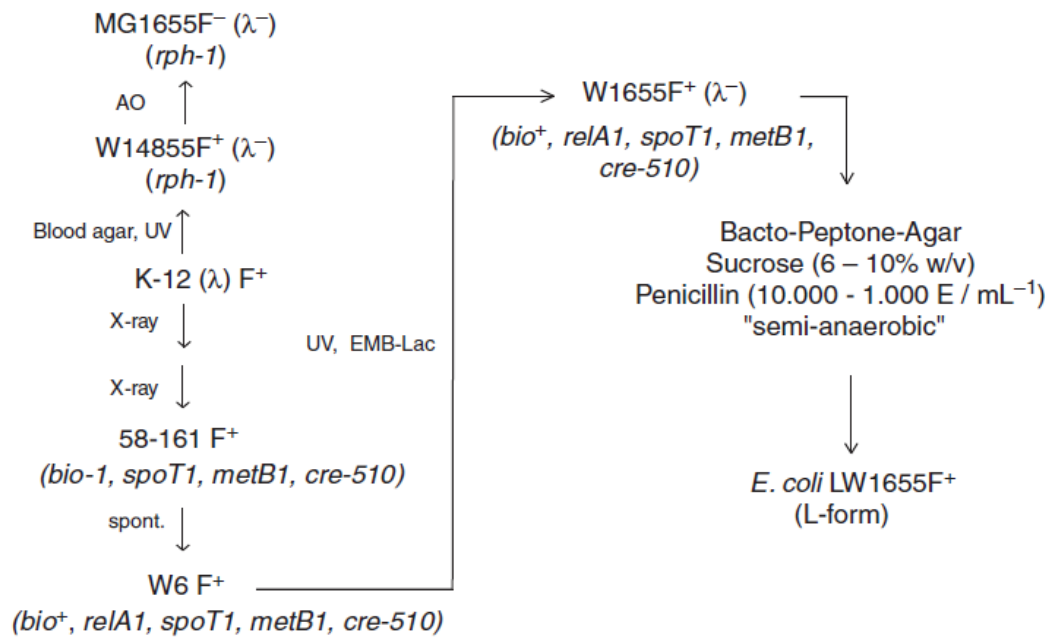
Figure 1.1 Isolation of protoplast type L-form LW1655F+ from *Escherichia coli* K12 strain. Acridine orange is used for curing the F (fertility)-plasmid, Spont. stands for spontaneous mutation, UV stands for ultraviolet treatment, EMB-Lac stands for Eosin Methylene Blue-lactose selection (Siddiquiet al., 2006).

Conversion of *E. coli* K12 strain to protoplast type L-form LW1655F$^+$ includes X-ray treatment which leads to spontaneous mutations in the genome. Sequencing studies demonstrate that some cell division and cell wall synthesis genes are mutated during conversion of *E. coli* K12 strain to protoplast type L-form LW1655F$^+$. Cell wall synthesis genes that are mutated during this process are *mraY, murG,* and mutated cell division genes during the process are *ftsW, ftsQ, ftsA* and *ftsZ*. It is clearly known that *ftsQ* becomes non-functional because of a nonsense mutation which leads to an early stop codon. On the other hand, *ftsZ* function is not affected because sequencing studies show that *ftsZ* gene has a silent mutation. However, some gene functions are still not clear. For example, *mraY* has a premature stop codon and its last 62 amino acids are ceased, so that it is still not certain whether remaining part of *mraY* is functional or not (Siddiqui*et al.*, 2006).

L-form cells can be used as important tools which give us a better understanding about structural and functional re-arrangement of bacterial cells. For biotechnological purposes, they provide unique expression systems. Moreover, interaction of L-form organism with animals as pathogenic, symbiotic or commensal agents can be used for disease therapy (Allan *et al*., 2009).

1.2 Minicells

Minicells are small cell-like structures that do not have genomic DNA and they are produced during the logarithmic phase of growth (Figure 1.2). Division event does not occur for minicells and they can be isolated via density gradient centrifugation from rod-shaped *E .coli* culture (Adler *et al.,* 1967).

Figure 1.2 Thin section electron microscopic image of minicells and minicell producing *E. coli* K12 P678-54 strain X 66,000 (Adler *et al.,* 1967).

Minicells were named as "anucleate" cells and atypical cell division can be considered as the cause of minicell production. Electron microscopy and phase – contrast microscopy studies demonstrated that the area which has normally not had nuclear structure is used to form minicells. Moreover it has thought that minicells contain little or no DNA (Shull *et al.,* 1971). Early studies showed that single stranded DNA can be inserted into the minicells via conjugation and complementary strand can be produced *in vivo*. Moreover early studies were failed to show *in vivo* protein synthesis in conjugated or non-conjugated minicells (Fralick *et al.,* 1969).

Minicell phenotype is the result of mutation in the gene called *minB*. Studies demonstrate that septation of dividing *minB* mutant has equal probability where septum would be formed either at a cell pole or internal localization. Internal localization of septum leads to normal division, however cell pole localization of septum leads to minicell production (Boer *et al.,* 1989). It was first suggested that *minA* and *minB* genes had to be mutated for the production of minicell. Chromosomal regions that include these genes are *lac-purE* and *pyrC-trp* regions for *minA* and *minB,* respectively (Sydnor and Rothfield, 1984). Necessity of *minB* mutation for the production of minicell phenotype was demonstrated, yet whether *minA* mutation is essential for the minicell production (Schaumberg and Kuempel, 1983) represents a question. It has been shown that mutation at *minA* is not required for production of minicell, whereas *minB* alone can produce minicell phenotype with studies conducted via Hfr- and P1-mediated genetic transfer experiments (Sydnor and Rothfield, 1984). For a normal replacement of division septum, *minC, minD* and *minE* genes' coordinated expression is essential which are altogether forming *minB* locus. Localization of division septum is affected by gene products of those three genes separately. Gene products of *minC* and *minD* work together to produce cell division inhibitor which blocks septum formation at all potential division sites when it is necessary. Gene product of *minE* establishes topological specificity to the cell division inhibitor so *minE* gene product makes the cell division inhibitors work at the polar division sites. Therefore, three genes products together guarantee that the septum formation occurs only at midcell as the proper division site (Boer *et al.,* 1989).

Membrane phospholipid composition of minicells was studied by Koppelman *et al.* (2001) in order to compare phospholipid composition of *E. coli* minicells and wild type cells. It was found that they are more or less the same. Minicells extract and minicell producing mutant cells were compared and it seemed that cardiolipin (CL) amount has increased from 1.9 mol% to 6.5 mol% and the amount of phosphatidylglycerol (PG) has decreased from 18.4 mol% to 12.8 mol%. It is hypothesized that increased cardiolipin level might be a consequence of activation or stabilization of divisome proteins (Koppelman *et al.,* 2001).
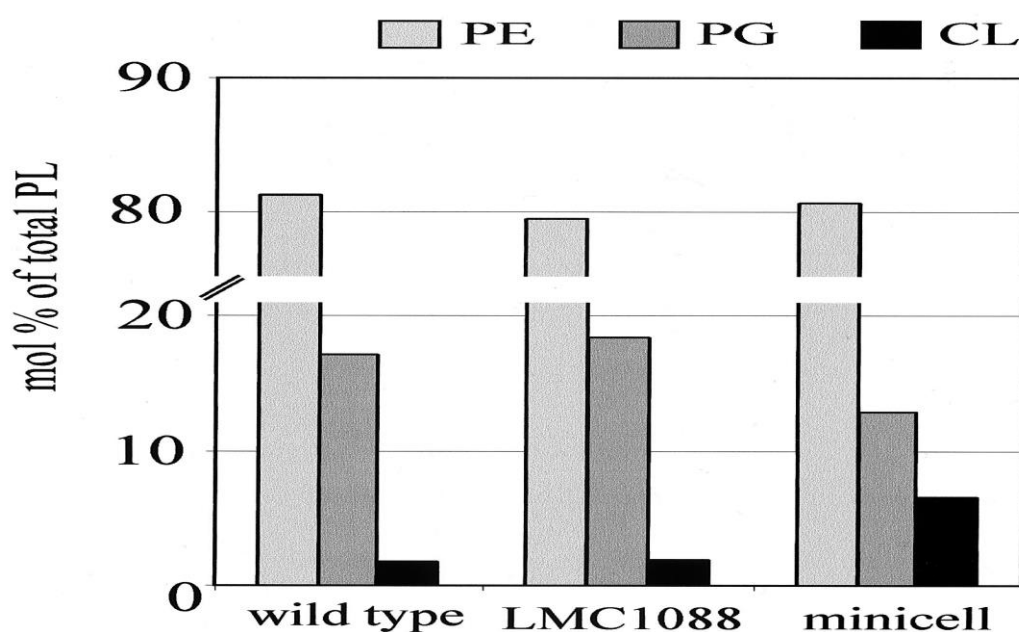
Figure 1.3 Phospholipid (PL) head group comparison between wild type, minicell producing strain LMC1088 and minicell, PE stands for phosphatidylethanolamine, PG stands for phosphatidylglycerol and CL stands for cardiolipin**.** (Koppelman *et al.,* 2001).

1.2.1 Plasmid segregation into minicells

It has been postulated that minicells do not contain any chromosomal DNA; however they can include plasmid DNA which segregate from parent cell to the minicell during abnormal cell division. Moreover plasmids that have resistance factors can be

transferred from minicells to normal cells as efficient as cell to cell transfer. Studies on minicell DNA content have demonstrated that 95% of DNA in minicells is pure R-factor DNA (plasmid) and half of those DNA are in the state of covalent circular molecule. Moreover, more than one plasmid can be found in a minicell (Levy, 1971).

At the polar region of the mother cell, minicells are formed. High efficiency of plasmid segregation might be the consequence of some mechanisms in that plasmid replication site in the cell may affect the efficiency of a plasmid to segregate into minicell. Studies have shown that plasmids can be attached to the minicell membrane although replication is prevented. Plasmids that segregate into the minicells were found to be membrane-bounded; therefore it was proposed that membrane binding is essential for the segregation process (Rashtchian *et al.,* 1986). By using thymidine incorporation method, DNA synthesis in minicells has been quantified, however it was found to be less than actively replicating DNAs' score and only more than repair score. Plasmids that segregate into the minicells convert minicells to a so called active form by synthesizing RNA and protein. All minicells do not have to contain plasmids to form R-minicells and they do not synthesize RNA or protein because they lack RNA polymerase. On the other hand, R+ minicells have RNA polymerase; therefore they can synthesize protein and RNA which was shown in a study using rifampin sensitivity. Rifampin studies demonstrated that origin of polymerase in the minicells is bacterial, however less than 10% of polymerase could be produced by plasmid segregated into the minicell (Levy, 1971). Minicells can be formed from both poles of the cell and plasmids can be segregated from the both poles into the minicells. Different types of plasmids are more likely to segregate into different minicells as expected from membrane association of plasmids in such cells (Rashtchian *et al.,* 1986).

1.2.2 The use of bacterial minicells

Minicells are very suitable for analyses of the biological processes and products of cloned genes (Christen *et al.,* 1983). Minicells can be used as gene delivery vehicles in order to prevent genetic reversion or other safety concerns. All of the molecular

components of the parent cell are included into the minicell except for chromosome. Moreover, a minicell producing parental strain transformed with a desired recombinant plasmid will produce minicells with desired plasmid inside and gene products of that plasmid. Therefore, plasmid DNA packaged with minicells could be segregated eukaryotic cells the efficiency of which can be enhanced with minicell delivery system. This can be exemplified by a study in which the integrin targeting domain of the *Yersinia pseudotuberclosis* invasin protein (Tinv) was produced in minicell as encoded by a recombinant plasmid which was used to target and transfer DNA into cultured eukaryotic cells (Giacalone *et al.,* 2006). For the purpose of observation of newly synthesized macromolecules, plasmid containing minicells can be used. Moreover, minicells can be used for producing viable T4 bacteriophages which constitutes another biological use and importance (Roozen *et al.,* 1971). Minicells are used for new technologies like targeting cancer cells and delivering therapeutic agents with the relevant concentrations of chemotherapeutics. Bispecific antibodies can be used for homing drug-bearing minicells to cancer cells. Moreover, small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) can be packed in minicells and targeted to the cancer cell via bispecific antibodies to suppress important cell cycle associated proteins expression taking part in tumor cell proliferation. Bispecific antibodies targeted minicells can be used for therapies involving intravenous applications of cytotoxic drugs (MacDiarmid *et al.,* 2009).

1.3. *FtsZ* gene

FtsZ gene is a necessary cell division gene that forms a ring structure at division site under the control of cell cycle event. *FtsZ* gene functions early in the division pathways in between other division proteins. Moreover, it is the target of two known division inhibitors SulA and MinCD (Mukherjee *et al.,* 1993). It was shown via immunoelectron microscopy that FtsZ protein is placed at the site where the division starts in a ring structure that is called FtsZ ring (Bi and Lutkenhaus, 1991). *FtsZ* gene codes a hydrophilic 40-kDa protein which is highly conserved among eubacteria (Dai and Lutkenhaus, 1991). Intracellular level of the FtsZ protein can alter the topology, frequency and timing of division (Carballes *et al.,* 1999). Gene fusion experiments

demonstrated that *ftsZ* gene has at least two promoters that one of them is placed in the coding sequence of the *ftsA* gene. Transcription of the *ftsZ* gene can be high enough via both promoters (Sullivan and Donachie, 1984). The other promoter of *ftsZ* gene is located within *ddl* gene and both promoters are essential and required for sufficient level of FtsZ protein in the cell (Dai and Lutkenhaus, 1991). There were some questions about the function of FtsZ ring: (*i*) What is the required cell cycle signal for the FtsZ ring formation? and (*ii)* how is the FtsZ ring localized, assembled and disassembled? It was suggested that FtsZ is a cytoskeletal element which can self-assemble into the ring structure during division process and it can disassemble after the completion of division (Bi and Lutkenhaus, 1991). Localization dynamics of FtsZ resembles tubulin and actin in eukaryotic cells which use a nucleoside triphosphate as a cofactor. Tubulin contains a 7-amino acid segment (GGGTGSG) which is homologous to FtsZ that contains (GGGTGTG) segment and it is suggested that this is the segment for the interaction of tubulin with guanine nucleotides. When the FtsZ was isolated and purified, it was found that FtsZ is a GTP/GDP binding protein which shows GTPase activity (Mukherjee *et al.,* 1993). A block in cell division can be achieved by preventing DNA replication in *E. coli*. SulA is an inducible inhibitor which is an element of SOS response which induces DNA damage rapidly and it prevents division via interfering FtsZ. However, MalE-SulA fusion blocks division by binding to FtsZ while it does not inhibit GTPase activity of FtsZ (Lutkenhaus and Addinall, 1997). Assembly and disassembly of FtsZ rings are regulated to happen only once per cell. During the late stage of cytokinesis, FtsZ ring condensation is the result of FtsZ polymers dissociation as well as the force that is applied by the daughter cell walls which are falling apart (Sun and Margolin, 1998). Mutation studies demonstrated that *ftsZ* gene mutation led to lemon morphology resulting from the failure of the start of the septation process, showing that *ftsZ* is an early acting gene. However, *ftsA, ftzQ* and *ftsI* mutations led to a sausage morphology which suggested that septation has already been started; hence they are later acting genes (Lutkenhaus and Addinall, 1997). Localization of FtsZ gives some clue that FtsZ is polymerized at the division site to form Z ring. Polymerization requires GTP and it might be analogous of tubulin polymerization into microtubules. There are some significant differences between FtsZ and tubulin polymerization in

