UNIVERSITI PUTRA MALAYSIA

CONSTRUCTION AND CHARACTERIZATION OF A LACTOCOCCUS LACTIS IN-TRANS SURFACE DISPLAY SYSTEM HARBORING MURINE GLYCOSYLATED TYROSINASE RELATED PROTEIN-2

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CONSTRUCTION AND CHARACTERIZATION OF A *LACTOCOCCUS LACTIS IN-TRANS* SURFACE DISPLAY SYSTEM HARBORING MURINE GLYCOSYLATED TYROSINASE RELATED PROTEIN-2

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Food and commensal lactic acid bacteria (LAB) surface display system exploitation for bacterial, viral, or protozoal antigen delivery has received immense interest currently. The Generally Regarded as Safe (GRAS) status of LAB such as *Lactococcus lactis* coupled with non-recombinant strategy of *in-trans* surface display system, provide a safe platform for therapeutic drug and vaccine development. However, therapeutic proteins fused with cell-wall anchoring motif production are predominantly limited to prokaryotic expression system. This presents a major disadvantage in surface display system particularly when glycosylation has been recently identified to significantly enhance epitope presentation. In this study, glycosylated murine Tyrosinase related protein-2, mTRP-2, tumor associated antigen anchoring to *L. lactis* cell wall was attempted. The *mtrp-2-cA* (AcmA, peptidoglycan anchoring motif) fusion gene expression in Chinese Hamster Ovary, CHO cells was carried out. Initial CHO cell expression of both native *mtrp-2* and *mtrp-cA* was a failure. Codon optimized *mtrp-2* and *cA* genes also did not result in target protein production. In order to investigate post-translational modification interruption, expression of codon optimized *mtrp-2*$_{125\text{-}276}$ epitope devoid of *mtrp-2* native maturation signal peptide was performed which resulted in misfolded plus aggregated mTRP-2$_{125\text{-}276}$ and mTRP-2$_{125\text{-}276}$ –cA protein production. Successful expression of both *mtrp-2*$_{125\text{-}276}$ and *mtrp-2*$_{125\text{-}276}$ *-cA* genes suggest CHO cell's endoplasmic reticulum signal peptidase inability to recognize mTRP-2 signal peptide cleavage site. The following substitution of native mTRP-2 signal peptide with Chinese Hamster TRP-2 signal peptide, CHsp resolved this issue by successful expression of soluble mTRP-2 and mTRP-2-cA by CHO cells in both intracellular and extracellular fraction. A total amount of 40 µg of mTRP-2-cA protein from 2.7 g in wet weight of CHO cells was purified and detected to be glycosylated by glycoprotein staining. Subsequent mTRP-2-cA anchoring to the cell wall of *L. lactis* showed excitation of FITC conjugate on secondary antibody which signified successful binding of glycosylated TRP-2 on the surface of *L. lactis*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

PEMBINAAN DAN PECIRIAN SISTEM PEMPAMERAN PERMUKAAN *IN-TRANS LACTOCOCCUS LACTIS* YANG MEMBAWA PROTEIN BERKAITAN TIROSINASE-2 TIKUS TERGLIKOSILAT

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Sejak kebelakangan ini, eksploitasi sistem paparan permukaan bakteria makanan dan komensal seperti Bakteria Laktik Asid (LAB) untuk penyajian antigen bakteria, virus dan protozoa semakin menerima perhatian. Status LAB yang secara umumnya dianggap selamat, (GRAS) seperti *Lactococcus lactis* dan strategi bukan rekombinasi sistem paparan permukaan *in-trans*, mewujudkan satu platform yang selamat bagi penghasilan dadah dan vaksin terapeutik. Walau bagaimanapun, strategi penggabungan protein terapeutik dengan motif pautan dinding sel kebanyakannya terhad kepada sistem pengekspresan prokariot. Ini merupakan satu kelemahan sistem paparan permukaan bakteria terutamanya apabila glikolisasi baru-baru ini dikenal pasti meningkatkan kebolehan pempameran epitop. Dalam penyelidikan ini, pautan antigen berkaitan dengan tumor, *Protein berkaitan Tirosinase-2*, tikus, mTRP-2 yang terglikosilat, pada dinding sel *L. lactis* dikaji. Pengekspresan gen gabungan *mtrp-2-cA* (motif pautan peptidoglikan AcmA) dalam sel Ovari Hamster Cina, CHO dijalankan. Pada permulaannya, sel CHO gagal mengekspres gen *mtrp-2* dan *mtrp-2-cA* yang asli. Pengoptimuman kodon bagi gen *mtrp-2* dan *cA* juga tidak menghasilkan protein sasaran. Bagi menyiasat gangguan modifikasi pasca translasi, pengekspresan epitop *mtrp-2*$_{125\text{-}276}$ yang tidak mempunyai peptida isyarat kematangan asli, dijalankan. Strategi ini menghasilkan protein mTRP-2$_{125\text{-}276}$ dan mTRP-2$_{125\text{-}276}$ −cA yang tergumpal dan tersalah lipat. Kejayaan pengekspresan gen *mtrp-2*$_{125\text{-}276}$ dan *mtrp-2*$_{125\text{-}276}$*-cA* mencadangkan kegagalan enzim peptidase isyarat retikulum endoplasma sel CHO mengenal pasti tapak pemotongan peptida isyarat. Sehubungan dengan itu, penukaran peptida isyarat mTRP-2 asli kepada peptida isyarat TRP-2 Hamster Cina, CHsp dijalankan. Strategi ini berjaya menyelesaikan masalah pengekspresan gen melalui penghasilan mTRP-2 and mTRP-2-cA yang terlarut oleh sel CHO dalam bahagian intrasel dan ekstrasel. Sejumlah 40 µg mTRP-2-cA protein daripada berat basah 2.7 g sel CHO telah berjaya ditulenkan dan hasil glikosilasi dikesan melalui kaedah pewarnaan glikoprotein. Ini dikuti dengan analisa pautan mTRP-2-cA pada dinding sel *L. lactis* yang menunjukkan pengujaan konjugat FITC pada antibodi sekunder, menandakan kejayaan pautan TRP-2 terglikosilat pada permukaan *L. lactis.*

