IN SILICO UNRAVELLING OF THE *Lactococcus lactis* SIGNAL RECOGNITION PARTICLE AND SIGNAL RECOGNITION PARTICLE RECEPTOR PROTEIN INTERFACIAL INTERACTION

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In memory of my *late* mother,
Che Rahani binti Taib.

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

To
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family and friends,
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ABSTRACT

The signal recognition particle (SRP) protein targeting pathway is highly conserved across all kingdoms of life. In bacteria, gene conservation and experimental data show that the *Lactococcus lactis* has the simplest version of protein secretion system compared to *Escherichia coli* and *Bacillus subtilis* which have more complex systems. *L. lactis* only possess the SRP pathway, where the specific interaction of its signal recognition particle protein (Ffh) and its SRP receptor protein (FtsY) is known to be essential for the efficiency and fidelity of its protein targeting. On this basis, modeling and characterization study of Ffh-FtsY will allow structural analysis and identification of crucial region and amino acids that are critical in Ffh-FtsY interaction during protein targeting. This work is the first attempt to model the *L. lactis* Ffh-FtsY complex using the blind docking approach. Modelled Ffh-FtsY showed that the complex interface was predominantly stabilized by hydrophobic interactions and hydrogen bonding with putative binding interfaces mostly confined at the motifs II (5' TFRAGAIDQL 3') and III (5' DTAGR 3') in each G domain of Ffh and FtsY. Three amino acids of Ffh-NG (Y141, L196 and I198) interact with four amino acids of FtsY-NG (V113, F141, L196 and A236) to form four hydrophobic interactions. 11 amino acids of Ffh-NG (D139, Y141, R142, A144, K168, G194, E197, I198, D199, E205 and Q232) interact with 12 amino acids of FtsY-NG (N111, R142, A143, G144, A165, D168, R195, Q197, N201, E205, N235 and Q239) to form 17 hydrogen bonds. Several highly conserved amino acids in motif II, amino acids D139, R142 and A144 of Ffh-NG and D139, R142 and A143 of FtsY-NG were expected to play important roles in initiating or regulating guanosine triphosphate hydrolysis. Mutations were introduced to the insertion binding domain (IBD) loop R142 of the putative GTP binding site. Mutations R142A and R142E of FtsY-NG significantly disrupted the predicted hydrogen bond that was required for GTP binding. *L. lactis subsp. cremoris* MG1363 Ffh and FtsY were successfully cloned, expressed and purified by three-step column chromatography. However, ineffective mass spectrometry analysis data of Ffh-FtsY NG has inhibited computational results validation. This structural information will allow for the rational design of *L. lactis* Ffh-FtsY association in the future.