that tubulin lattice is skewed but FtsZ lattice is a flat sheet (Figure 1.4.) (Lutkenhaus and Addinall, 1997). It was shown that division of *B. subtilis* L-form does not require FtsZ protein (Leaver *et al*., 2009)
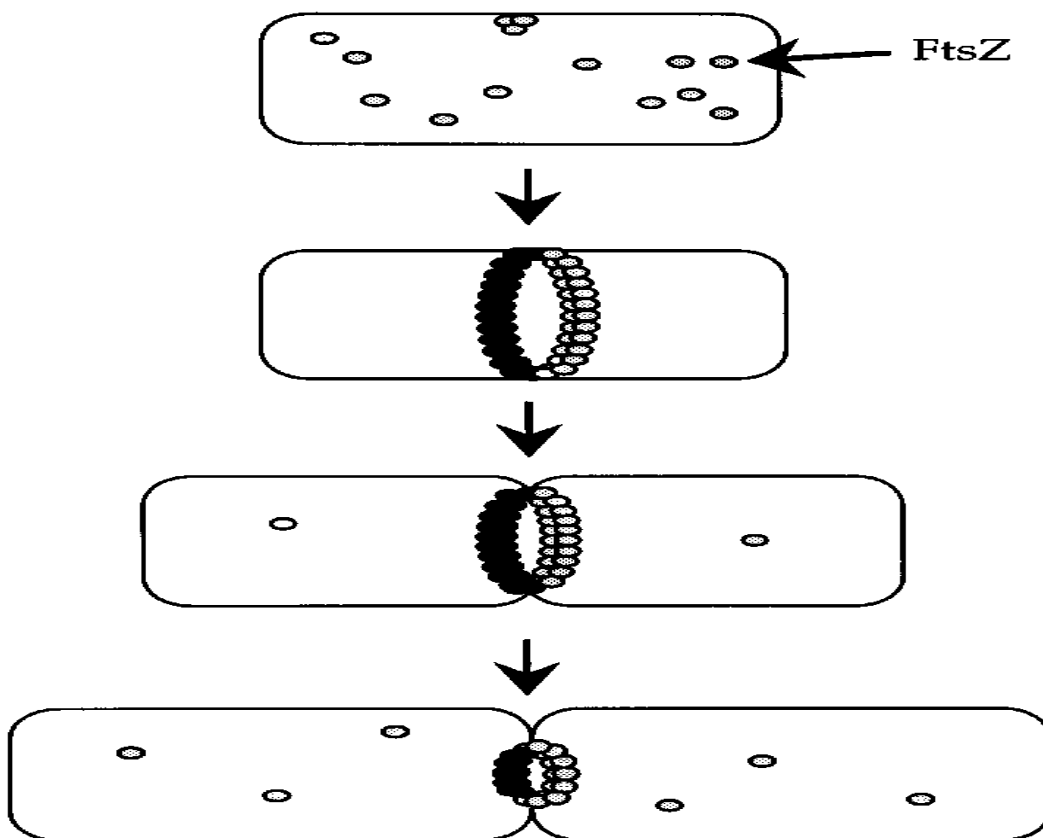
Figure 1.4 FtsZ dynamic behavior during the cell division cycle. It is thought that Z ring formation is started from a single spot which is rapid, happening in less than a minute. Z ring decreases in a diameter at the edge during division. Enough FtsZ is present in *E. coli* to form nearly 20 rings (Lutkenhaus and Addinall, 1997).

1.4 Overexpression of *ftsZ* gene in *E. coli*

The experiments on *ftsZ* gene product dosage revealed sufficient explanation about the function of FtsZ in the bacterial cells. It has been suspected that *ftsZ* gene function, on the basis of its role in the cell division process, can lead to minicell

phenotype when *ftsZ* gene dosage is increased. It would then be logical to suggest that minicell formation is an enhanced process of bacterial cell division (Lutkenhaus and Addinall, 1997). This hypothesis was tested via plasmid expression studies which contained *ftsQ, ftsA* and *ftsZ* genes, separately or together (Ward and Lutkenhaus, 1985). All possible combinations were tested because all genes take part in cell division process. It seemed that multicopy plasmids containing *ftsZ* gene induced minicell phenotype when it was transformed *E. coli* cells that are not normally forming minicell. Multicopy plasmids carrying *ftsA* and *ftsQ* genes but not intact *ftsZ* gene did not lead to minicell phenotype (Ward and Lutkenhaus, 1985). It was also confirmed that cell division process is very sensitive to the dosage of FtsZ. Decreasing the level of FtsZ leads to cell division process to stop and/or to be inhibited (Dai and Lutkenhaus, 1991). Average cell length of classical minicell mutant population is increased because minicell division occurs at the expense of normal division (Teather *et al.,* 1974). Minicells that are produced by overexpression of *ftsZ* gene are not formed at the expense of normal division (Ward and Lutkenhaus, 1985). Therefore, cells demonstrate same frequency of normal division with plasmids that induce minicell phenotype. FtsZ-induced minicell division happens in addition to normal divisions, so high level of FtsZ leads to more division per doubling of cell mass (Figure 1.4.). Moreover, mean cell length of the population slightly changed because of minicell production by multicopy plasmid in *E. coli*. Plasmid that contains cloned *ftsZ* increases total septa 1.7-fold while 10% decrease of the mean cell length occurs. Smaller cells appeared in plasmid containing population when compared to control population. Therefore, overproduction of FtsZ leads to an increase in the frequency of cell division, production of minicell and occurrence of the cell division earlier in the cell cycle (Ward and Lutkenhaus, 1985). Antisense RNA for *ftsZ* gene encoded by a defective phage is used for bacterial chromosome silencing. Under the control of *lac* promoter, expression of antisense RNA could reduce the expression level of FtsZ by 80%. Decreased level of FtsZ led to delay in division (Lutkenhaus and Addinall, 1997). Moreover, high level of expression of *ftsZ* gene seems to inhibit normal cell division. Fivefold increase of FtsZ leads to increasing number of minicell septa; however, higher concentration of FtsZ production leads to inhibition of cell division and minicell formation. Cell division

inhibition occurs when the FtsZ level increases 12-fold or more (Ward and Lutkenhaus, 1985).
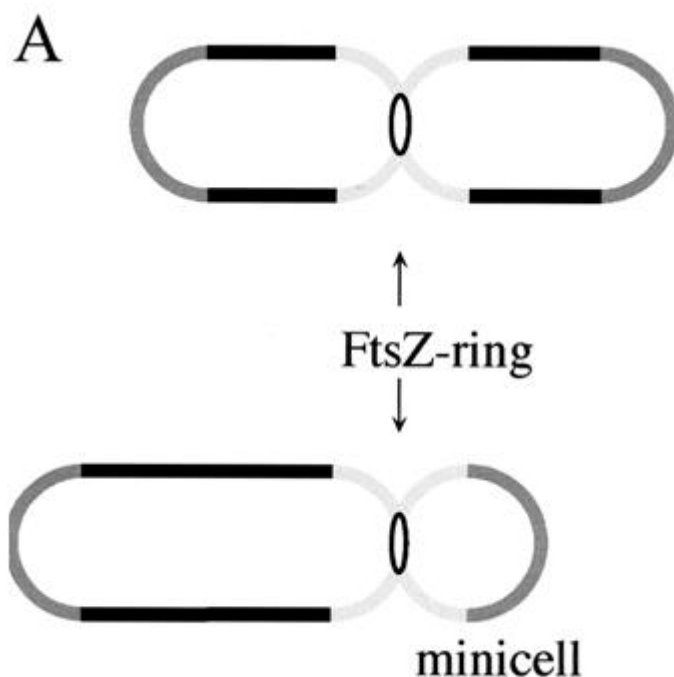
Figure1.5 Schematic representation of normal division vs minicell formation (Koppelman *et al.,* 2001).

1.5. The present study

The aim of this study is to overproduce FtsZ in L-form *E. coli* and to show whether it is possible to control minicell formation by this way in this strain. Former studies demonstrated that overproduction of FtsZ induce minicell formation in *E. coli* K-12 strain (Ward and Lutkenhaus, 1985). A thorough literature search showed that production of minicell in L-form *E. coli* has not yet been investigated. As it was mentioned before, some strains of *E. coli* can produce minicells due to the mutations in *minB* gene (Boer *et al.,* 1989). Moreover, there have been no reported mutations in *minB, minC, minD* and *minE* genes as based on sequencing studies (Siddiqui*et al*., 2006). Therefore, it was hypothesized that overproduction of FtsZ in L-form *E. coli*

can lead to production of minicells. Techniques applicable to minicell detection included flow cytometry and scanning electron microscopy (SEM).

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial strains and plasmids

Bacterial strains used in this study and their sources are listed in Table 2.1. Plasmids that were used in cloning experiments are shown in Table 2.2. Plasmid structures and size markers are given in Appendix A.

Table 2.1 Bacterial strains used in the present study.

Strain	Characteristic	Source and Reference
E. coli LW1655F$^+$	Stable protoplast cell wall-less *E. coli*	Leibniz Institute (Siddiqui *et al.,* 2006)
E. coli DH5α	U169 *sup*E44λ$^-$ *thi*-1 *gyr*A *rec*A1 *rel*A1 *end*A1 *hsd*R17 F' φd*lac*ZΔ(*lac*ZY A-argF)	American Type Culture Collection
E. coli BL21 (DE3)	F$^-$ *ompT gal dcm lon* hsdS$_B$(r$_B^-$ m$_B^-$) λ(DE3 [*lac*I *lac*UV5-T7 gene 1 ind1 sam7 nin5])	Novagen, Merck Germany
E. coli LW1655F$^+$ FtsZ	*E. coli* LW1655F$^+$/ pET28a-*ftsZ*	This study

Table 2.2 Cloning and expression vectors.

Plasmid	Size	Antibitic markers	Source
pGEM®-T Easy	3.0 kb	*amp (*Amp^r), *lacZ*	Promega Inc. (Madison, WI)
pET-28a(+)	5.3 kb	*kan* (Kan^r)	Novagen, Merck Germany
pET28a-*ftsZ*	6.5 kb	*kan* (Kan^r)	This study

2.2 Culture media

Preparation and composition of culture media are given in Appendix B.

2.3. Buffers and solutions

Composition of buffers and solutions used in the study are documented in Appendix C.

2.4. Enzymes and chemicals

Suppliers of enzymes and chemicals are given in Appendix D.

2.5. Growth conditions and maintenance of bacterial strains

E. coli BL21 and *E. coli* DH5α strains were grown in Luria Broth (LB, Appendix B) and maintained on Luria agar (LA, Appendix B). *E. coli* BL21 and *E. coli* DH5α strains were grown for 16 h at 37^{0}C with shaking (200 rpm). For short term storage, LA media were stored at 4^{0}C and subcultured monthly. For long term storage, LB cultures that included *E. coli* BL21 or *E. coli* DH5α cells were grown until mid-log phase and then mixed with 50% glycerol at a 1:1 ratio. Brain Heart Infusion (BHI) Broth (Appendix B) supplemented with yeast extract (Appendix B) was used to grow L-form *E. coli* LW1655F$^+$ cells. BHI Broth with yeast extract supplemented with 10% Horse serum (Appendix B) and Bacto Agar (Appendix B) was used as the solid agar medium (BHIA) for L-form *E. coli* LW1655F$^+$ cells which were grown for 48 h

to 96 h at 37^0C (200 rpm). For short term storage, BHIA cultures were stored at 4^0C and subcultured monthly. For long term storage, cells were harvested from BHIA media and mixed with skim milk prior to freeze-drying (lyophilization). Lyophilizated cells were stored at -80^0C until use. Whenever necessary, the media were supplemented with appropriate antibiotics including ampicillin; 100 µg/mL and kanamycin; 30 µg/mL. Growth curves were constructed after seed culture inoculation into fresh media and absorbance was measured at OD_{600} at 30 min-1 h intervals.

2.6 Primer Design

Primers of *ftsZ* gene were designed according to the *E. coli* K-12 substr. MG1655 complete genome sequence with EcoGene accession number EG10347. Templates of PCR primers are based on L-form *E. coli* LW1655F^{+} genome (Table2.3). The ftsZF primer and the ftsZR primers were designed to include the *Bgl*II and *Bam*HI restriction enzyme cut sites, respectively.

Table 2.3 Primers of PCR reaction. Underlined sequences are restriction enzyme cut sites

Gene name	Primer Name	Primer Sequence	Size of PCR product
ftsZ	ftsZF	CC<u>AGATCT</u>ATGTTTGAACCAATGGAACTTACC	1152 bp
ftsZ	ftsZR	CC<u>GGATCC</u>TTAATCAGCTTGCTTACGCAGGAA	1152 bp

2.7. Polymerase chain reaction (PCR)

PCR mixture was prepared to include the components with the following final concentrations: 0.2 mM dNTP mix (Thermo Scientific), 0.5 mM of each primer, 1X PCR buffer (Thermo Scientific), 2 units of Phire Hot Start II DNA Polymerase (Thermo Scientific) and template DNA (10 ng pure) or a single colony from BHIA medium. dH_2O was added to the PCR mixture to complete the final volume to 50μL. Table 2.4 demonstrates PCR conditions that were used to amplify the *ftsZ* gene. 1% agarose gel was used for running PCR amplicons after PCR reaction and the desired products were cut from the gel and extracted using the Qiagen Gel Extraction Kit.

Table 2.4 PCR conditions to amplify *ftsZ* gene

Product	Primers used	PCR conditions (35 cycle)
ftsZ	ftsZF and ftsZR	Initial denaturation: 1 min at 98 ^{o}C Denaturation: 20 s at 98 ^{o}C Annealing: 15 s at 55 ^{o}C Extension: 20 s at 72 ^{o}C Final extention: 1 min at 72 ^{o}C

2.8. Agarose gel electrophoresis

A horizontal submarine electrophoresis apparatus was used for agarose gel electrophoresis. TAE buffer (Appendix C) was used to prepare 1% agarose gel and the gel was run at 100 Volts for 40-50 min. Ethidium bromide solution (0.5 μg/mL in TAE buffer) was used to stain the gels. Shortwave UV transilluminator (UVP, Canada) was used to visualize the DNA bands in gels. Molecular weights of DNA bands were determined using the *Pst*I digested Lambda DNA marker (Fermentas, Appendix A).

2.9 Adding "A" overhangs to the blunt PCR product

Phire Hot Start II DNA Polymerase's (Thermo Scientific) blunt end PCR products were 3'-adenylated using *Taq* polymerase. 1X PCR buffer (Fermentas), 2.5mM MgCl$_2$, 0.2 mM dATP, 5 units of *Taq* polymerase and Phire Hot Start II DNA Polymerase's (Thermo Scientific) blunt end PCR products were mixed to a final volume of 50 µL. The mixture was then incubated at 72 $^{\circ}$C for 30 min.

