UNIVERSITI PUTRA MALAYSIA

CONSTRUCTION OF A RECOMBINANT PLASMID FROM NEWCASTLE DISEASE VIRUS ANTIGEN, HAEMAGGLUTININ-NEURAMINIDASE AND LACTOCOCCAL N-ACETYLMURAMIDASE AND ITS EXPRESSION

NUR ADEELA BINTI YASID

FBSB 2009 26

CONSTRUCTION OF A RECOMBINANT PLASMID FROM NEWCASTLE DISEASE VIRUS ANTIGEN, HAEMAGGLUTININ-NEURAMINIDASE AND LACTOCOCCAL N-ACETYLMURAMIDASE AND ITS EXPRESSION

By

NUR ADEELA BINTI YASID

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements of the Degree of Master of Science

February 2009

Specially dedicated to,

My beloved hubby, Nuha Alia, Adam Aqil and families

For their invaluable love, understanding, patience, support and care.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science.

CONSTRUCTION OF A RECOMBINANT PLASMID CARRYING THE CODING SEQUENCES OF N-ACETYLMURAMIDASE AND HAEMAGGLUTININ-NEURAMINIDASE AND ITS EXPRESSION IN CHINESE HAMSTER OVARY CELLS

By

NUR ADEELA BINTI YASID

Februari 2008

Chairman: Professor Raha Abdul Rahim, PhD

Faculty: Biotechnology and Biomolecular Sciences

The anchoring of proteins to the cell surface of Lactococcus using recombinant DNA techniques is an exciting and emerging research area that holds great promise for a wide variety of biotechnological applications. Presently available anchoring systems are based on recombinant bacteria displaying proteins or peptides on the cell surface. This research is focused on using lactococcal cell wall binding (cwb) region for display of viral epitopes and antigenic determinants. Newcastle disease virus (NDV) is the aetiological agent of Newcastle disease which can result in 100% morbidity and mortality in chicken. Immune response of haemagglutinin-neuraminidase (HN) protein antigens of NDV plays an important role in the prevention of viral infection. A lactococcal domain coding region, *acmA'* (300 bp) was cloned into the mammalian expression vector, pcDNA3.1/His. The 1.7 kb HN gene was then inserted at the 5'-end of the *acmA'* coding region. Plasmid extraction and restriction enzyme digestion analyses showed that cloning

was carried out successfully. Sequencing results indicated that the inserts (*acmA'*, HN and *acmA'*HN) were 99%, 97% and 98% homologous to the published sequence of *Lactococcus lactis* and NDV strain AF2240. The condition for transfection was optimised by testing various amounts of transfected DNA (1 µg, 2 µg and 3 µg) and different charge ratio of liposome reagent (µl): DNA (µg) in a 6-well plate. Transient expression of the fusion protein in Chinese hamster ovary (CHO) cells was analysed by SDS-PAGE and Western blotting. The results indicated that the best combination of DNA used for transfection was 2 µg in 1:3 charge ratio of the liposome:DNA. This study showed that a recombinant *acmA'*, HN and *acmA'*-HN have been successfully constructed, transfected and expressed in CHO cells. This work could contribute towards the development of a recombinant vaccine delivery system for NDV.

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

PENGHASILAN PLASMID REKOMBINAN MEMBAWA JUJUKAN KOD N-ACETYLMURAMIDASE DAN HAEMAGGLUTININ-NEURAMINIDASE DAN PENGEKSPRESAN DI DALAM SEL CHINESE HAMSTER OVARY

