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CLONING AND EXPRESSION OF THE XYLANASE GENE FROM *BACILLUS COAGULANS* AND THE M GENE OF NEWCASTLE DISEASE VIRUS IN *LACTOCOCCUS LACTIS*

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By

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CLONING AND EXPRESSION OF THE XYLANASE GENE FROM *BACILLUS COAGULANS* AND THE M GENE OF NEWCASTLE DISEASE VIRUS IN *LACTOCOCCUS LACTIS*

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Chairman: **Associate Professor Khatijah Mohd. Yusoff, Ph.D.**

Faculty: **Science and Environmental Studies**

Lactococcus lactis is being developed as a vaccine delivery system as it bears no threat to animal and human health. It has been used for centuries in the fermentation of foods and is generally recognised as safe. However, a safety factor pertaining to the type of selectable marker present on the vector system poses to be of concern. Chances of the transfer of antibiotic resistance genes, usually employed by vectors as selectable markers, into the natural environment become a possible risk. Therefore, there is a need for the development of ideal vectors without such selectable markers (Dertzbaugh, 1998).

The activity of the xylanase gene of *Bacillus coagulans* ST-6 can be detected on Remazol Brilliant Blue-Xylan (RBB-Xylan) as a clear halo zone against a dark blue background. This characteristic allows xylanase to be used as a selectable chromogenic marker on any vector system. On the other hand, the matrix or membrane (M) protein of Newcastle disease virus (NDV) strain AF 2240 can be useful as an antigen to generate antibody response towards NDV infection in chickens.

Both, the xylanase gene (0.8 kb) of *B. coagulans* ST-6 and the M gene (1.1 kb) of NDV strain AF 2240 were cloned in lactococcal expression vector pMG36e and transformed into *Escherichia coli* XLI-blue MRF'. The recombinant plasmids, pMG36e-X and pMG36e-X-M were sub-cloned into *L. lactis* MG1363 via electroporation. The insertion and orientation of the xylanase gene was confirmed using restriction enzyme analysis and PCR amplification. Xylanase activity and expression on RBB-Xylan agar plates further confirmed its presence in the recombinant plasmid pMG36e-X. In addition, the enzyme activity was also quantitatively showed using the Somogyi-Nelson assay. The sequence of the M gene obtained in clone pMG36e-X-M from *L. lactis* MG1363 was found to be 99% homologous to the established sequence (Jemain, 1999). Expression of the fusion M protein was studied at the transcriptional level. RT-PCR was used to detect the transcription of the gene using RNA of *L. lactis* MG1363 containing the recombinant plasmid pMG36e-X-M as template. The size of the RT-PCR product correlated with the size of the cloned M gene (1.1 kb). In addition, the RT-PCR product was sequenced to confirm the presence of the M gene.

Based on the results obtained, recombinant plasmids pMG36e-X and pMG36e-X-M were successfully constructed and introduced into *E. coli* XLI-blue-MRF' and *L. lactis* MG1363. The recombinant DNA pMG36e-X is capable of expressing the xylanase gene and can be further developed as a chromogenic selection marker for a new and improved food-grade shuttle vector for *E. coli* and *L. lactis*. On the other hand, expression of the fusion M gene was detected at transcriptional level in recombinant clone pMG36e-X-M.

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

PENGKLONAN DAN PENGEKSPRESAN GEN XILANASE DARIPADA *BACILLUS COAGULANS* DAN GEN M DARIPADA VIRUS PENYAKIT NEWCASTLE KE DALAM *LACTOCOCCUS LACTIS*

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Lactococcus lactis dikaji sebagai agen pengangkutan vaksin kerana ia tidak membahayakan kesihatan haiwan dan manusia. Ia digunakan dalam proses fermentasi makanan sejak beratusan tahun dahulu dan sehingga kini dianggap selamat. Isu tentang faktor keselamatan berkenaan jenis penanda pilihan dalam sistem vektor diberikan perhatian. Gen penahanan antibiotik selalu digunakan dalam vektor sebagai penanda pilihan dan pemindahannya ke dalam keadaan semulajadi mungkin mendatangkan risiko. Dengan itu, pembinaan vektor yang tidak menggunakan penanda pilihan sebegini adalah memanfaatkan (Dertzbaugh, 1998).

Enzim xilanase daripada *Bacillus coagulans* ST-6 boleh dikesan sebagai zon cerah terhadap latar belakang yang biru menggunakan Remazol Brilliant Blue-Xylan (RBB-Xylan). Sifat ini menjadikan gen xilanase berpotensi sebagai penanda pilihan jenis kromogenik dalam sebarang sistem vektor. Sebaliknya, matrix atau membran (M) protein yang immunogenik daripada virus penyakit Newcastle (NDV) strain AF2240 boleh digunakan sebagai antigen untuk menghasilkan tindakbalas antibodi terhadap jangkitan NDV di dalam ayam.