2.10. Sequencing reactions

Sequencing reactions were performed by RefGen Biotechnology Inc. (Ankara, Turkey) using the chain termination method and the BigDye Cycle Sequencing Kit V3.1 (Applied Biosystems) in ABI 3130x1 Genetic Analyzer (Applied Biosystems). Sequencing data were analyzed via National Center for Biotechnology Information (NCBI) database by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST).

2.11 Ligation reactions

PCR products were ligated to pGEM-T Easy vector (Promega) according to the supplier's recommendation. pET28a(+) vector and insert DNA was ligated by mixing 5 Weiss units of T4 DNA ligase (Fermentas), 1 µL of 10x ligase buffer (Fermentas), and 1:3 molar ratio of vector to DNA insert up to 10 µL. The mixture was then incubated at 4°C for 16h.

2.12 Transformation of *E. coli* cells

 E. coli competent cells were prepared and stored at -80^{0}C according to the protocol described by Hanahan (1985). 100 µL aliquots of *E. coli* competent cells were thawed on ice for 5 min. The ligation product was then mixed with the competent cells and incubated for 30 min on ice. The mixture was incubated at 42°C for 60 s (heat shock) and then on ice for 5 min. Following adjustment of the final volume to 1 ml by addition of LB medium, cells were incubated at 37°C for 80 min. Cells were then centrifuged for 10 min at 3000 rpm and resuspended in 100 µL of LB. Resuspended cells were spread on selective medium that contained the appropriate antibiotic and incubated at 37°C for 16 h. LA medium containing 80 mg/mL X-gal, 0.5 mM IPTG and 100 µg/mL ampicillin were used for blue-white colony selection.

2.13. Transformation of L-form *E. coli* cells

L-form cells were grown for 48 h in BHIB medium supplemented with 5 g/L yeast extract. 0.1 mL of an L-form culture was mixed with 0.15 mL of PEG solution (MW 6000, 30% [w/v] in 0.4 M sucrose) and 20 µL of plasmid DNA. The mixture was then incubated on ice for 10 min followed by incubation at 37°C by shaking at 200 rpm for 5 min. Final volume was completed to 1 mL with BHIB supplemented with 5 g/L yeast extract and 1 g/L sucrose and then incubated at 37°C by shaking at 200 rpm for 2 h. 0.1 mL of this mixture was spread on BHIA supplemented with 10% Horse serum and appropriate antibiotics and incubated at 37°C for 2-4 days.

2.14. Plasmid isolation

For the isolation of *E. coli* and L-form *E. coli* plasmids, QIAprep Spin Miniprep Kit (Qiagen) and GeneJET Plasmid Miniprep Kit (Fermentas) were used according to the manufacturer's recommendations.

2.15. Restriction enzyme digestion

Restriction enzyme digestions were carried out in a suitable buffer system using 1 Unit of restriction enzyme for 1 µg of DNA. Mixtures were incubated at 37°C for 2-3 h and stored at -20°C until use.

2.16. Recombinant plasmid construction

ftsZ gene was amplified with PCR using chromosomal DNA of L-form *E. coli* as the template and the ftsZF and ftsZR primers. Single 3'-deoxyadenosine was added to the end of amplified blunt end PCR products. PCR products were ligated to pGEMT Easy vector according to manufacturer's recommendations and transformed into *E. coli* DH5α cells. Restriction enzyme analysis and nucleotide sequence analysis were conducted and recombinant plasmids were verified. After verification of recombinant pGEMT Easy vector-*ftsZ*, *ftsZ* gene was cut out from the vector via restriction enzyme digestion of *Bgl*II and *Bam*HI and linear *ftsZ* gene was then cloned into pET28a vector from the *Bam*HI restriction enzyme cut site. All cloning steps were carried out in *E. coli* DH5α cells. Recombinant plasmid was next isolated from *E. coli* DH5α cells and transformed into L-form *E. coli* cells.

2.17. His-tagged protein purification

Recombinant pET28a-*ftsZ* plasmids were inserted into both *E. coli* BL21 and L-form *E. coli* and expression studies were carried out for both strains. *E. coli* BL21 cells that carry recombinant pET28a-*ftsZ* plasmid were grown in LB (Merck) supplemented with 30 µg/mL kanamycin. After the cell density reached an OD_{600} of 0.6, expression was induced by the addition of 1mM isopropyl-β-D-galactopyranoside (IPTG, Sigma) and cultures were incubated for 5 h at $37^{o}C$ 200rpm. Cells were harvested by centrifugation at 6,000 *g* at $4^{o}C$ for 15 min, and then resuspended in 8M urea containing LEW buffer (Appendix C). L-form *E. coli* strain with recombinant pET28a-*ftsZ* plasmids was grown in 10 mL BHIB with 5 g/L yeast extract (Appendix B) for 2-4 days and then inoculated in 100 mL BHIB with 5 g/L yeast extract. When the cell density reached on OD_{600} of 0.6, expression was induced by the addition of 2mM isopropyl-β-D-galactopyranoside (IPTG, Sigma) and cultures were incubated for 48 h at $37^{o}C$ 200rpm. Cells were harvested by centrifugation at 6,000 *g* at $4^{o}C$ for 15 min, and then were resuspended in 8M urea containing LEW buffer (Appendix C). Sonication was carried out using a CP70T Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL) for 6x10 sec at 60% amplitude and centrifugation was carried out to remove the cellular debris at 15,000 *g* for 15 min. FtsZ proteins were purified from the supernatants by using Protino Ni-TED 2000 packed columns (Macherey-Nagel, Germany) according to the manufacturer's recommendations.

2.18. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gels were prepared according to Laemmli (1970). Sample loading buffer (Appendix C) was mixed with the desired amount of protein (10 µg) and the gel was run at 16-18 mA in 1X running buffer (Appendix C) on a Mini-Protean electrophoresis (Bio-Rad) chamber.

2.19. Coomassie blue R-250 staining of polyacrylamide gels

Following electrophoresis, gels were placed into fixation solution (Appendix C) for 60 min. Protein bands were then stained with Coomassie Brilliant Blue R-250 (Neuhoff *et al.*, 1988) (Appendix C) and visualized.

2.20 Western bloting

Six Whatman® papers which were cut the same size with SDS gel were saturated with the Transfer Buffer (TB) (Appendix C) and laid out on a semi-dry blotter (Cleaver Scientific Ltd, Warwickshire, UK) base one by one taking care to avoid bubble formation between Whatman® papers and between other layers during the layering process. Nitrocellulose membrane, having 0.2 µm pore size (Bio-Rad, Hercules, CA) was soaked in TB and placed on top of the Whatman® papers. The SDS-Polyacrylamide gel was placed onto the membrane and then was covered with the remaining TB wetted Whatman® papers (6 sheets) before closing the upper part of the semi-dry blotter (Figure 2.1).
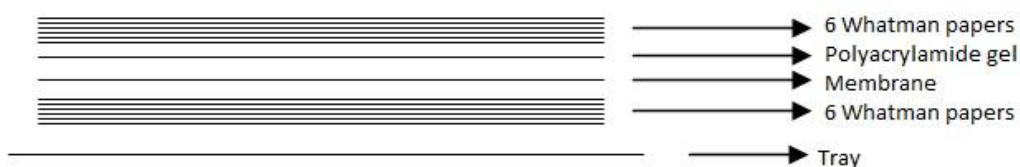
Figure 2.1 Transfer set up for Western blot.

The current that would be applied to the system was based on the membrane size and was kept at 1.5 µA per cm^2 for 1 h. Following the termination of transfer, the membrane was blocked for 2 h at 37°C using skimmed milk (10% w/v in 1xTBS), prepared in. The membrane was then washed using 1x Tween-Tris-Buffered Saline (TTBS) (0.5% Twin in 1X TBS) for 10 min. Following washing, 1/400 diluted primary antibody (Rabbit Anti-FtsZ Lyophilized serum, Agrisera) was added in %5 w/v skimmed milk–1x TBS solution and the membrane was incubated for 1 h at

room temperature (RT). After 1X TTBS washing for 10 min, the membrane was incubated in %5 w/v skimmed milk-1X TBS solution containing the secondary antibody (Goat pAb to Rb IgG (AP), Abcam) at a dilution of 6 μL/100mL at RT for 1 h. Final washing step involved treatment of membrane with 1X TBS. To visualize the bands, AP conjugated substrate (Bio-Rad, Hercules, CA) was added until the bands on the membrane was clearly seen. Equal amounts of proteins were used from all culture lysates.

2.21 Sample preparation for SEM

Morphological analysis of minicells or membrane vesicles harvested from culture supernatants were carried out using Scanning Electron Microscopy (SEM) according to the protocol described by De *et al*. (2008). For this, minicells or membrane vesicles were pelleted at 15,000 g (at $4^{o}C$ for 15 min). Samples were then dried on slides and then coated with 10 nm Au/Pd and visualized under high vacuum using a FEI Quanta 200 FEG scanning electron microscope equipped with ETD detector.

2.22 Sample preparation for flow cytometry

Number of minicells/membrane vesicles secreted by L-form *E. coli* or L-form *E. coli* overproducing FtsZ was assessed from culture supernatants using flow cytometry (Accuri™ C6, BD Biosciences, US). For this, following an initial centrifugation step at 500 g at $4^{o}C$ for 5 min, OD of cultures supernatants were equalized. Samples were then acquired on an Accuri™ C6 flow cytometer (50 μL volume, FSC threshold set at 80,000). Blank medium without any bacteria served as the negative control.

2.23 Statistical analyses of flow cytometry results

Flow cytometry data was collected from two different samples: uninduced (negative control) and IPTG induced *E. coli* LW1655F$^+$ cells. Basic descriptive statistics of these two groups were calculated and independent samples t-test for difference of

their means was conducted using SPSS (SPSS 20 Software, IBM, US). Also bar graphs showing the means of the two groups with standard errors were constructed using the same software.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Handling and growth of L-form *E. coli*

Generation time which is also known as doubling time refers to the rate of exponential growth of a bacterial culture (Harvey, 1972). Therefore, calculation of generation time has to be done during the exponential growth phase of specific bacteria. K-12 strain of *E. coli* from which our model organism L-form *E. coli* has originated has a generation time of about 20.9 min (Wang *et al.,* 2010) which is quite typical of *E. coli* strains grown in complex media. According to our results, L-form *E. coli* had a generation time of about 240 min, a doubling time that is significantly higher than that expected parental *E. coli* K-12 strain (Figure 3.1). It is worth noting that the growth of L-form *E. coli* could only be determined indirectly by measuring the turbidity since viable cell count (direct measurement) was not applicable to this organism that tended to form slime rather than single colonies on agar plates.
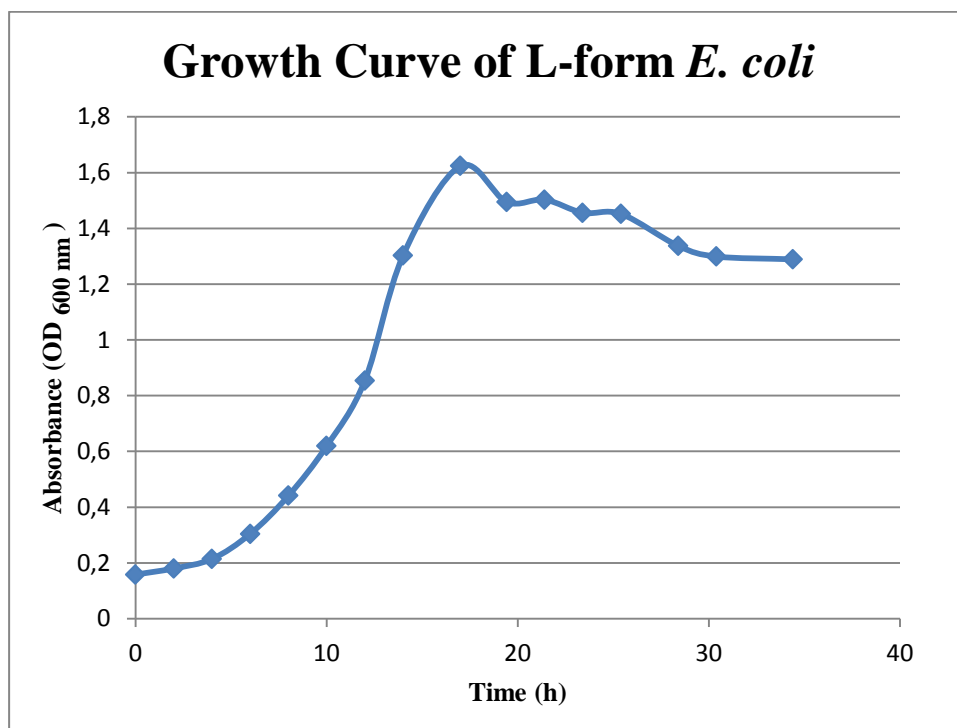
Figure 3.1 Growth curve of L-form *E. coli.*

The delayed growth pattern of L-form *E. coli* makes this organism difficult to work with. It is known that a change in generation time is a one of the several factors that could lead to variations of the rate of adaptation in different lineages (Rosenheim *et al*., 1991). In our case, significantly increased generation time resulted in a decrease in proliferation of bacteria. This might account for poor survival of L-form *E. coli* and had a negative impact on the success of other laboratory techniques applied to this organism. For instance, it was not possible to maintain L-form *E. coli* as a -80^{0}C glycerol stock. The frozen cells could never been activated upon thawing even a day after their freezing. Therefore, our stocks had to be lyophilized to generate viable cells via resuspension. Furthermore, inoculum size had to be significantly increased. For instance, a 50 mL liquid culture of L-form *E. coli* required inoculation of at least 5 mL of seed culture whereas for the parental *E. coli* strain a 50 µL seed culture was enough. L-form *E. coli* could not been grown on solid medium to form separate colonies which constituted another inconvenience. Transfer of L-form *E. coli* grown on agar medium onto a fresh agar medium could only be achieved by scraping and transferring a piece of agar instead of picking a single colony. In order to have a lawn

growth of bacteria on an agar medium, this agar piece transfer scraping procedure had to be repeated at least three to four times, a process which takes at least six to eight days, as practiced also by Allan *et al.*, 2009.

The lack of cell wall in L-form *E. coli* makes it vulnerable to chemical and physical influences of the environment and metabolic end products. There are important issues that have to be taken into consideration when studying with L-form *E. coli* such as prevention of contamination and prevention of cell lysis. In order to avoid contamination, addition of high concentrations of β-lactam antibiotics into the medium is preferred since these agents do not prevent L-form *E. coli* growth and antibiotic concentration may be kept even above 1,000 U mL^{-1}. Detergent contamination of glassware (i.e Triton X-100) can destroy the cytoplasmic membrane of L-form *E. coli* even in trace amounts. Thus, all glassware must be washed adequately prior to inoculation of L-form *E. coli*. Osmolarity is another important factor for growth of L-form *E. coli* as the cells are vulnerable to osmotic pressure. Taken together, L-form *E. coli* growth medium has to be prepared with special care in order to maintain cell integrity (Allan *et al.*, 2009).