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

~	approximately
°C	degree Celcius
µg	microgram
µl	microlitre
AcmA	N-acetylglucosamidase
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
bp	base pairs
$CaCl_2$	calcium chloride
cDNA	complementary deoxynucleotide acid
CHO	Chinese Hamster Ovary
CWBD	Choline binding domain
Da	Dalton
DCT	DOPAchrome tautomerase
dH_20	distilled water
DHI	5,6-dihydroxyindole
DHICA	5,6-dihydroxyindole-2-carboxylic acid
DNA	deoxyribonucleotide acid
dNTP	deoxyribonucleotide triphosphate
DOPA	L-3,4 –dihydroxyphenylalanine
DQ	DOPAquinone
EDTA	Ethylenediaminetetraacetic acid
EJC	Exon junction complex
ER	Endoplasmic reticulum
eV	electron volt
g	gravity force
GAD	glutamate decarboxylase
GEM	Gram-positive Enhancer Matrix
GlcNAc	N-acetyl-D-glucosamine
GM17	M17 supplemented with 0.5% glucose
GRAS	Generally Regarded as Safe
h	hour
HIF	hypoxia inducible factors
HRP	Horse Radish Peroxidase
hRNP	heteronuclear ribonuclear protein
kb	kilo base pairs
kDa	kilo Dalton
kV	kiloVolt
l	litre
LAB	Lactic Acid Bacteria
LB	Luria-Bertani
LysM	Lysin Motif
M	Molar
mA	milliampere
MBP	Membrane Bound Protein

min	minute
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
$MgCl_2$	magnesium chloride
mRNA	messager Ribonucleic acid
MurNAc	N-acetylmuramic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
ng	nanogram
NICE	Nisin Controlled Gene Expression
OD	Optical Density
PCR	Polymerase Chain Reaction
PTM	Post Translational Modifications
RE	Restriction enzymes
RNAPII	RNA Polymerase II
rpm	revolutions per minute
RT	retention time
SAm	Surface Anchoring motif
SCWP	Secondary cell wall polymers
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
sec	seconds
SLHD	S-layer Homology Domain
SRP	Signal Recognition Particle
TAA	Tumour associated antigen
Ta	annealing temperature
TBP	TATA-binding protein
TCA	Trichloroacetic acid
TF	Transcription Factor
TGN	Trans-golgi network
Tm	melting temperature
TRP-1	Tyrosinase-related protein 1
TRP-2	Tyrosinase-related protein 2
TSA	Tumour specific antigen
V	volt
v/v	volume per volume
VTC	vasicular tubular complexes
W	Watts
w/v	weight per volum

CHAPTER 1

INTRODUCTION

The utilization of food and commensal lactic acid bacteria (LAB) as cellular vehicles for vaccine delivery has received immense interest over the past decade. Besides their GRAS (generally regarded as safe) status compared to their attenuated pathogenic counterparts, the LAB have the ability to colonize animal and human gastrointestinal tracts or genital mucosa with probiotic and immunomodulatory properties. This has made LAB an excellent candidate for oral and intranasal vaccine development (Pontes *et al.,* 2011; Raha *et al.,* 2005). Therefore, the *Lacotococcus lactis* can be genetically engineered to become an efficient recombinant cell factory for DNA delivery as well as production and presentation of antigens (Pontes *et al.,* 2011; Morello *et al.,* 2008). Such presentation of antigens through surface display or secretion by *L. lactis* in numerous studies utilizes the well understood and characterized surface binding protein domain such as transmembrane domains, lysin M, LysM and LPXTG motifs (Bahey-El-Din *et al.,* 2010; Raha *et al.,* 2005).