Oleh

NUR ADEELA BINTI YASID

Februari 2009

Pengerusi: Profesor Raha Abdul Rahim, PhD

Fakulti: Bioteknologi dan Sains Biomolekul

Perlekatan protein pada permukaan sel *Laktokokus* menggunakan teknik rekombinan DNA merupakan satu bidang penyelididkan yang menarik serta menjanjikan harapan yang tinggi kepada pelbagai penggunaan teknologi. Sistem perlekatan sedia ada adalah berdasarkan rekombinan bakteria yang mempunyai protein atau peptida yang melekat pada permukaan selnya. Kajian ini difokuskan kepada sistem laktokokal untuk perlekatan epitop virus dan penentu antigen. Virus penyakit Newcastle (NDV), adalah agen atiologi bagi penyakit Newcastle yang boleh menyebabkan 100% morbiditi dan mortaliti pada ayam. Reaksi keimunan terhadap antigen protein hemaglutinin-neuraminidase (HN) NDV memainkan peranan yang penting dalam pencegahan jangkitan. Domain pengikatan dinding sel (cwb) dari *L. laktis*, gen *acmA'* (300 bp) diklonkan ke dalam vektor penzahiran mamalia, pcDNA3.1/His (5.5 kb). Gen HN (1.7 kb) pula disubklonkan di hujung 5'-domain pengikatan *acmA'*. Pada masa yang sama, gen HN diklonkan ke dalam vektor yang kosong untuk kajian perbandingan. Pengekstrakan

plasmid dan analisis enzim pembatas menunjukkan pengklonan berjaya dilakukan. Analisis jujukan DNA menunjukkan bahawa rekombinan *acmA'*, HN dan *acmA'*HN mempunyai 99%, 97% dan 98% persamaan dengan jujukan *L. laktis* dan NDV yang telah diterbitkan. Keadaan transfeksi dioptimakan dengan mengkaji pelbagai amaun DNA (1 µg, 2 µg dan 3 µg) dengan nisbah cas reagen liposome (µl) kepada DNA (µg) di dalam piring 6 lubang. Pengekspresan sementara telah diasai dalam sel CHO dengan SDS-PAGE dan Pemblotan western. Keputusan menunjukkan nilai kombinasi DNA terbaik untuk transfeksi adalah 2 µg pada nilai cas liposome kepada DNA 1:3. Kajian ini juga menunjukkan rekombinan *acmA'*HN berjaya dibina, ditransfeksi dan dizahirkan dalam sel CHO. Kajian ini boleh menyumbang kepada penghasilan sistem rekombinan vaksin untuk NDV.

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation and thanks to my main supervisor, Prof. Dr. Raha Abdul Rahim, for her never ending guidance and encouragement throughout this project. Her patience and dedication have inspired me to give my very best in completion of this study and the thesis.

My sincere thanks to my co-supervisor, Prof. Datin Paduka Dr. Khatijah Yusoff for her invaluable advice and constructive criticism during the entire progress of this project. Her suggestions and comments definitely helped in the success of this thesis.

My heartiest appreciation goes to my beloved husband, Shahrul Ezhar, my little daughter and son, Nuha Alia and Adam Aqil and families for their continual support and understanding for me to proceed completion.

A special note of appreciation also goes out to the staff from Faculty of Biotechnology and Biomolecular Sciences and everyone who have contributed in one way or another to this study.

I am truly indebted to my lab mates, Kak Yan, Sab, Lina, Alai, Boon Hooi, Wei Yeng, Anu, Dr. Varma, Dr. Elida, Dr. Prema, Dr. Yiap and Kak Ernie for their helpful collaboration and discussion. Also not forgetting my friends, Wawa, Fazu, Surini, Taznim and a bunch of others that have really helped me in any possible way there could be. May god bless you all.

I certify that an Examination Committee has met on 4.2.09 to conduct the final examination of Nur Adeela Yasid on her degree thesis entitled "Construction of a Recombinant Plasmid Carrying The Coding Sequences of N-Acetylmuramidase and Haemagglutinin-Neuraminidase and Its Expression in Chinese Hamster Ovary Cells" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the Master of Science degree.

Members of the Examination Committee were as follows:

Raja Noor Zaliha Raja Abdul Rahman, PhD
Professor,
Faculty of Biotechnology and Biomolecular Studies
Universiti Putra Malaysia
(Chairman)

Tan Wen Siang, PhD
Professor,
Faculty of Biotechnology and Biomolecular Studies,
Universiti Putra Malaysia.
(Internal Examiner)

Sieo Chin Chin, PhD
Dr,
Faculty of Biotechnology and Biomolecular Studies,
Universiti Putra Malaysia.
(Internal Examiner)

Mohd Nazalan Mohd Najimudin, PhD
Professor,
School of Biological Sciences,
Universiti Sains Malaysia,
Malaysia
(External Examiner)

BUJANG KIM HUAT, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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:

Raha Abdul Rahim, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Khatijah Yusoff, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

HASANAH MOHD GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I hereby declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently submitted for any other degree at Universiti Putra Malaysia or at any other institutions.