3.2. Cloning of *ftsZ* gene

For PCR amplification of *ftsZ* gene from the genomic DNA of L-form *E. coli* LW1655F^{+}, primer design was the first step. For primer design, the genome of *E. coli* K-12 strain was used as a reference obtained from National Center for Biotechnology Information (NCBI) database. However, for PCR amplification, genomic DNA of L-form *E. coli* LW1655F^{+} was used as a template. Amplification of an 1170 bp DNA product of expected size was confirmed by agarose gel electrophoresis (Figure 3.2).
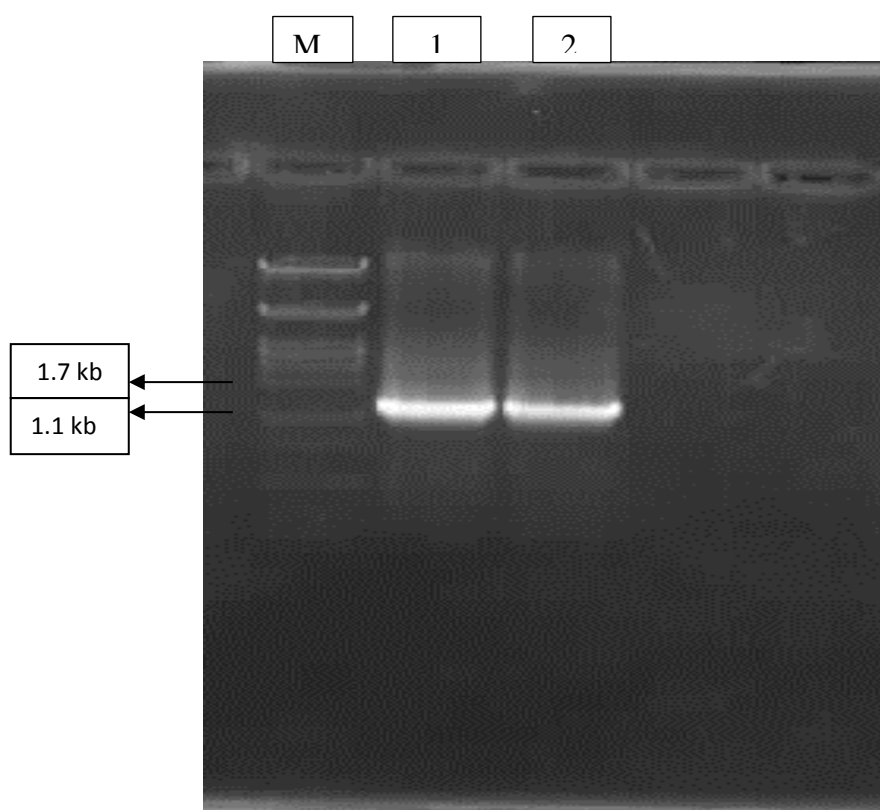
Figure 3.2 PCR amplification of *ftsZ* gene. M stands for Lambda DNA/*PstI* marker. The band shown on lane 1 and lane 2 represents the amplicon corresponding to *ftsZ* gene.

It is known that the *ftsZ* gene of L-form *E. coli* LW1655F^{+} has a silent mutation which does not change the primary structure of FtsZ (Siddiqui *et al*., 2006). Although the structure of encoded proteins is not changed by silent mutations, gene expression can be affected. Global translation efficiency and fitness of cells are affected by codon bias which can be a result of silent mutations (Kudla *et al*., 2009). Therefore, in our study, we chose to clone the *ftsZ* gene of L-form *E. coli* LW1655F^{+} in order not to affect translation efficiency and cellular fitness. The forward primer of *ftsZ* gene includes a start codon and the reverse primer of the *ftsZ* gene includes a stop codon in order to amplify the whole gene by PCR and clone it into an expression vector. It is important that in expression studies the whole sequence of the desired gene has to be included to enable the synthesis of the intact functional protein.

Amplification of the PCR products was accomplished using the Phire Hot Start II DNA Polymerase (Thermo Scientific) which is not suitable for TA cloning since this polymerase does not add dATP to the end of PCR products. Therefore, in order to make TA cloning possible dATP was added manually at the end by using *Taq* polymerase. After PCR amplifications, amplicons were extracted from the agarose gel followed by the addition of dATP at the 3' ends. The inserts prepared as such were then ligated to commercially available pGEM-T Easy vector (Promega) which was used as the cloning vector. *E. coli* DH5α competent cells were used as the host organism to transform the ligation products. To identify the transformed colonies, plasmids were isolated from the colonies and then digested by *Bgl*II and *Bam*HI restriction enzymes (Figure 3.3).
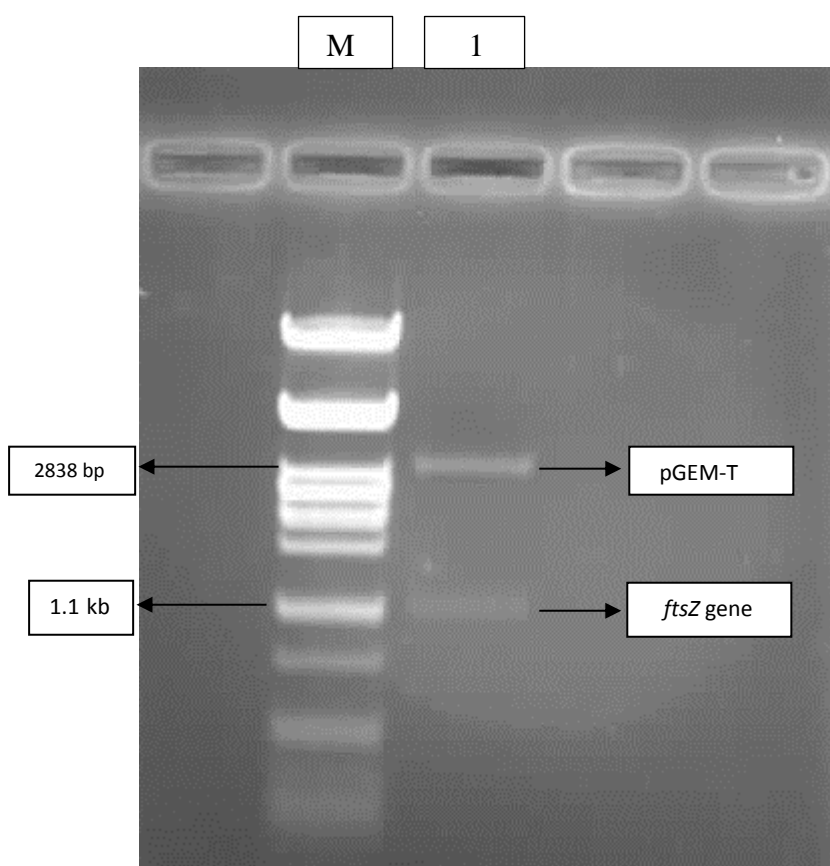
Figure 3.3 Restriction enzyme digestion of pGEMT-*ftsZ* with *Bgl*II and *Bam*HI for verification of cloning. M stands for Lambda DNA/*PstI* marker. Lane 1 stands for double digested pGEMT-*ftsZ* vector.

Double digestion of pGEMT-*ftsZ* vector was expected to yield two bands: a 3015 bp long vector and a 1160-1170 bp long insert. The agarose gel in Figure 3.3 demonstrates the presence of 2 bands (3015 and 1160 bp, respectively) and was consistent with the expected result.

PCR cloning vectors are generally used for facile cloning of PCR products. The concentration of the desired gene can be increased and stable inserts can be produced using such vectors. Restriction enzyme digestion of double stranded PCR products can be inefficient which makes cloning them directly into expression vectors very difficult. Although using PCR cloning vectors may seem like an additional step, in general it is a time-saving process (Green *et al*., 2012).

3.3. Sequence analysis of *ftsZ* gene from *E. coli* LW1655F+

The restriction enzyme digested and verified pGEMT-*ftsZ* vector was then sent for sequence analysis. The result of this sequence analysis was compared with the NCBI database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

In contrast to Siddiqui *et al*.'s (2006) findings, our sequencing data showed the absence of a silent mutation at the reported site. It is conceivable that this may be caused by a reverse mutation at that site (Figure 3.4). When our amino acid sequence of FtsZ was compared to that of *E. coli* K12 strain, it can be seen that they are the same according to NCBI database (Figure 3.5).

CCAGATCTATGTTTGAACCAATGGAACTCACCAATGACGCGGTGATTAAAGTCA
TCGGCGTCGGCGGCGGCGGCGGTAATGCTGTTGAACACATGGTGCGCGAGCGCA
TTGAAGGTGTTGAATTCTTCGCGGTAAATACCGATGCACAAGCGCTGCGTAAAA
CAGCGGTTGGACAGACGATTCAAATCGGTAGCGGTATCACCAAAGGACTGGGCG
CTGGCGCTAATCCAGAAGTTGGCCGCAATGCGGCTGATGAGGATCGCGATGCAT
TGCGTGCGGCGCTGGAAGGTGCAGACATGGTCTTTATTGCTGCGGGTATGGGTG
GTGGTACCGGTACAGGTGCAGCACCAGTCGTCGCTGAAGTGGCAAAAGATTTGG
GTATCCTGACCGTTGCTGTCGTCACTAAGCCTTTCAACTTTGAAGGCAAGAAGCG
TATGGCATTCGCGGAGCAGGGGATCACTGAACTGTCCAAGCATGTGGACTCTCT
GATCACTATCCCGAACGACAAACTGCTGAAAGTTCTGGGCCGCGGTATCTCCCT
GCTGGATGCGTTTGGCGCAGCGAACGATGTACTGAAAGGCGCTGTGCAAGGTAT
CGCTGAACTGATTACTCGTCCGGGTTTGATGAACGTGGACTTTGCAGACGTACGC
ACCGTAATGTCTGAGATGGGCTACGCAATGATGGGTTCTGGCGTGGCGAGCGGT
GAAGACCGTGCGGAAGAAGCTGCTGAAATGGCTATCTCTTCTCCGCTGCTGGAA
GATATCGACCTGTCTGGCGCGCGCGGCGTGCTGGTTAACATCACGGCGGGCTTC
GACCTGCGTCTGGATGAGTTCGAAACGGTAGGTAACACCATCCGTGCATTTGCTT
CCGACAACGCGACTGTGGTTATCGGTACTTCTCTTGACCCGGATATGAATGACG
AGCTGCGCGTAACCGTTGTTGCGACAGGTATCGGCATGGACAAACGTCCTGAAA
TCACTCTGGTGACCAATAAGCAGGTTCAGCAGCCAGTGATGGATCGCTACCAGC
AGCATGGGATGGCTCCGCTGACCCAGGAGCAGAAGCCGGTTGCTAAAGTCGTGA
ATGACAATGCGCCGCAAACTGCGAAAGAGCCGGATTATCTGGATATCCCAGCAT
TCCTGCGTAAGCAAGCTGATTAAGGATCCGG

Figure 3.4 Nucleotide sequence of cloned *ftsZ* gene. Red nucleotides stand for *Bgl*II
cut site, Blue nucleotides stand for *Bam*HI cut site, Green nucleotides stand for start
codon and purple nucleotides stand for stop codon.

MFEPMELTNDAVIKVIGVGGGGGNAVEHMVRERIEGVEFFAVNTDAQALRK
TAVGQTIQIGSGITKGLGAGANPEVGRNAADEDRDALRAALEGADMVFIAA
GMGGGTGTGAAPVVAEVAKDLGILTVAVVTKPFNFEGKKRMAFAEQGITEL
SKHVDSLITIPNDKLLKVLGRGISLLDAFGAANDVLKGAVQGIAELITRPGLM
NVDFADVRTVMSEMGYAMMGSGVASGEDRAEEAAEMAISSPLLEDIDLSG
ARGVLVNITAGFDLRLDEFETVGNTIRAFASDNATVVIGTSLDPDMNDELRV
TVVATGIGMDKRPEITLVTNKQVQQPVMDRYQQHGMAPLTQEQKPVAKVV
NDNAPQTAKEPDYLDIPAFLRKQAD

Figure 3.5 Amino acid sequence of FtsZ protein encoded by the cloned *ftsZ* gene.

3.4. Expression of *ftsZ* gene in *E. coli* BL21 (DE3)

pGEMT-*ftsZ* plasmid was digested with *Bgl*II and *Bam*HI restriction enzymes. This resultant *ftsZ* gene segment had a *Bgl*II sticky end at the 5' end and *Bam*HI sticky end at the 3' end of the gene which was ligated to the *Bam*HI restriction cut site of the pET28a(+). When sticky ends of *Bgl*II and *Bam*HI unite, the product of this union is not recognized by either enzyme, therefore creating a new site that is not digested by these restriction enzymes. The ligation product of pET28a-*ftsZ* was transformed into *E. coli* BL21 (DE3) which contains the phage T7 RNA polymerase and is hence an ideal host for expression of the gene cloned into pET28a(+) (Kothari *et al*., 2006).
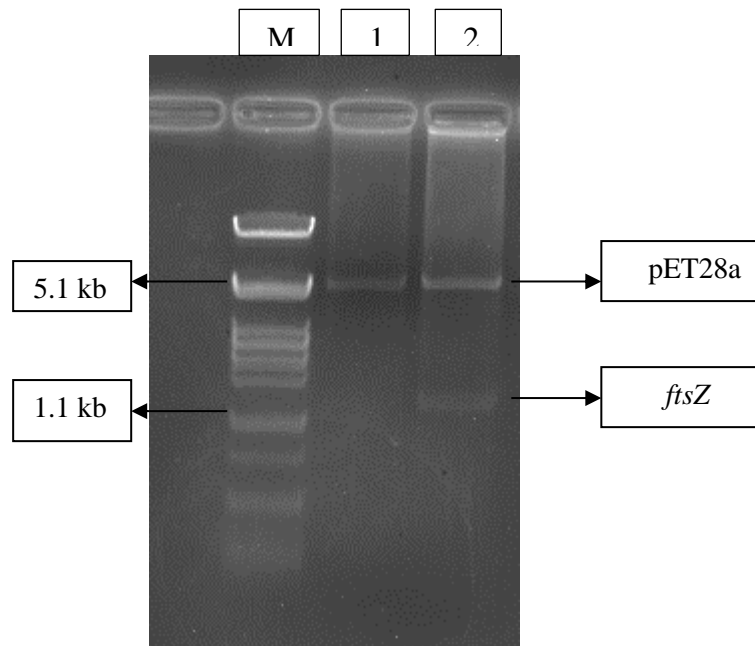
Figure 3.6 Agarose gel electrophoresis of *Bgl*II and *Bam*HI cut pET28a-*ftsZ* isolated from recombinant *E. coli* BL21 (DE3). M stands for Lambda DNA/*PstI* marker, Lane 1 stands for pET28a(+) cut with *Bgl*II and *Bam*HI, and Lane 2 stands for pET28a-*ftsZ* cut with *Bgl*II and *Bam*HI (1 µg DNA/well).

The full pET28a(+) size is about 5369 bp (Appendix A). When the expression vector pET28a(+) was cut with the *Bgl*II and *Bam*HI restriction enzymes, the fragment size decreased to 5166 bp (Figure 3.6). The fragment in between the *Bgl*II and *Bam*HI cut sites was 203 bp. When the pET28a-*ftsZ* was cut with the same restriction enzymes, the presence of the expected bands of 5166 bp and 1370 bp were verified on the agarose gel (Figure 3.6).