ABSTRAK

Partikel pengecaman signal (SRP) laluan sasaran protein adalah sangat terpelihara di dalam semua bentuk kehidupan. Dalam bakteria, pemuliharaan gen dan data eksperimen menunjukkan bahawa *Lactococcus lactis* mempunyai versi sistem rembesan protein yang paling mudah berbanding *Escherichia coli* dan *Bacillus subtilis* yang mempunyai sistem lebih rumit. *L. lactis* hanya mempunyai laluan SRP, di mana interaksi khusus di antara protein partikel pengecaman signal (Ffh) dan protein penerima SRPnya (FtsY) diketahui adalah penting bagi kecekapan dan ketepatan sasaran proteinnya. Atas dasar ini, pemodelan dan pencirian Ffh-FtsY akan membolehkan analisa struktur dan pengenalpastian kawasan genting dan asid-asid amino yang kritikal dalam interaksi Ffh-FtsY semasa sasaran protein. Kajian ini merupakan percubaan pertama untuk memodelkan kompleks *L. lactis* Ffh-FtsY menggunakan pendekatan dok rambang. Model Ffh-FtsY menunjukkan bahawa antaramuka kompleks kebanyakannya distabilkan oleh interaksi hidrofobik dan ikatan hidrogen dengan ikatan antaramuka kebanyakannya terletak pada motif II (5' TFRAGAIDQL 3') dan III (5' DTAGR 3') dalam setiap domain G Ffh dan FtsY. Tiga asid amino Ffh-NG (Y141, L196 dan I198) berinteraksi dengan empat asid amino FtsY-NG (V113, F141, L196 dan A236) untuk membentuk empat interaksi hidrofobik. 11 asid amino Ffh-NG (D139, Y141, R142, A144, K168, G194, E197, I198, D199, E205 dan Q232) berinteraksi dengan 12 asid amino FtsY-NG (N111, R142, A143, G144, A165, D168, R195, Q197, N201, E205, N235 dan Q239) untuk membentuk 17 ikatan hidrogen. Beberapa asid amino yang sangat konservatif di dalam motif II, asid amino D139, R142 dan A144 bagi Ffh-NG dan D139, R142 dan A143 bagi FtsY-NG dijangka memainkan peranan penting dalam memulakan atau mengawal hidrolisis trifosfat guanosin. Mutasi diperkenalkan kepada gelung domain ikatan pemasukan (IBD) R142 dari tapak pengikat GTP. Mutasi-mutasi R142A dan R142E bagi FtsY-NG secara signifikan mengganggu ikatan hidrogen yang diperlukan untuk pengikatan GTP. *L. lactis subsp. cremoris* MG1363 Ffh dan FtsY berjaya diklonkan, dinyatakan dan ditulenkan melalui tiga langkah kromatografi turus. Walau bagaimanapun, ketidak berkesanan data analisis spektrometri jisim bagi Ffh-FtsY NG telah merencatkan pengesahan keputusan pengkomputeran. Maklumat struktur ini akan membolehkan reka bentuk rasional bagi pembentukan *L. lactis* Ffh-FtsY pada masa akan datang.

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LIST OF SYMBOLS

% v/v	-	volume per volume percent
% w/v	-	weight per volume percent
%	-	percentage
°C	-	unit of temperature, degree Celcius
µg	-	micrograms
µg/ml	-	micrograms per mililitre
µg/ml	-	micrograms per mililitre
µl	-	microlitre
µm	-	micrometre
µM	-	micromolar
Å	-	unit of resolution, Angstrom
g/l	-	grams per litre
K	-	unit of temperature, Kelvin
L	-	litre
M	-	molar
mg	-	milligram
mg/l	-	milligrams per litre
mg/ml	-	milligrams per mililitre
mM	-	millimolar
rpm	-	round per minute
U	-	Unit
α	-	alpha
β	-	beta
φ	-	phi
ψ	-	psi

LIST OF ABBREVIATIONS

3D	-	three-dimensional
A_{280}	-	absorbance at 280 nm
A_{595}	-	absorbance at 595 nm
A_{600}	-	absorbance at 600 nm
aa	-	amino acids
ABC	-	ATP-binding cassette
ATPase	-	adenosine triphosphate enzyme
B. subtilis	-	*Bacillus subtilis*
BCIP	-	4-chloro-3-indolyl phosphate
BLAST	-	Basic Local Alignment Search Tools
bp	-	base pair
BSA	-	bovine serum albumin
DNA	-	deoxyribonucleic acid
DOPE	-	Discrete Optimized Protein Energy
DSS	-	disuccinimidyl suberate
E. coli	-	*Escherichia coli*
EM	-	energy minimization
FDA	-	Food and Drug Administration
FFT	-	Fast Fourier Transform
GC	-	guanine-cytosine
GMPPNP	-	5'-guanylyl imidodiphosphate
GRAS	-	generally recognized as safe
GROMACS	-	Groningen Machine for Chemical Simulations
His-Tag	-	histidine-tags
ID	-	identity
IPTG	-	isopropyl β-D-1-thiogalactopyranoside
L. lactis	-	*Lactococcus lactis*
LAB	-	lactic acid bacteria
LB	-	Luria-Bertani
LC-MS	-	liquid chromatography-mass spectrometry