_____

NUR ADEELA BINTI YASID

Date:

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Aa	amino acid
AcmA	N-acetylmuramidase
Amp	Ampicillin
anti-His	antibody against His tag
Bp	Basepair
BLAST	basic local alignment search tool
B	Beta
CO_2	carbon dioxide
$CaCl_2$	calcium chloride
dH_2O	distilled water
DMSO	Dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotides triphosphates
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetate
EtBr	ethidium bromide
F	fusion protein
GRAS	generally regarded as safe
H	Hour
His	Histidine
HRP	horseradish peroxidise
Kb	kilobase pair

kDa	Kilo Daltons
LAB	lactic acid bacteria
LB	luria bertani
L. lactis	*Lactococcus lactis*
M	Molarity
mA	Milliampere
MCS	multiple cloning site
Mg	Milligram
$MgCl_2$	magnesium chloride
Min	Minute
Ml	Millilitre
mM	Millimolar
mRNA	messenger RNA
NaCl	sodium chloride
N-terminal	the amino-terminal (NH_2) of a polypeptide
nm	Nanometer
NDV	Newcastle disease virus
ºC	degrees centrigrade
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	potential of Hydrogen
PVDF	polyvinylidene difluoride
RE	restriction enzyme

RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse-transcriptase PCR
s	Second
SDS-PAGE	Sodium dodecyl polyacrylamide gel electrophoresis
sp.	Species
subsp.	Subspecies
TAE	tris-acetate EDTA
Taq	*thermus aquaticus*
TEMED	Tetramethyl-ethylene diamine
U	Unit
UV	Ultraviolet
v	Volt
µg	microgram
µl	microlitre
µm	micromolar
v/v	volume per volume
w/v	weight per volume
%	Percentage
x *g*	centrifugal force
X-gal	5-bromo-4-chloro-3-indol-β-D-galactosidase

CHAPTER 1

INTRODUCTION

Heterologous surface display of proteins in bacteria has become an increasingly important study. Cell-surface display allows peptides and proteins to be displayed on the surface of bacterial cells by fusing them with anchoring motifs such as Leu-Pro-Xaa-Thr-Gly- (LPXTG), bacterial fimbriae and S-layer proteins (Lee *et al*., 2000; Sang *et al*., 2003). The most common application for bacterial surface display is the development of live bacterial vaccine delivery systems, since cell surface display of heterologous antigenic determinants has been considered advantageous in order to induce antigen-specific antibody responses when live recombinant cells were used for immunisation (van Roosmalen *et al*., 2005).

Many studies have been carried out to evaluate the effect of antigen-presenting systems by Gram-positive bacteria. This is because Gram-positive bacteria have certain properties that potentially make them more suitable for bacterial surface display applications. First, the surface receptors of Gram-positive bacteria seem to be more permissive for the insertion of extended sequences of foreign proteins (Fischetti *et al*., 1993) compared to the different Gram-negative systems. Another advantage is the translocation proud in which only a single membrane is required to achieve proper surface exposure of the heterologous peptides. In addition, the thicker cell wall of Gram-positive bacteria is more rigid

and allows various laboratory procedures to be carried out without extensive cell lysis. Expression systems for the mouth commensal bacterium *Streptococcus gordinii* (Pozzi *et al*., 1992) and the nonpathogenic bacterium *Staphylococcus xylosus* (Hansson *et al*., 1992) have been developed based on the fibrillar M6 protein from *Streptococcus pyogenes* and protein A from *Staphylococcus aureus*, respectively. Staphycoccal protein A (SpA) has often been used as a model system to study the anchoring mechanisms of surface proteins in Gram-positive bacteria (Ton-That *et al*., 1997; Marraffini *et al*., 2004). It is because the sorting signals of SpA include LPXTG motif contains a cleavage site for sortase (SrtA). The hydrophobic domain is a membrane-spanning region. The charged tail serves as a retention signal to prevent secretion of the polypeptide chain into the surrounding medium.