Next, to validate that the pET28a-*ftsZ* construct was functional, the *ftsZ* gene was first overexpressed in *E. coli* BL21 (DE3). For overproduction of FtsZ, the plasmid was introduced into *E. coli* BL21 (DE3) cells that were grown in Luria Broth medium supplemented with IPTG to a final concentration of 1 mM (IPTG was added and maintained in the culture for 5 h after an OD_{600} of 0.6 was reached) (Kothari *et al*., 2006). The 5 h exposure to IPTG for overproduction of the desired gene product was optimized in former studies in our laboratory. The IPTG-induced *E. coli* BL21 (DE3) cells were then resuspended in the Lysis-Elution-Wash (LEW) buffer

containing 8M urea and then cells were sonicated, followed by centrifugation in order to eliminate cellular debris. The supernatant containing the overproduced protein was harvested and subjected to SDS-polyacrlamide gel electrophoresis (Figure 3.7) to verify over production of FtsZ.
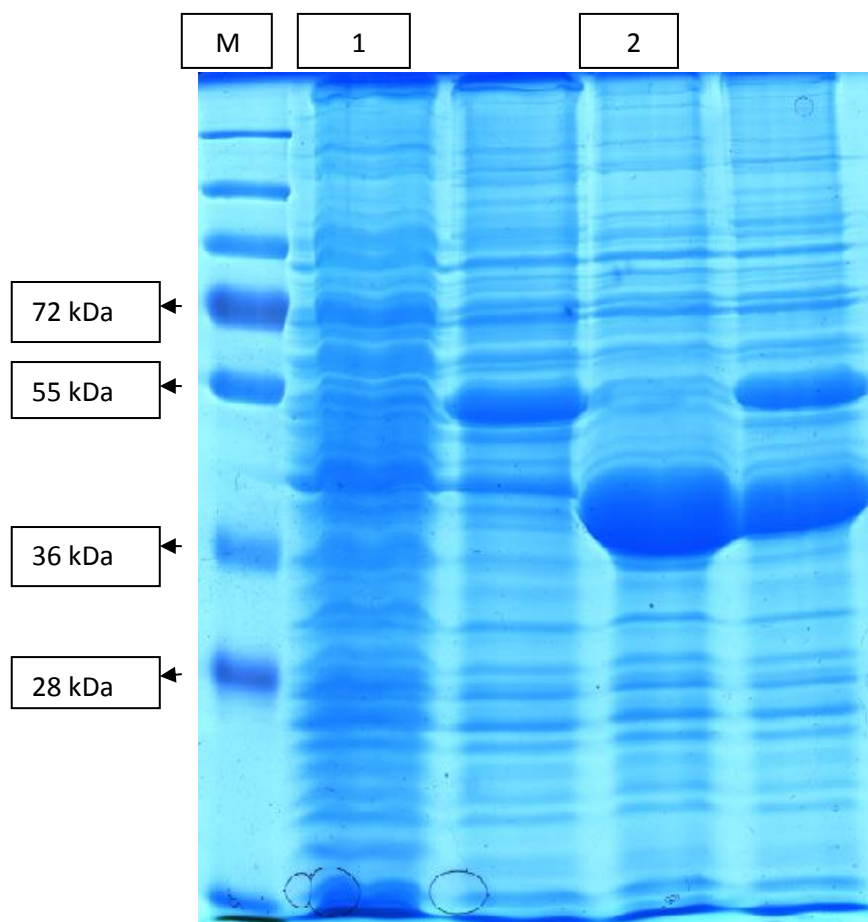
Figure 3.7 Total protein isolation from *E. coli* BL21 (DE3) cells that bear pET28a-*ftsZ*. M: Prestained protein marker, Lane 1 stands for uninduced culture lysate, Lane 2 stands for IPTG induced culture lysate (10 μg protein/well) (other lanes stand for samples from a different study).

It is known that the *ftsZ* gene codes for a 40 kDa hydrophilic protein (Dai and Lutkenhaus, 1991). The molecular weight of overproduced FtsZ protein is expected to be 40 kDa-43.5 kDa. Presence of this expected band was verified in the SDS-PAGE gel (see the dark blue band in lane 2, Figure 3.7) indicating overproduction of

FtsZ protein. Although these results are suggestive of FtsZ overproduction, they are not conclusive requiring further examination as shown in Section 3.5.

3.5. Expression of *ftsZ* gene in *E. coli* LW1655F+

Expression vector pET28a-*ftsZ* was introduced in *E. coli* LW1655F⁺ cells using PEG transformation (Allan *et al*., 2009). Transformant cells were harvested and passaged three or four times to generate sufficient numbers of *E. coli* LW1655F⁺ cells and then were grown in liquid media supplemented with 30 µg/mL kanamycin. Next, transformed cells were analyzed following *Bgl*II and *Bam*HI restriction enzyme digestion (Figure 3.8).
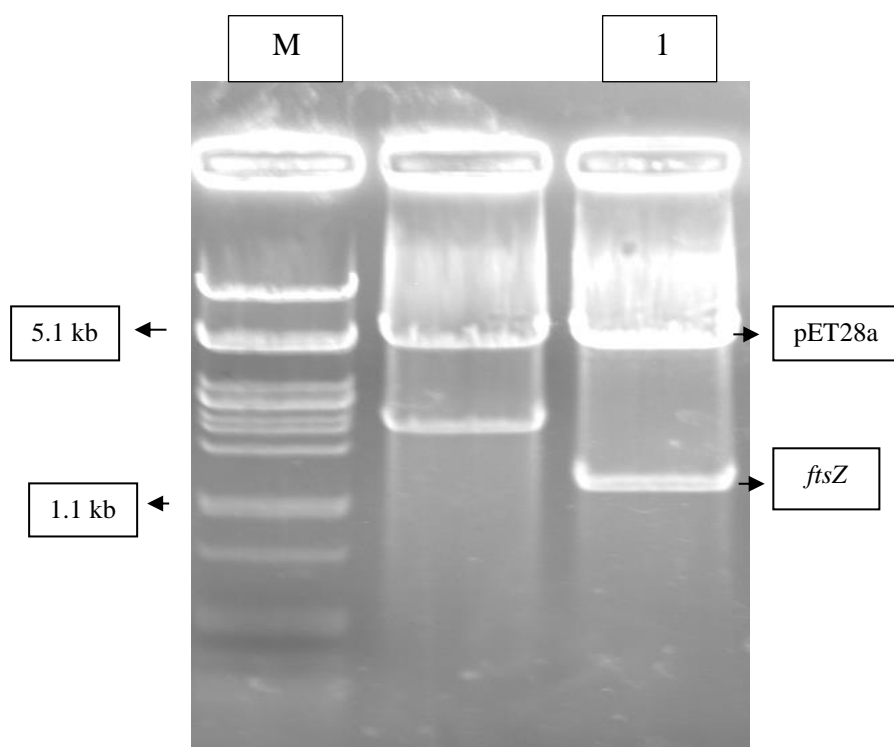
Figure 3.8 Agarose gel photo of *Bgl*II and *Bam*HI digested pET28a-*ftsZ* vector isolated from recombinant *E. coli* LW1655F⁺. M stands for Lambda DNA/*Pst*I marker, Lane 1 stands for pET28a-*ftsZ* cut with *Bgl*II and *Bam*HI (other lane is from a different study) (1 µg DNA/well).

The results showed that the restriction enzyme digestion of pET28a-*ftsZ* isolated from L-form *E. coli* gave fragments identical to those observed with the recombinant *E. coli* BL21 (DE3 (Figure 3.6 and Figure 3.8). Since no modification has occurred

on the construct isolated from recombinant L-form *E. coli* cells as the new hosts, we proceeded to the expression studies.

Overproduction of FtsZ in L-form *E. coli* proved to be another challenging step. As mentioned before, *E. coli* LW1655F$^+$ cells were derived from the *E. coli* K-12 parental strain which can be considered as wild type *E. coli* (Siddiqui*et al*., 2006). Unlike the genetically optimized hosts like *E. coli* BL21 (DE3), overproduction of desired genes in wild type *E. coli* is difficult due to the lack of genetic modifications which are present in *E. coli* BL21 (DE3). The BL21 strain of *E. coli* lacks Lon protease and OmpT outer membrane protease that degrade proteins during isolation and purification steps. Therefore overproduced proteins in *E. coli* BL21 strain are more stable as compared with those from other host strains that contain these proteases. Moreover, the DE3 strain of *E. coli* BL21 has the *lacI^q* repressor that is more advantageous than the wild type repressors since it provides tighter control over basal expression (adopted from pET system manual of Novagen, 1999). On the other hand, it is known that expression vectors that have constitutive promoters of *sak, speA* and inducible promoters like *lac, tac, tetA, T7*and origin of replication like ColE1, pBR322, p15A or pSC101 can be used for over production studies in L-form *E. coli* (Allan *et al*., 2009). Our expression vector pET28a has a pBR322 origin of replication and a *lac* inducible promoter, and therefore our choice of using this vector for the overproduction study in L-form *E. coli* was justified. However, the recombinants had to be compared with each other for the level of overproduction and the colonies best serving this purpose had to be chosen for further studies.
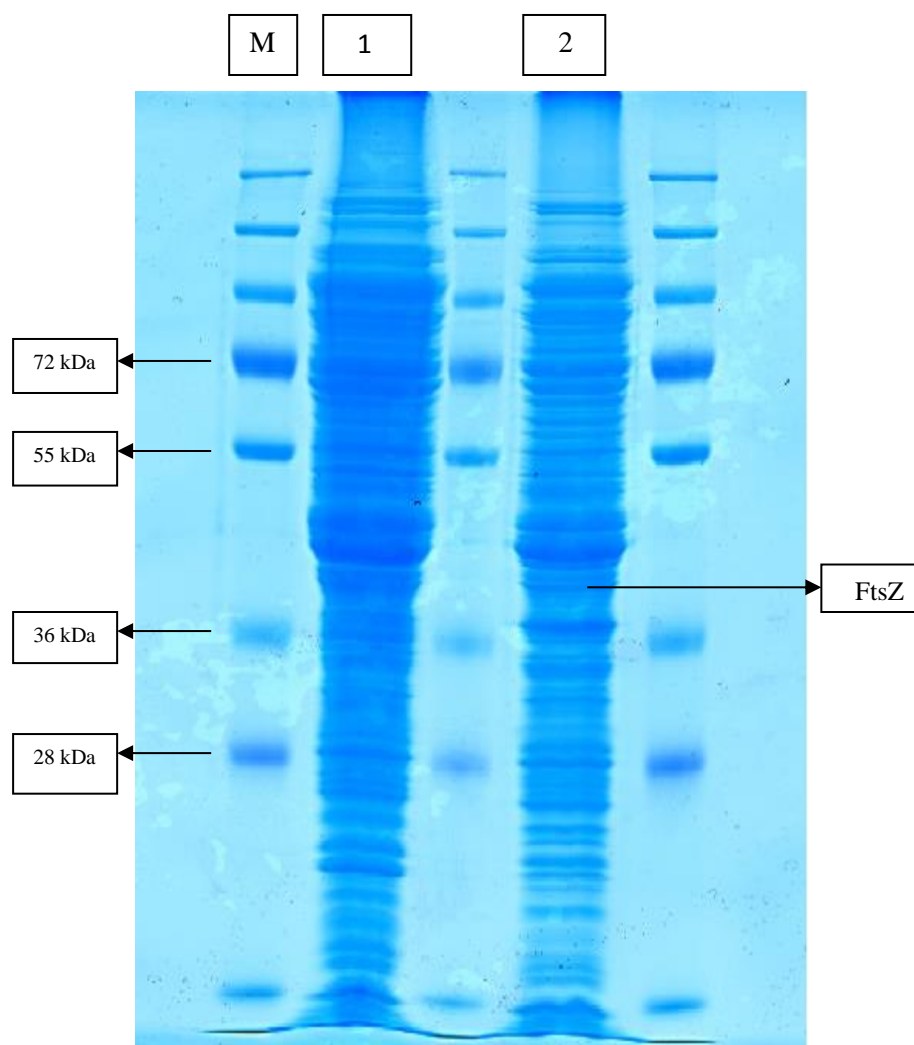
Figure 3.9 SDS-PAGE image of total protein isolation from *E. coli* LW1655F^{+} cells that bear pET28a-*ftsZ*. M: Prestained protein marker, Lane 1 stands for uninduced culture lysate of the cells that bear only pET28a(+)-*ftsZ*, Lane 2 stands for IPTG induced culture lysate of the cells that bear pET28a-*ftsZ* (10 µg protein/well).

As stated before, L-form *E. coli* is not an optimal host for our expression vector pET28a(+) so that we did not expect to see very high levels of FtsZ expression which was achieved with *E. coli* BL21 (DE3) (Figure 3.9). Therefore we switched to a more sensitive method that would enable us to assess FtsZ expression level. For this, Western blot analysis of FtsZ expression was performed (Figure 3.10). Position of the FtsZ on SDS-PAGE gel was calculated by constructing traditional log of MW versus RF plot.
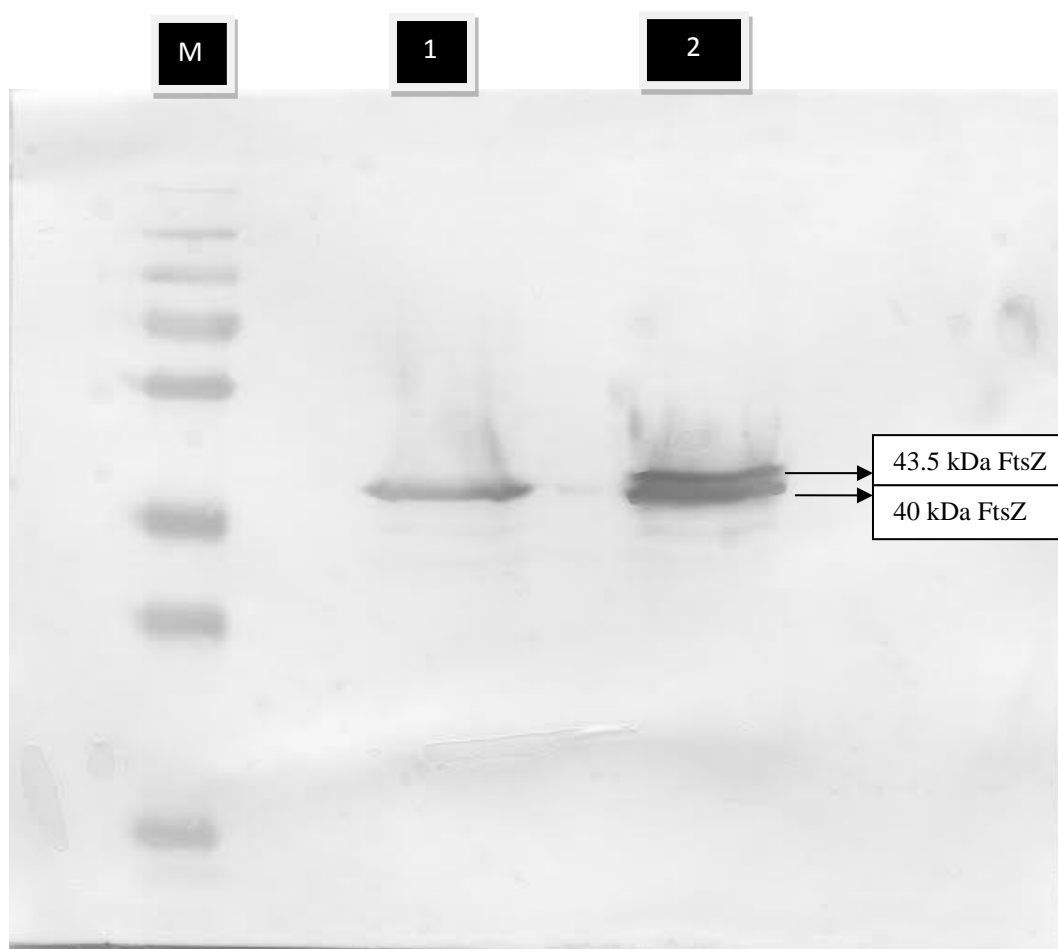
Figure 3.10 Western blot analysis of *E. coli* LW1655F$^+$ cells that bear pET28a-*ftsZ*. M: Prestained protein marker, Lane 1 stands for uninduced culture lysate of the cells, Lane 2 stands for IPTG-induced culture lysate of the cells.