LPS	-	lipopolysaccharides
MD	-	molecular dynamics
MFP	-	membrane fusion protein
molpdf	-	molecular Probability Density Functions
MS	-	mass spectrometry
NBT	-	nitro-blue tetrazolium chloride
NCBI	-	National Center for Biotechnology Information
NLE	-	non-local environment
NMR	-	nuclear magnetic resonance
OMP	-	outer membrane protein
PCR	-	polymerase chain reaction
PDB	-	Protein Data Bank
PIC	-	Protein Interactions Calculator
PME	-	Particle Mesh Ewald
PMF	-	proton-motive force
PPIase	-	peptidyl-propyl-cis/trans-isomerase
PSI-BLAST	-	position-specific iterated BLAST
R_g	-	radius of gyration
RMSD	-	root mean square deviation
RMSF	-	root mean square fluctuation
RNC	-	ribosome nascent chain
SDS-PAGE	-	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sec	-	secretion
sp.	-	species
SPC	-	simple point charge
SRP	-	signal recognition particle
subsp.	-	subspecies
T. aquaticus	-	*Thermus aquaticus*
Tat	-	Twin-arginine-translocation
TSS	-	transformation and storage solution
UV	-	ultraviolet

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Bacteria have been used as cell factories for a long time. Danchin (2004) reported, bacteria such as *Streptomyces lividans*, *Corynebacterium glutamicum*, *Bacillus subtilis*, *Escherichia coli* and *Zymomonas mobilis*, are used not only as research models but also as large-scale production factories, mostly small molecules such as antibiotics, vitamins and amino acids. Thus, in biotechnology, efficient protein secretion is very important as it provides an active and stable production of enzyme essential for successful biocatalysis. Secretion is always preferable to cytoplasmic production due to its advantages such as providing N-terminal authenticity of the expressed protein, allowing continuous culture (Lv *et al*., 2012), simplifying purification process, avoiding proteolysis, enhancing biological activity, and giving high product stability and solubility (Mergulhão *et al*., 2005).

For decades, the universal model *E. coli* was industrially explored as a ubiquitous cell factory. As early as in the early eighties, many proteins of medical interest were produced using *E. coli* as the factory. For example, in 1982, recombinant human insulin was successfully produced by *E. coli* as the first host to produce a recombinant DNA pharmaceutical (Swartz, 2001). To date, *E. coli* still remains as one of the most attractive and preferable prokaryotic hosts for industrial heterologous protein production, due to its long-term experience and extensive documentation with the United States Food and Drug Administration (FDA) and other regulatory bodies, its well-characterized genetic tools, ease in genetic handling, and its ability to grow rapidly in high density on low-cost substrates (Baneyx, 1999). Despite these advantages, the high production of protein in the cytoplasm subsequently leads to the

formation of inclusion bodies. Thus, protein secretion to the extracellular milieu is a new approach to overcome such a problem. However, the extracellular secretion in *E. coli* always correlates with no-specific leakage and cell lyses (Jonet *et al*., 2012; Ismail *et al*., 2011; de Marco, 2009). There are papers reporting that the production of some proteins has often proven to be challenging in *E. coli* because of the proteolytic degradation of cloned gene products by its host-specific proteases (Mergulhão *et al*., 2004; Belagaje *et al*., 1997; Gottesman, 1996). Several proteases have been recognized in the cytoplasm and periplasm of *E. coli* (Narayanan and Chou, 2009; Gottesman, 1996; Gottesman and Maurizi, 1992; Maurizi, 1992).

Besides *E. coli*, another well-known host for expression and secretion of heterologous proteins is *Bacillus*, another generally recognized as safe (GRAS) organisms, AT-rich Gram-positive bacteria. Researchers pay more attention to this alternative host with the target of commercial exploitation of *B. subtilis* as major "cell factories" since it has a complete genome sequence. Besides its transparent genome, there are advantages in employing *B. subtilis* as an expression host. A large variety of genetic tools have been developed for the *B. subtilis*. It has a huge capacity to secrete proteins directly into the extracellular medium, thus it is widely used for the industrial enzyme production. Other than that, *B. subtilis* is of non-biased codon usage, non-pathogenic and free of endotoxins (Li *et al*., 2004). Some heterologous genes have been expressed successfully in the *B. subtilis* expression systems. However, there are two drawbacks, plasmid instability and low level of protein production, which limit its application potential (Westers *et al*., 2004b; Bolhuis *et al*., 1999).