Based on the above, the LAB has the potential to be developed as a tumour antigen carrier for therapeutic or prophylactic cancer vaccines. Such cancer vaccines would able to mount sustainable immune response to eradicate primary tumour as well as prevent cancer relapses (Pejawar-Gaddy and Finn, 2008). Nevertheless, despite the early discovery of probiotic antitumour activity (Kelkar *et al.,* 1988), the utilization of LAB in anticancer therapy has been limited to cytotoxicity reduction of drugs used in chemotherapy and radiation therapy (Mego *et al.,* 2005). A part from that, the LAB has only been manipulated as prophylactic adjuvants in the prevention of colorectal cancer (Satonaka *et al.,* 1996). Cancer antigen delivery by the LAB, on the other hand, has not been widely explored and was only limited to surface displaying viral antigens from the human papillomavirus type-16 (HPV-16) E7 antigen on *L. lactis* , *Lactobacillus plantarum* and *Lactobacillus casei* for cervical cancer treatment (Ribelles *et al.,* 2013; Cortes-Perez *et al.,* 2005).

The TRP-2 (Tyrosinase related protein-2) is a tumor-associated antigen involved in melanin biosynthesis of both melanocytes and melanoma. TRP-2 has also been intensely studied as a viable therapeutic and prophylactic vaccine candidate for melanoma and glioblastoma (Yamano *et al.,* 2005; InSug *et al.,* 2003). The TRP-2 peptide vaccination alone only resulted in weak T cell response with insignificant tumouricidal effect (Jia *et al.,* 2005). Subsequent attempts to improve the TRP-2 immunogenecity and antigen presentations through plasmid DNA vaccination has been relatively inefficient in inducing antibody response and cellular mediated immunity toward TRP-2 (Yamano *et al.,* 2005). Nevertheless, the TRP-2 DNA vaccination for glioblastomamultiforme treatment has resulted in tumour regression and immunological targeting to increase chemotherapeutic drugs sensitivity (Liu *et al.,* 2005; InSug *et al.,* 2003). Therapeutic effects for melanoma by alphavirus replicon (Avogadri *et al.,* 2010), cytomegalovirus (CMV) (Xu *et al.,* 2013), attenuated *Salmonella typhimurium* (Zhu *et al.,* 2010) and *Listeria Monocytogenes* (Bruhn *et al.,* 2005) carrying TRP-2 have also been reported. Surprisingly, despite well documented adjuvancy of LABs in mucosal immunogenicity (Kajikawa *et al.,* 2010; Mercenier *et al.,* 2000), these GRAS status bacteria have yet to be manipulated to introduce TRP-2 gene for both therapeutic and prophylactic settings. In addition,

common autoimmunity side effect of hypopigmentation (vitiligo) resulting from TRP-2 (self-antigen) immunization have been observed to be dependent on the vaccine strategies (Avogadri *et al.*, 2010; Steitz *et al.*, 2000) suggesting the unknown possibility of GRAS bacteria carrying TRP-2 in generating autoreactive T-cells.

In this study, construction of live *L. lactis* surface displaying TRP-2 was attempted. The novel concept of introducing post-translationally modified TRP-2, *in-trans* to *L. lactis* peptidoglycan was explored. The prospect of using non-recombinant prokaryotes to deliver glycosylated eukaryotic protein, particularly in vaccine application is an attractive one. Recently, N-glycosylation has been identified to significantly enhance epitope presentation of MHC class I molecules by using tyrosinase as model antigen (Ostankovitch *et al.*, 2009). However, the surface display strategy for glycosylated proteins has been restricted to the yeast system which has been a key advantage over other surface display strategies (Boder *et al.*, 1997). Despite such advantage, different linkage of carbohydrate moieties (primarily mannose) to the core glycosyl unit as well as hyperglycosylation have rendered the preference of utilizing mammalian cells against yeast in generating therapeutic glycoproteins (Romanos, 1995; Stratton-Thomas *et al.*, 1995). Therefore a new antigen delivery system is crucial to avoid using carrier at the expense of antigen quality. Non-recombinant, *in-trans* binding of heterologous protein emerge to be an exciting solution for expression host restriction in surface display system. It can be hypothesized that therapeutic proteins such as TRP-2 can be produced in the mammalian cell system and then anchored to the bacterial *L. lactis* cell surface by fusing the cell wall anchoring motif, cA to the aforementioned therapeutic protein.
Therefore, the main objectives of this study is to express and purify a fusion protein comprising TRP-2 and C-terminal cell wall anchoring motif of *L. lactis* N-acetylmuramidase, cA in Chinese Hamster Ovary (CHO) cell system as well as to analyse its anchoring to live *L. lactis* cell wall.

The specific objectives are:

1. To construct vectors for the expression of *trp-2-cA* fusion gene and *trp-2* genes in mammalian CHO expression system.

2. To express the *trp*-2-*cA* and *trp*-2 genes in CHO cells and purify target TRP-2-cA protein;

3. To anchor purified TRP-cA fusion protein on the *L. lactis* cell wall.

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