For Western blot analysis, commercially available polyclonal antibody raised against FtsZ was used (Rabbit Anti-FtsZ Lyophilized serum, Agrisera). Single band on the first lane of the Western blot image demonstrates the basal level of FtsZ protein which is present in normal L-form *E. coli* cytoplasm. The second lane of the Western blot image demonstrates both the basal and recombinant FtsZ introduced by the expression vector. Genomic FtsZ is a 40 kDa hydrophilic protein (Dai and Lutkenhaus, 1991). However overproduced FtsZ originates from the expression vector pET28a-*ftsZ* and contains the His.Tag and T7.Tag amino acid sequences in front of the cloned *ftsZ*. The overall sequence of additional amino acids is: Met-Gly-Ser-Ser-His-His-His-His-His-His-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly-Arg (adopted from pET system manual

of Novagen, 1999). The presence of these extra 32 amino acids increased the molecular weight of FtsZ to 43.5 kDa (ProtParam, ExPASy). Therefore, we have expected to see two bands on lane 2 of the Western blot (Figure 3.10). Moreover, the upper band in this lane was found to be 43.5 kDa, quite consistent with the expected size.

To address the question of "How many times was FtsZ expression increased over the control?" band intensities were compared using the Bio 1-D module of Quantum ST4 (Vilber Lourmat). Results show that FtsZ level was increased by 2.25 fold in 48 h cultures following IPTG induction of L-form *E. coli* containing the pET28a-*ftsZ* plasmid. It is known that two to seven fold increase in FtsZ is sufficient to induce minicell formation in *E. coli* K-12 strain of HB101 and W3110 (Ward and Lutkenhaus, 1985). Higher levels of expression could not been achieved using our expression vector pET28a(+) in L-form *E. coli* even though at least twenty different recombinant colonies were analyzed for overproduction. However, we still expected to see minicell production since the 2.25 fold increase in FtsZ levels was within the sufficient range described by Ward and Lutkenhaus (1985).

3.6. Scanning electron microscopy (SEM) and flow cytometry analyses of cultures
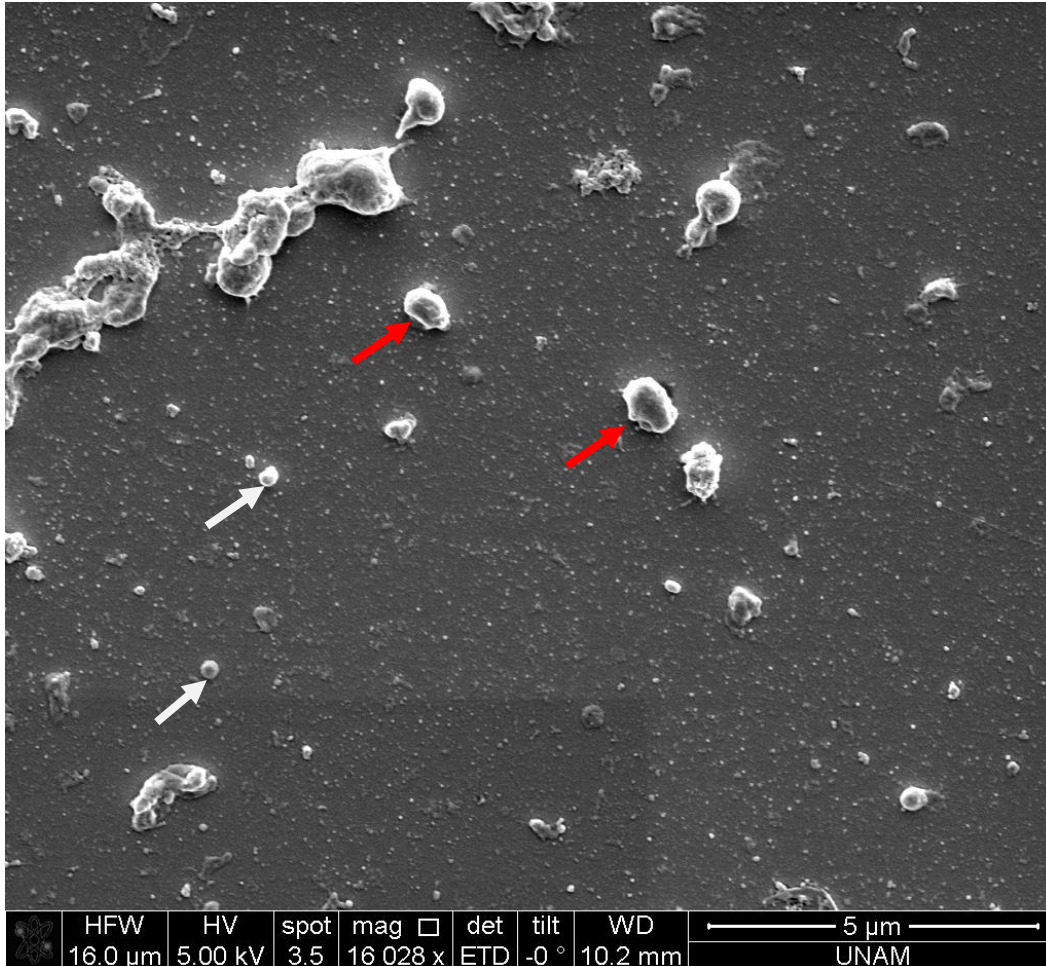
Figure 3.11 SEM image of uninduced control of L-form *E. coli*. Red arrows demonstrate L-form *E. coli* and white arrows demonstrate vesicle-like structures.

As mentioned before, L-form *E. coli* has spherical shapes as could be recognized on our SEM images (Figure 3.11, red arrows). However, the vesicle-like structures (indicated using white arrows in Figure 3.11) were unexpected since they resembled minicells. This was unexpected since the L-form *E. coli* genome did not include any minicell phenotype mutations (Siddiqui *et al*., 2006). Comprehensive literature search demonstrated that some types of L-form organisms like *Listeria*

monocytogenes, Enterococcus faecalis, Enterococcus faecium, Enterococcus hirae and *Enterococcus durans* can form vesicles as a part of their L-form multiplication process (Briers *et al*., 2012). The L-form bacteria first produce intracellular vesicles, the mother cell collapse and the vesicles are thus released into the medium. Only a fraction of these vesicles demonstrate cellular viability after their release from the mother cell (Briers *et al*., 2012). There were no published results on the production of intracellular vesicles by L-form *E. coli.* In this context, our results suggest that there occurs vesicle production also in L-form *E. coli* (Figure 3.11).
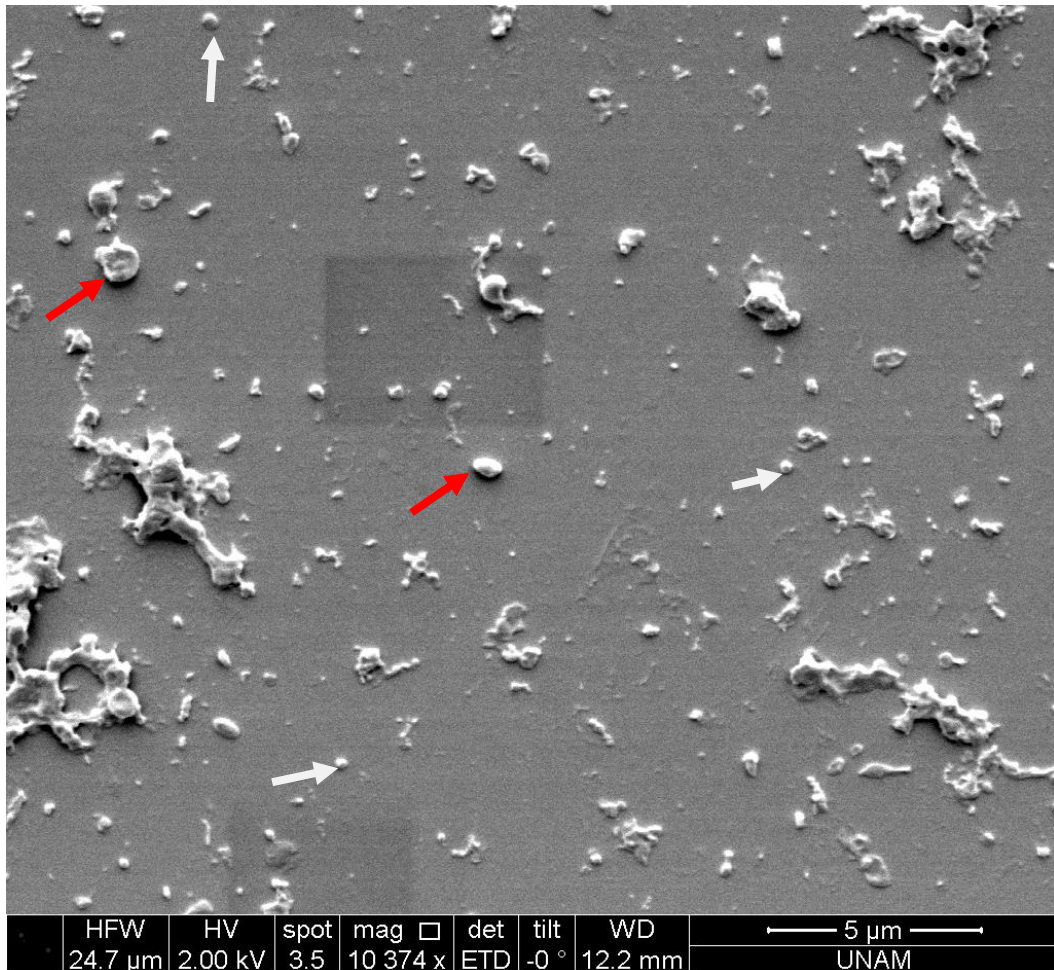
Figure 3.12 SEM image of induced L-form *E. coli* that express the pET28a-*ftsZ* plasmid. Red arrows demonstrate L-form *E. coli* and white arrows demonstrate vesicle-like structures.

SEM analysis of L-form *E. coli* expressing the pET28a-*ftsZ* plasmid also showed vesicle-like structures, but in much higher amounts (Figure 3.12). Our expectation was that overproduction of FtsZ may lead to the production of minicells. However since our control also showed these vesicle like structures, it was not possible to distinguish between minicells and vesicles morphologically based only on SEM analysis. To address this issue, we next conducted flow cytometric analysis of vesicles/minicells and intended to quantitate the number of particles generated in uninduced versus induced samples (Figure 3.13).
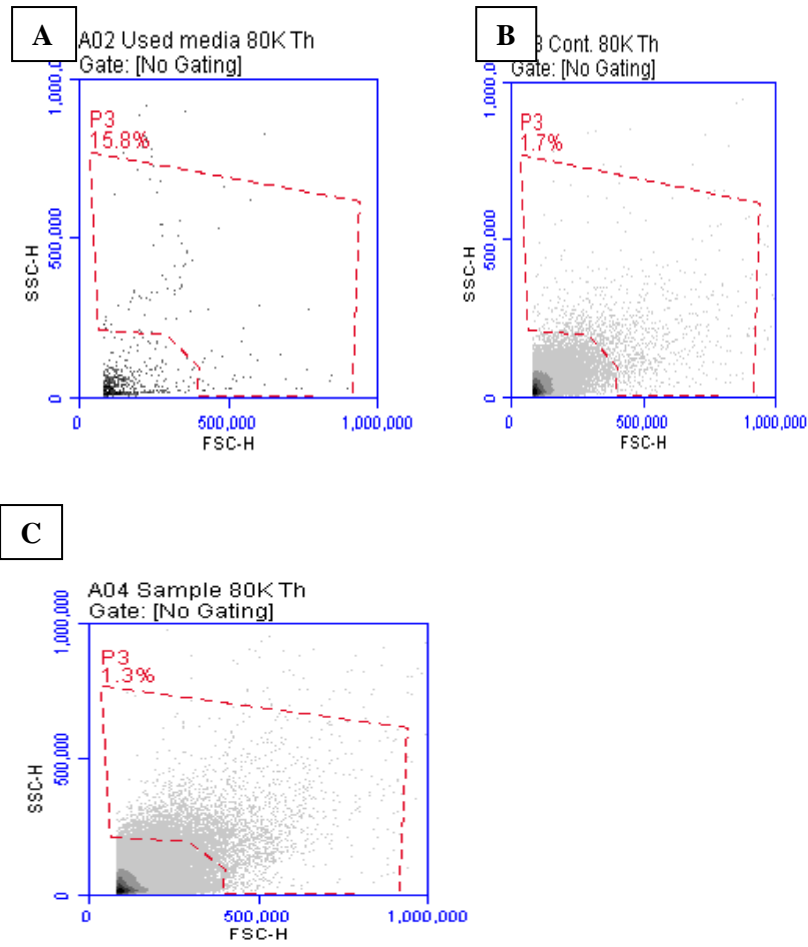
Figure 3.13 Flow cytometry analysis of FtsZ overproducing and control cultures for minicell production. A: Medium only as negative control, B: Uninduced culture of L-form *E. coli* containing pET28a-*ftsZ,* C: IPTG-induced culture of L-form *E. coli* that bear pET28a-*ftsZ.* Values of events for overall were 603, 59624 and 377266 for A, B and C respectively.
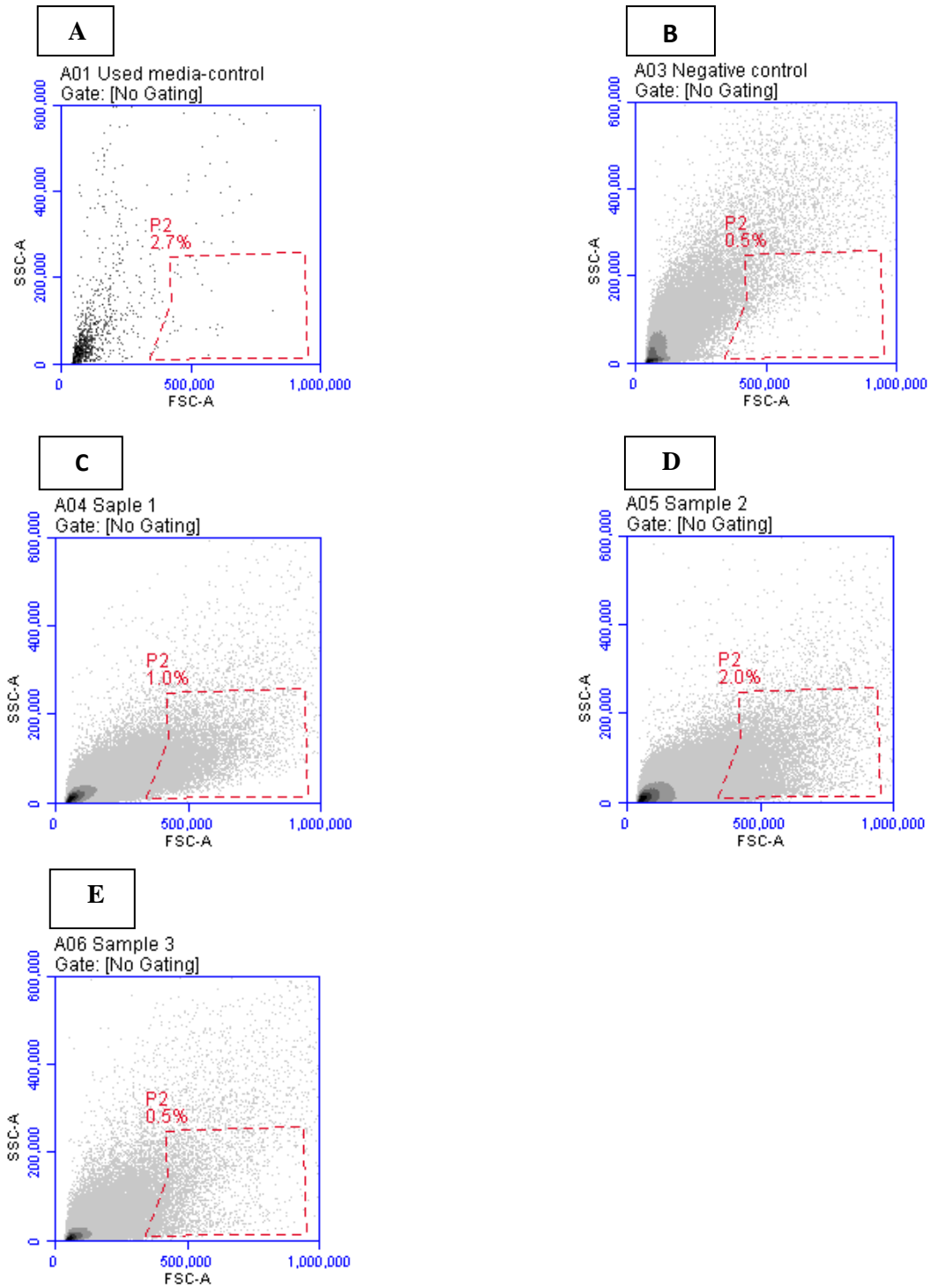
Figure 3.14 Flow cytometry analysis of FtsZ overproduced medium and control medium for minicell production. A: Medium only as negative control, B: Uninduced culture of L-form *E. coli* containing pET28a-*ftsZ,* C, D, E: Biological replicates of IPTG-induced cultures of L-form *E. coli* recombinants that bear pET28a-*ftsZ.*