Since secretion yields of heterologous proteins in the *B. subtilis* system is generally much lower (Westers *et al*., 2004a; 2004b), therefore, the possibility to secrete heterologous proteins in other Gram-positive bacteria, lactic acid bacteria (LAB) have been addressed in several studies (Mathiesen *et al*., 2008; Oh *et al*., 2007; Steidler *et al*., 2003; Le Loir *et al*., 2001). LAB belongs to a group of Gram-positive anaerobic bacteria that excretes lactic acid as their main fermentation products into the culture medium. LAB was among the first organisms to be used in food manufacturing. Today LAB plays crucial roles in the manufacturing of fermented milk products, vegetables and meat, as well as in the processing of other products such as wine.

An increase of interest has been shown on LAB *Lactococcus lactis* as an expression host for recombinant protein production. *Lactococcus lactis* has several advantages over conventional cell factories like *E. coli* and *B. subtilis*. The bacterium has a well-established safety profile and a GRAS status. This feature makes it suitable for delivery vehicles in pharmaceuticals and in industrial manufactures of fermented food product (Liang *et al*., 2007). Interestingly, in terms of heterologous protein production, when compared with other well-known protein producers is that *L. lactis* does not produce lipopolysaccharides (LPS) or any proteases as *E. coli* or *B. subtilis* do, respectively. Importantly, *L. lactis* can secrete proteins ranging from low to high (<10 kDa to >160 kDa) molecular mass through its Sec-dependent pathway (Le Loir *et al*., 2005). Experimental data and genomic analyses indicate that only one major protein, Usp45, is secreted into the medium thus simplifying downstream purification processes (van Asseldonk *et al*., 1990). In addition, *L. lactis* laboratory strains possess only one exported housekeeping protease, HtrA (Poquet *et al*., 2000).

For the past ten years, LAB had been studied extensively and are now among the best-characterized microorganisms with respect to their genetics, physiology and applications (Konings *et al*., 2000). Many studies have used *L. lactis* to produce recombinant proteins. However, low secretion level of heterologous proteins by *L. lactis* becomes a bottleneck for its application in industry. Therefore, a variety of strategies, genetic tools and modifications have been explored and developed to enhance the secretion efficiency in *L. lactis*, thus to improve the production yields of secreted proteins such as, fusion of "protein of interest" to a heterologous or homologous signal peptide for translocation recognition (Ng and Sarkar, 2012; Ravn *et al*., 2003), overexpression of intracellular chaperone for secretion competency (Martínez-Alonso *et al*., 2010) and overexpression of extracellular chaperone to improve folding of the secreted protein (Lindholm *et al*., 2006). Besides, cultivation strategies were also identified as a factor that contributes to the expression of the recombinant proteins in *L. lactis* host cell. The production yield of heterologous proteins has shown a significant increase in large-scale applications when culture conditions such as culture medium composition, addition of medium supplements, pH medium, incubation temperature, inducer concentration and time of induction and/or aeration are optimized (Berlec *et al*., 2008; Oddone *et al*., 2007; Mierau *et al*., 2005).

As for reference, production of 300 mg/l of *Staphylococcus simulans* biovar lysostaphin protein was reached in *L. lactis* when the cultivation parameters were optimized (Mierau *et al*., 2005). Despite of this improvement, the production yields in *L. lactis* still have not reached the same level as for aerobic bacteria, which can grow to cell densities above 100 g/l of dry cell mass in conventional culture tanks and produce several grams of protein per litre of culture (Knoll *et al*., 2007; Riesenberg and Guthke, 1999).