There is an interest in developing an alternative vaccine carrier system based on harmless and nonpathogenic Gram-positive bacteria, such as *Lactococcus lactis*. The bacterium has a long history of safe use in food fermentation (Sorensen *et al*., 2000; Hansen, 2002) and possesses both GRAS (generally regarded as safe) and food grade status. It is able to survive in the gastrointestinal tract of humans and other animals for 2-3 days, but it does not invade or colonise the mucosal surface, nor does it evoke an immune response against itself. The fact that it is non-colonizing, non-invasive and has low innate immunogenic makes it a desirable model as a recombinant vaccine delivery system.

However, this model has one major drawback in the delivery of eukaryotic epitopes from the bacterial system. For example, lactococcal processing does not assure post-translational modifications such as glycosylations, and disulfide bond formation may be limited due to the lack of disulfide isomerase. This results in a very low quantity of the active form of the protein of interest, due to a low rate of passive formation of disulfide bonds as observed with IL-2 in *L. lactis* (Bermudez-Humaran *et al*., 2003a) and ovine omega interferon (Bermudez-Humaran *et al*., 2003b).

Previous research has shown the capability of targeting cell wall binding protein to *L. Lactis* to the surface of bacterial cells (Steen *et al*., 2003; van Roosmalen *et al*., 2006; Moorthy and Ramasamy, 2007). A strategy to use recombinant cell wall of *L. lactis* as an antigen carrier and display of proteins or peptides in an eukaryotic expression system has been developed to try and overcome the above problem. The cell wall anchor motif (protein anchor) of the *L. lactis* cell wall hydrolase AcmA is a versatile tool for the display of heterologous proteins on the surface of Gram-positive bacteria. It consists of three domains. The N-terminal signal sequence is followed by an active domain and C-terminal membrane anchor. In the C-terminal part of the protein, three 44-amino acid repeated regions are present, and these are separated by intervening sequences. A system displaying heterologous proteins to the C-terminus of AcmA has been established by Raha *et al*. (2005) and Okano *et al*. (2008). They successfully expressed the protein in *E. coli* and *L. lactis*, and also demonstrated the display of target protein on *L. lactis*.

Haemagglutinin-neuraminidase (HN) gene encodes for a surface glycoproteins of Newcastle disease virus (NDV) possesses both haemagglutinin (HA) and neuraminidase (NA) activities. It also has an undefined role in fusion as well as the major antigenic determinant of the virus (Bousse *et al*., 2004). In this study, a constructs were developed containing the HN recombinant protein that can be expressed together with the anchor motif in an animal culture system. Since the expression of AcmA protein in mammalian cells has not been demonstrated so far, the main objective of this work was to determine the ability of a bacterial cell wall binding protein to fuse with HN gene and express in a mammalian cell system. This system would allow for the expression of viral epitopes that needs post-translational system to be displayed on the surface of *L. lactis*.

The objectives of this study were:

a. To isolate and amplify the *acmA'* gene of *L. lactis* and HN gene of NDV strain AF2240;
b. To construct specialized vectors for expression of recombinant HN proteins; and
c. To study the expression of the above genes in Chinese hamster ovary (CHO) cells.

CHAPTER 2

LITERATURE REVIEW

2.1 SURFACE DISPLAY OF HETEROLOGOUS PROTEINS

2.1.1 Cell Wall of Gram-Positive Bacteria

Gram-positive bacteria are simple cells. On the basis of morphological criteria, three distinct cellular compartments can be distinguished: the cytosol, a single cytoplasmic membrane and the surrounding cell. The cell envelope of lactic acid bacteria consists of a cytoplasmic membrane and a cell wall. The cell wall protects the underlying protoplast, resists turgor and maintains the shape of the cell. Structured protein layers (S-layer) or polysaccharide Gram-positive bacteria also serves as an attachment site for proteins. Protein attachment involves either the covalent binding of LPXTG-carrying proteins to the peptidoglycan pentaglycine cross-bridge or the noncovalent binding of protein to the peptidoglycan. It requires a C-terminal sorting signal which an LPXTG motif, a hydrophobic domain interacting with the cytoplasmic membrane, a charged tail preventing secretion and a sortase, which catalyses the transpeptidation reaction (Schneewind *et al*., 1992; Mesnage *et al*., 2000). In general, the cell wall of Gram-positive bacteria is composed of a thick layer of peptidoglycan, accessory polysaccharides, teichoic acid, lipoteichoic acid (LTA) and proteins