Sample preparation for flow cytometric analysis generally includes centrifugation of cells and resuspension of the pellet in an appropriate buffer for two or three times in order to get rid of debris (Berney *et al*., 2007). However, for our model organism this procedure did not give very successful results because resuspension of bacterial pellet of L-form *E. coli* generated cell clumps interfering with our analysis. Such large cell clumps can be easily observed in SEM images (Figure 3.12). Therefore, another procedure was followed that omitted centrifugation and resuspension of L-form *E. coli*. In this protocol, an initial centrifugation step (500 g at 4°C for 5 min) was used to get rid of large cells and other debris and vesicles/minicells present in supernatants were then analyzed by flow cytometry (Koppelman *et al.,* 2001). The analysis began with choosing an appropriate area to count events (indicated by the red gates on the dot plots). The rationale behind this gating strategy was to focus on larger particles (defined as having larger Forward Scatter values) to enable the discrimination of the larger minicells from the smaller vesicular structures (Figure 3.14). The events counted from the gated regions were 31, 981, 8306, 13636 and 2839 for A, B, C, D and E, respectively. Total number of events (ungated) were 1106, 207564, 835477, 690319 and 546512 for A, B, C, D and E, respectively (Figure 3.14). These results show that the number of particles present in the FtsZ overproducing culture was approximately 6.33 fold higher for the Figure 3.13 data and 4.025, 3.326 and 2.63 fold higher for the Figure 3.14 data of C, D, and E, respectively when compared to their negative control.

Basic descriptive statistics of flow cytometry data and results of independent samples were analyzed using t-test and are shown in Table 3.1 and Table 3.2, respectively. The average number of particles quantitated from these groups is given in Figure 3.15.

Table 3.1 Basic Descriptive Statistics of Flow Cytometry Data

Group Statistics

	Groups	N	Mean	Std. Deviation	Std. Error Mean
Events	Negative Control	2	133594,0000	104609,3772	73970,00000
	Induced Cells	4	612393,5000	196183,5406	98091,77031

Table 3.2 Results of Independent Samples t-test.

		Levene's Test for Equality of Variances		t-test for Equality of Means						95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Events	Equal variances assumed	1,257	,325	-3,110	4	,036	-478799,5	153952,3	-906240	-51359,3
	Equal variances not assumed			-3,897	3,7	,020	-478799,5	122855,8	-829179	-128420
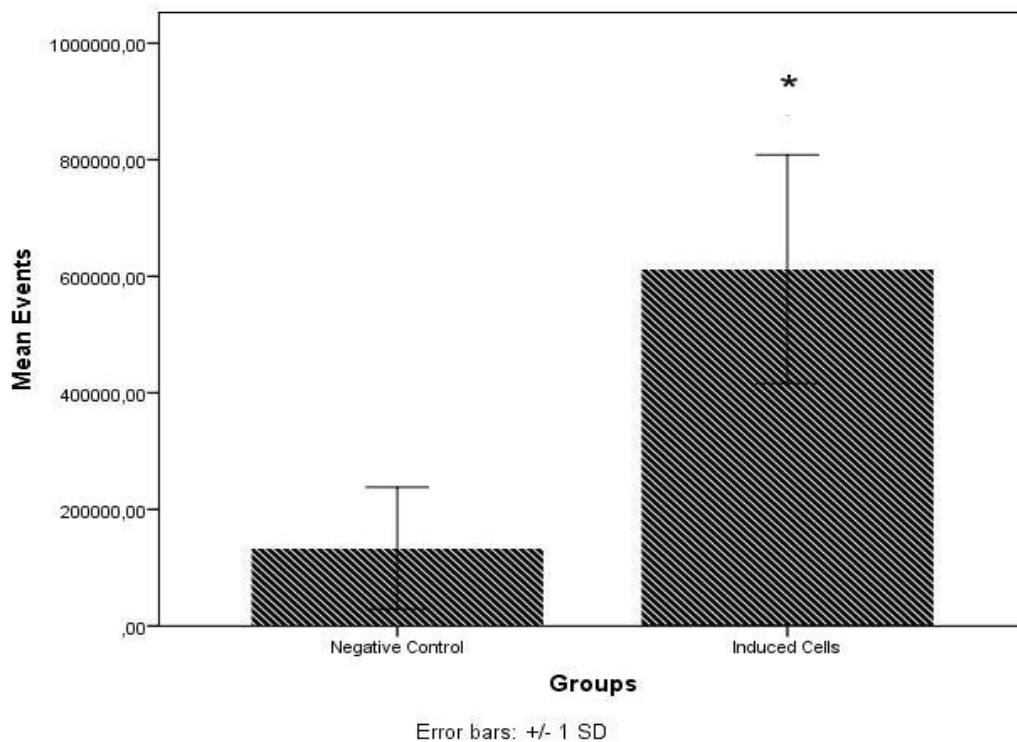
Figure 3. 15 Histogram for the means of uninduced negative control and IPTG-induced cells

The results of the basic descriptive statistics showed that the mean number of events for induced samples was 4.58 fold higher than the negative control samples. In order to determine if this difference was significant or not, results of the t-test was interpreted. First the results of the Levene's Test (Table 3.2) showed that variances of these two groups can be equal (p=0.325). Assumption of equal variances in t-test is crucially important since the results of the test may differ dramatically according to this assumption. Table 3.2 summarizes the results for both groups t-test (equal variances or not). The mean number of particles observed in these two groups were statistically different from each other and were significant (p=0.036). Even if the result of Levene's Test was wrong, the difference would still be significant having a p value of 0.020. Also for both of the cases 95 % confidence intervals for the difference of means did not contain zero which means that they cannot be equal. The significant difference observed between the means of these two groups is depicted schematically as bar diagrams in Figure 3.15.

This significant increase in the total number of events for induced samples can be interpreted based on Forward Scatter (FSC) information. According to Figure 3.14 results, events within the gated area demonstrated that FSC of the events are increased without a change in the SSC parameter. This indicates that larger particles were counted in the gated area in Figure 3.14. This gated area indeed includes both minicells and vesicles. When the two samples with equivalent OD_{600} values were analyzed the event counts should correlate with the number of vesicles/minicells. Based on the above information, these results suggest that the number of particles within the gated region stem mostly from the larger minicells and not from the smaller vesicles and that this number was found to be increased in the induced population. Although our SEM and flow cytometry results did not directly prove minicell production in L-form *E. coli*; flow cytometric analysis showed that over production of FtsZ increased the total event number. The number of particles increased 8.46, 13.9 and 2.89 folds for samples C, D and E, respectively (Figure 3.14, gated population). Based on these results one can speculate that the control and the induced sample both produce vesicles and the differences in flow cytometric analysis of particle count is due to additional minicell production in the induced sample. However it is also possible that the increased number of the events in the induced sample may be due to an increase in vesicle production. As a result, although a direct proof of overproduction leading to minicell production in L-form *E. coli* is absent, flow cytometric analysis indicates that the increased number of events may be due to the consequence of increased number of minicell formation or vesicle formation. Whether the former or the latter is true should be pursued in further studies. However, the minicells and vesicles are both spherically shaped and their sizes are not uniform (Briers *et al*., 2012). In contrast, minicells and vesicles have different formation mechanism which can be used to identify them separately. Minicells are "anucleate" cells and atypical division of cell near the parent cell can be considered as cause of production of minicells (Shull *et al.,* 1971). However, it was shown that for *Listeria monocytogenes* L-form, the vesicles was first appear in the enlarged L-form structure and then are released into the medium after the collapse of the mother cell (Dell'Era *et al*., 2009). Based on the SEM results, our study shows for the first time that L-form *E. coli* also produces such vesicles.

Whether vesicle production in L-form *E. coli* and *Listeria monocytogenes* have similar mechanism, the same purpose as being reproductional units, or the same internal components, remains to be determined. Briers *et al.* (2012) stated that *Enterococcus* L-forms have the same phenotype and multiplication properties with *Listeria* L-forms. However, it cannot be known whether L-form *E. coli* vesicles have these multiplication properties and phenotype. It can just be assumed that they serve similar purposes. If it is assumed that L-form *E. coli* vesicles have similar multiplication properties to those observed with *Listeria monocytogenes* L-forms, then it can be said that their vesicles should similarly contain genomic DNA (Briers *et al.*, 2012). In order to be reproduction units, these vesicles must have genomic DNA, and therefore it would be possible to discriminate between minicells and vesicles based on the presence or absence of their genomic DNA content. It is mentioned before that minicells do not include genomic DNA (Adler *et al.,* 1967). Therefore genomic DNA labeling studies can be utilized in order to differentiate vesicle like structures from minicells. Other identification strategies may focus on the formation mechanism of minicells. As it was mentioned before, minicells are the results of "atypical" division near the parent cell (Shull *et al.,* 1971). FtsZ over production was thought to cause this "atypical" division event. However it was clearly stated that intracellular vesicles first appear inside the cell (Dell'Era *et al.*, 2009). Therefore, labeling of FtsZ ring with fluorescent proteins or other techniques can be helpful in understanding the formation mechanism of minicell like structures. In order to understand formation mechanism of vesicle-like or minicell-like small spherical structures, FtsZ ring labeling has to be followed by live imaging via high resolution microscopes. Counter staining of genomic DNA and cytoplasm of bacterial cell or cellular membrane of bacterial cell may be an alternative technique that can be applied in order to differentiate minicell from mother cell or vesicle like structures. Fluorescence dyes can be used for these purposes. However inadequate resolution power of optical microscopy for structures smaller than 0.5 μm would still constitute a difficulty to differentiate mother cells from minicells.

Fluorescence and confocal microscopic analyses were carried out in order to differentiate minicell and L-form *E. coli*; however, sensitivity of the techniques was not sufficient for this differentiation. Moreover, sucrose density gradient

centrifugation was also conducted to separate minicells from L-form *E. coli*. Both negative control and induced sample included minicell and vesicle-like structures in the low density part of the sucrose solution; therefore, we concluded that traditional sucrose density gradient centrifugation was not suitable for the separation of minicells and L-form *E. coli*.

CHAPTER 4

CONCLUSION

The present study optimized the conditions enabling the growth of L-form *E. coli*. The generation time was found to be 240 min, duration much longer than those observed in other strains such a K-12 and MG1655 strains of *E. coli* having a generation time about 20.9 min. The current study also identified major factors that have to be strictly maintained for successful cultivation of L-form *E. coli*. These include the use of β-lactam antibiotics for prevention of contamination, avoidance of detergent based contamination like Triton X-100 on glassware and maintenance of osmolarity since L-form *E. coli* are devoid of cell walls and are vulnerable to osmotic pressure (Allan *et al*., 2009).

The gene for *ftsZ* was amplified from L-form *E. coli* LW1655F$^+$ genome and cloning of the *ftsZ* gene first into pGEM-T Easy PCR cloning vector and then into pET28a(+) (Novagen) expression vector was conducted. Western blot analysis which was utilized by polyclonal antibody of FtsZ demonstrated that FtsZ protein was produced 2.25 fold higher in IPTG-induced samples.

SEM image analysis for L- form *E. coli* (control cultures) showed that there were vesicle-like structures. Comprehensive literature search demonstrated that some types of L-forms can form intracellular vesicles possibly as a multiplication mechanism.

Flow cytometry analysis showed that there was significant increase in the events in overall rates of FtsZ overproduced cultures as compared to L-form *E. coli* (control). The number of minicells/vesicle-like particles increased 8.46, 13.90 and 2.89 folds for three independent FtsZ overproducing cultures and this increase was statistically significant.

In brief, the present study gives promising results about production of minicells and/or vesicle-like structures in L-form *E. coli*; however, it is still not known whether vesicle production of L-form *E. coli* and *Listeria monocytogenes* share the same

molecular mechanism, for the same purpose as being reproductional units or internal components.

Further studies must be conducted by applying techniques to separate minicells and vesicle-like structures in order to understand the nature of these structures formed much more extensively by L-form *E. coli* upon FtsZ overproduction.