The potential of *L. lactis* as a cell factory to produce foreign proteins and metabolites leads to questions concerning the host factors that affect protein production and secretion. Most studies focused on developing effective expression systems, strains optimization and optimum expression conditions, including the transformation protocol and vector construction to provide a platform for *L. lactis* as an efficient cell factory in heterologous protein production. However, a comparative analysis of the complete genome sequence of *L. lactis* revealed that the *L. lactis* secretion machinery comprised fewer components than the well-characterized *E. coli* and *B. subtilis* secretion machinery.

Basically, comparative genomics is a large-scale and holistic approach that compares two or more genomes to discover the similarities and differences between the genomes and to study the biology of the individual genomes. Comparative genomics applies to whole genomes or syntenic regions of different species, different subspecies, or different strains of the same species. The scientific impacts of the practical applications of comparative genomics are many and profound. In the past years, there has been an explosion of computational and biological advances in the field of comparative genomics. There are many challenges, however, comparative genomics is undoubtedly one of the most promising scientific fields today (Wei *et al*., 2002).

In this study, preliminary *in silico* comparative analysis allowed the identification of genes (components) specifically involved in protein secretion and production in *L. lactis*, via the signal recognition particle (SRP) secretion pathway. In the SRP pathway (also known as co-translational protein export), the cytosolic

targeting complex is directed to the translocation apparatus through the recognition between the SRP and its receptor. Previous studies claimed and proved that during the protein targeting to the membrane, Ffh (the protein component of SRP) and its specific interaction with FtsY (the SRP receptor) ensures the efficiency and fidelity of protein translocation in *L. lactis* (Morello *et al*., 2010; Chu *et al*., 2004; Tian and Beckwith, 2002; Ulbrandt *et al*., 1997). To date, *L. lactis* being a new attractive protein producer, however no structural or experimental data are yet available concerning these secretion components. This extensive knowledge has led to a detailed understanding and investigation of the intermolecular interaction of *L. lactis* Ffh-FtsY complex, through computational and experimental approaches. LAB, *L. lactis subsp. cremoris* MG1363 was used as a model organism throughout the study.

1.2 Problem Statements

Gram-positive bacteria are considered especially interesting as host organisms to produce heterologous secretory proteins due to its enormous secretion potential and considerable process advantages. However, attempts to use this group of bacteria for the production of the secretory proteins have often led to disappointing results. Most of the times, the yields obtained for heterologous proteins were significantly lower than those observed for homologous enzymes (Simonen and Palva, 1993). This phenomenon might be caused due to several bottlenecks during heterologous protein secretion such as poor targeting to the membrane, inefficient translocation, slow or incorrect post-translocational folding of the secretory protein, proteolysis problems and/or pre-translocational folding. In protein secretion studies, secretion machinery plays a major role in the three-stage protein secretion; protein targeting, protein translocation, and protein release and completion.

Within the *L. lactis* Sec-SRP protein export, gene conservation analysis and experimental data showed that the targeting and translocation processes via the complex Ffh and FtsY play a major role in the protein secretion, known to be essential to ensure the efficiency and fidelity of protein targeting and translocation (Morello *et*

al., 2010). Therefore, modeling and structural characterization study of Ffh-FtsY will give an idea of its crucial region and amino acids that are critical in Ffh-FtsY interaction during protein targeting.

1.3 Objective of the Study

The main objective of this study is to unravel the intermolecular interaction of *L*. *lactis* Ffh-FtsY complex through *in silico* structural characterization approaches.

1.4 Scopes of the Study

The approaches used to achieve the main objective are as follows:

a. Construction of the three-dimensional (3D) structure of Ffh and FtsY.
b. Construction of the Ffh-FtsY protein-protein docking.
c. Analysis of structure-structure interaction in Ffh-FtsY docked proteins to identify its key crucial region(s) and/or amino acid residue(s).
d. Cloning, expression and purification of recombinant Ffh and FtsY in *E*. *coli* expression system.
e. Formation and purification of Ffh-FtsY binary complex.
f. Determination of protein interfaces by intermolecular chemical cross-linking and mass spectrometry (MS).

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