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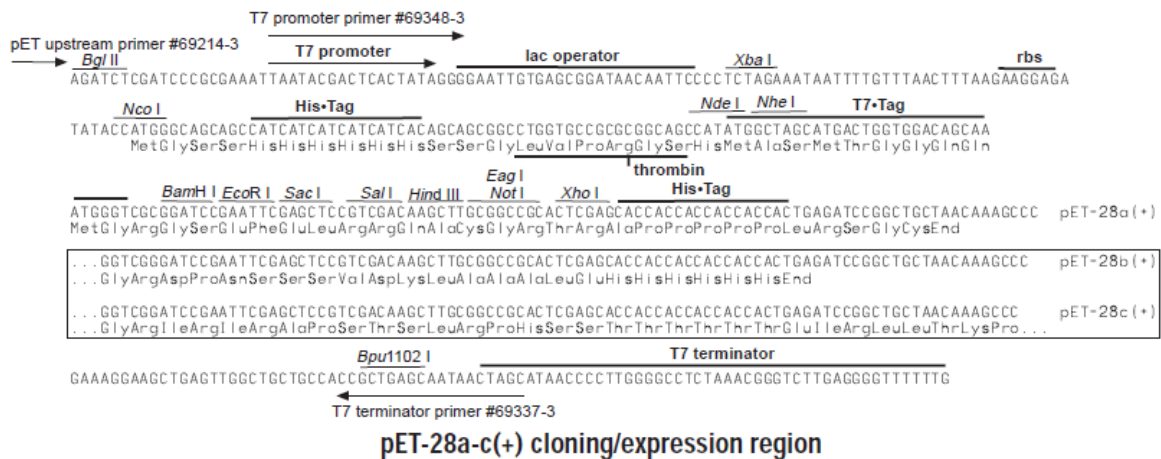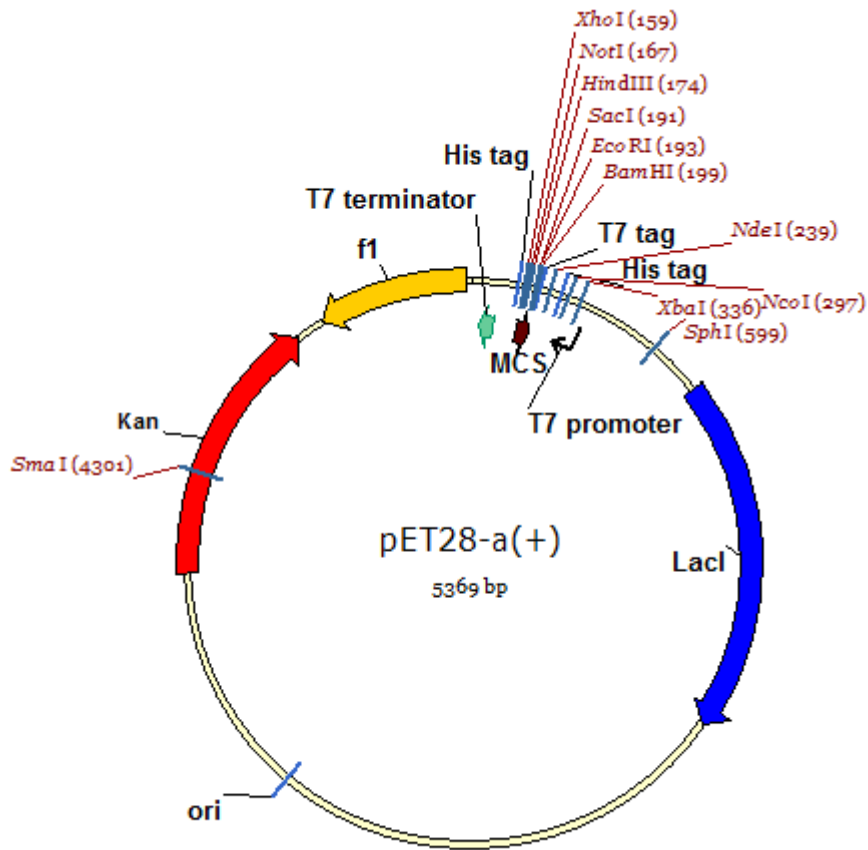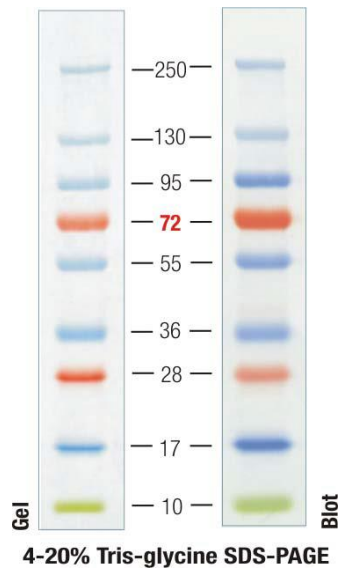
STRUCTURES OF PLASMID VECTORS AND SIZE MARKERS
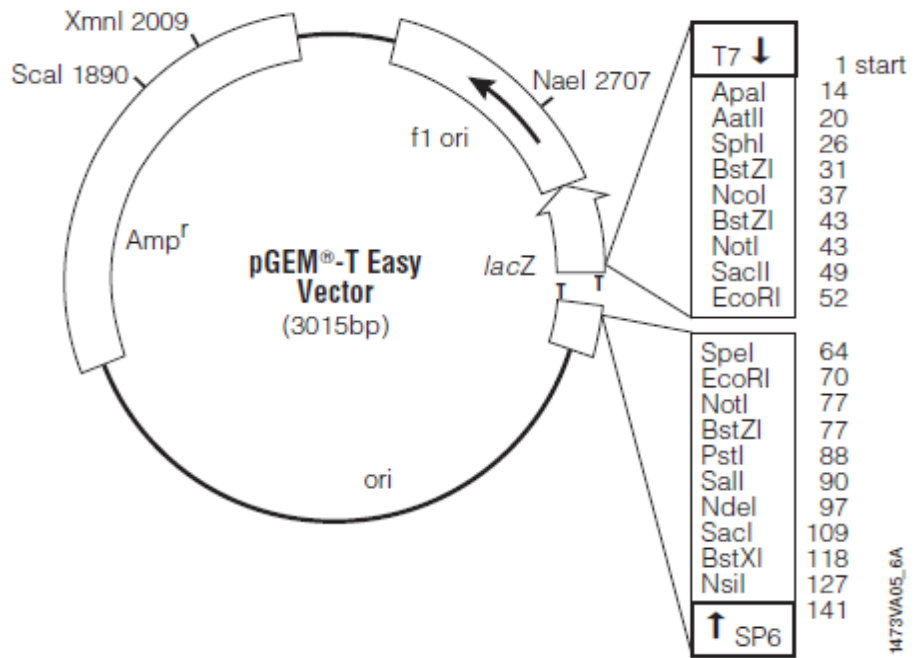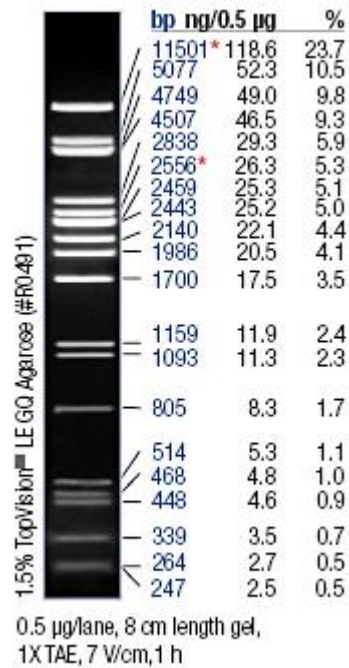

Figure A.1 pGEM®-T Easy Cloning Vector (Promega #A1360)

Figure A.2 pET-28a(+) His-tag Expression Vector (Novagen #69864-3)

kDa

60

Figure A.3 PageRuler™ Plus Prestained Protein Ladder (Fermentas #SM1811)

Figure A.4 Lambda DNA/*Pst*I Marker (Fermentas #SM0361)

APPENDIX B

COMPOSITION AND PREPARATION OF CULTURE MEDIA

Luria Broth:

- Tryptone   10 g
- Yeast Extract   5 g
- NaCl   10 g
- Distilled water   up to 1000 ml

Final pH is 7.0; sterilized at $121^{o}C$ for 15 min.

Luria Agar:

- Tryptone   10 g
- Yeast Extract   5 g
- NaCl   10 g
- Agar   15 g
- Distilled water   up to 1000 ml

Final pH is 7.0; sterilized at $121^{o}C$ for 15 min.

Brain-Heart Infusion Broth:

- Nutrient substrate   27.5 g
- D(+)Glucose   2 g
- NaCl   5 g
- Na_2HPO_4 2.5 g
- Yeast Extract   5 g
- Distilled water   up to 1000 ml

Final pH is 7.5; sterilized at $121^{o}C$ for 15 min.

Brain-Heart Infusion Agar:

- Nutrient substrate   27.5 g
- D(+)Glucose   2 g
- NaCl   5 g
- Na_2HPO_4 2.5 g
- Yeast Extract   5 g
- Bacto Agar   12 g
- 10 % Horse Serum
- Distilled water   up to 1000 ml

Final pH is 7.5; sterilized at $121^{o}C$ for 15 min.

APPENDIX C

SOLUTIONS AND BUFFERS

Agarose Gel Electrophoresis

TAE Buffer (50X)

- Tris-base   242 g
- Glacial acetic acid   57.1 mL
- EDTA (0.5 M, pH 8.0)   100 mL
- Distilled water   up to 1000 mL

Loading Buffer (10X)

- Bromophenol blue (w/v)   0.25%
- Xylene cyanol FF (w/v)   0.25%
- Sucrose (w/v)   40%

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Acrylamide/Bis

- Acrylamide   146 g
- N.N'-Methylene-bis acrylamide   4 g
- Distilled water   up to 500 mL

Filtered and stored at 4°C. Protected from light.

Tris HCl (1.5 M)

- Tris-base   54.45 g
- Distilled water   150 ml

pH is adjusted to 8.8 with HCl, distilled water to 300 ml and stored at 4°C.

Tris HCl (0.5 M)

- Tris-base   6 g
- Distilled water   60 ml

pH is adjusted to 6.8 with HCl, distilled water to 100 ml and stored at 4°C.

Running Buffer (10X)

- Tris-base   30 g
- Glycine   144 g
- SDS   10 g
- Distilled water   up to 1000 ml

Sample Loading Buffer (4X)

- Tris-HCl (1 M, pH 6.8)   2 ml
- EDTA (0.5 M)   1 ml
- Glycerol   4 ml
- SDS   0.8 g
- β-mercaptoethanol   0.4 ml
- Bromophenol blue   0.008 g
- Distilled water   up to 10 ml

Protected from light.

Fixation Solution

- Ethanol   40%
- Glacial acetic acid   10%
- Distilled water   50%

Coomassie Blue R-250 Stain

- Coomassie Blue R-250   0.25 g
- Methanol   125 ml
- Glacial acetic acid   25 ml
- Distilled water   100 ml

Protected from light.

Destaining Solution

- Methanol   100 ml
- Glacial acetic acid   100 ml
- Distilled water   800 ml

<u>Western Blot</u>

Transfer Buffer (1X)

- Methanol   200 ml
- Tris-base   3.63 g
- Glycine   14.4 g
- SDS   0.37 g
- Distilled water   up to 1000 ml

Tris-buffered Saline, TBS (1X)

- Tris-base   2.42 g
- NaCl   29.2 g
- Distilled water   up to 1000 ml

Protein Purification

LEW (Lysis-Elution-Wash) Buffer

- Urea   8 M
- NaCl   300 mM
- NaH_2PO_4 50 mM

pH is adjusted to 8.0

***E. coli* Competent Cell Preparation**

Buffer 1

- RuCl   100 mM
- KAc   30 mM
- $CaCl_2$ 10 mM
- Glycerol   15%

pH is adjusted to 5.8 with dilute acetic acid and filter sterilized.

Buffer 2

- CaCl$_2$ 75 mM
- RuCl   10 mM
- MOPS   10 mM
- Glycerol   15%

pH is adjusted to 6.5 with 0.2 M KOH and filter sterilized.

IPTG (Isopropyl-β-D-thiogalactoside) for Colony Selection

- IPTG   100 mg
- Distilled water   1 ml

The solution was filter sterilized and stored at –20°C.

X-Gal (5-bromo-4-chloro-3-indolyl-B-D-galactoside)

- X-Gal   20 mg
- Dimethylformamide 1 ml

The solution was stored at –20°C protected from light.

Plasmid Isolation

STE Buffer

- Sucrose (w/v)   10.3%
- Tris-HCl (pH 8.0)   25 mM
- EDTA (pH 8.0)   25 mM

Lysis Buffer

- NaOH   0.3 M
- SDS (w/v)   2%

APPENDIX D

SUPPLIERS OF CHEMICALS, ENZYMES AND KITS

Chemicals Suppliers

Acrylamide	Sigma
Agar-agar	Merck
Agarose	Biomax
(Prona)	
Ammonium persulfate	AppliChem
Ampicillin	Sigma
Anti-mouse IgG	Sigma
Bovine serum albumin	Sigma
Brain Heart Broth	Merck
Bromophenol blue	Merck
$CaCl_2.2H_2O$	Merck
Coomassie Blue G-250	Fluka
Coomassie Blue R-250	Fluka
Dimethylformamide	Merck
dNTPs	Fermentas
DTT	Fluka
EDTA	Sigma
Ethanol	Sigma

Ethidium bromide	Sigma
Fetal bovine serum	Biochrom
Formaldehyde	Merck
Glacial acetic acid	Merck
Glycerol	Merck
Glycine	Merck
H_2SO_4	Merck
HCl	Merck
IPTG	Sigma
Isopropanol	Merck
Kanamycin	Sigma
KCl	Merck
KH_2PO_4	Merck
Luria Broth	Merck
Methanol	Merck
$MnCl_2$	Merck
N.N'-Methylene-bis acrylamide	Sigma
Na_2CO_3	Merck
Na_2HPO_4	Merck
NaCl	Sigma
$NaHCO_3$	Merck

NaOH	Merck
Penicillin	Biochrom
Phenol/chloroform/isoamylalcohol	Amresco
Phosphoric acid	Merck
Potassium acetate	Merck
RuCl	Merck
SDS	Merck
Skim milk	Fluka
Sucrose	Merck
TEMED	Merck
Tris-base	Sigma
Tris-HCl	Fluka
Tween-20	Merck
Urea	Sigma
X-gal	Sigma
2-mercaptoethanol	Merck

<u>Enzymes</u>

Alkaline phosphatase	Roche
*Bam*HI	Fermentas
*Bgl*II	Fermentas
*Eco*RI	Fermentas
*Hin*dIII	Fermentas
*Not*I	Fermentas
T4 DNA ligase	Fermentas
Taq DNA polymerase	Fermentas
Phire Hot Start II DNA Polymerase	Thermo Scientific

<u>Kits</u>

AP Conjugate Substrate Kit	Bio-Rad
Gel Extraction Kit	Qiagen
Ni-NTA Spin Columns	Qiagen
pGEMT Easy Vector	Promega
Plasmid Midi Kit	Qiagen
Plasmid Mini Kit	Qiagen
Protino Ni-TED 2000 Packed Columns	Macherey-Nagel