Gen xilanase (0.8 kb) daripada *B. coagulans* ST-6 dan gen M (1.1 kb) daripada NDV strain AF 2240 telah diklonkan ke dalam vektor pengekspres pMG36e dan dipindahkan ke dalam *Escherichia coli* XLI-blue MRF'. Kedua-dua plasmid rekombinan pMG36e-X dan pMG36e-X-M ini dipindahkan ke dalam *L. lactis* MG1363 secara "electroporation". Pengesahan dan penentuan orientasi gen xilanase dibuat dengan penganalisasi enzim pembatas dan amplifikasi DNA secara PCR. Aktiviti dan ekspresi xilanase di atas RBB-Xylan mengesahkan kehadirannya dalam plasmid rekombinan pMG36e-X serta penentuan activiti enzim xilanase secara kuantitatif dilaksanakan dengan cara Somogyi-Nelson. Jujukan DNA gen M dalam klon pMG36e-X-M daripada *L. lactis* MG1363 menunjukkan bahawa ia mempunyai homologi sebanyak 99% berbanding dengan jujukan DNA yang telah ditentukan (Jemain, 1999). Pengekspresan gen M pada tahap transkripsi ditentukan. Cara yang digunakan untuk mengkajinya ialah secara RT-PCR menggunakan RNA daripada *L. lactis* MG1363 yang mempunyai plasmid rekombinan pMG36e-X-M. Produk yang diperolehi daripada RT-PCR adalah bersaiz 1.1 kb iaitu sama saiz dengan gen M yang diklonkan. Penentuan jujukan DNA daripada produk RT-PCR juga dilaksanakan untuk memastikan kehadiran gen M.

Berdasarkan kepada keputusan yang diperolehi, plasmid rekombinan pMG36e-X dan pMG36e-X-M telah berjaya direkabentuk dan dimaukkan ke dalam *E. coli* XLI-blue-MRF' dan *L. lactis* MG1363. Plasmid rekombinan pMG36e-X mampu megekspreskan enzim xilanase dan boleh digunakan sebagai penanda pilihan jenis kromogenik untuk menghasilkan vektor "food-grade" yang mempunyai ciri-ciri yang lebih baik untuk *E. coli* and *L. lactis*. Sebaliknya, pengekspresan gen M telah dipastikan pada tahap transkripsi mRNA.

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I certify that an Examination Committee met on 8th March 2001 to conduct the final examination of Chang Li Yen on her Master of Science thesis entitled "Cloning and Expression of a Xylanase Gene from *Bacillus coagulans* and an M Gene from Newcastle Disease Virus Strain in *Lactococcus lactis*" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

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

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Chang Li Yen

Date: 2 APRIL 2001

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LIST OF ABBREVIATIONS

Ω	-	ohms
°C	-	degrees centigrade
µF	-	microFarraday
A	-	adenine base nucleotide
BCIP	-	5-bromo-4-chloro-3-indoyl phosphate
bp	-	base pair
C	-	cytosine base nucleotide
$CaCl_2$	-	calcium chloride
cDNA	-	complementary DNA
Da	-	Dalton
DEPC	-	diethyl pyrocarbonate
DNA	-	deoxyribonucleic acid
dNTPs	-	deoxynucleotide triphosphate
DTT	-	dithiothreitol
EDTA	-	ethylenediamine tetraacetic acid
G	-	guanine base nucleotide
HCl	-	hydrochloride acid
kb	-	kilobase pair
kDa	-	kiloDalton
kV	-	kiloVolts
kV/cm	-	kiloVolts per centimetre
mA/gel	-	milliAmpere per gel
$MgCl_2$	-	magnesium chloride

mRNA	-	messenger RNA
N	-	nucleotide
NBT	-	nitro blue tetrazolium
pI	-	isoelectric point
PVDF	-	polyvinylidene difluoride
RNA	-	ribonucleic acid
rRNA	-	ribosomal RNA
sp.	-	species
spp.	-	species
subsp.	-	subspecies
T	-	thymine base nucleotide
TBS	-	Tris buffered saline
TBST	-	Tris buffered saline-Tween®20
TEMED	-	N,N,N',N'-Tetramethylethylenediamine
U	-	uridine base nucleotide
U/µg	-	unit per microgramme
U/mL	-	unit per millilitre
V/cm	-	Volts per centimetre
v/v	-	volume per volume
w/v	-	weight per volume

CHAPTER I

INTRODUCTION

The ability of the host immune system to recognise and neutralise invading foreign organisms is the basis of defense mechanism against infectious agents (Yong Kang, 1989). Vaccination has been practiced for over 2 centuries since Edward Jenner, who, in 1796 attributed the first scientific attempts to control an infectious disease (small pox) (Brown *et al.*, 1993). In addition, vaccination is the most cost effective means to control viral diseases in poultry and pigs industries. Outbreak of viral diseases may cause substantial economic losses. In general, conventional viral vaccines used for disease control have potential risks. Live attenuated viruses used as vaccine carries the risk that they might revert to a virulent state and establish persistent or latent infection. On the other hand, killed or inactivated virus vaccine may lead to disease outbreaks if inadequately inactivated or mutation of the wild-type virus will result in the existence of a highly resistance virus. Recent advances in biotechnology especially molecular biology and protein chemistry have offered prospects for the development of a new generation of vaccines that are safer, cheaper and more effective which are mainly genetically engineered vaccines.

Bacterial-based systems as live vectors for the delivery of heterologous antigens offer a number of advantages as a vaccination strategy. Both Gram-negative and Gram-positive bacteria have been investigated for the delivery of foreign antigens. The current knowledge of molecular biology and genetics as well as recombinant DNA techniques has allowed the insertion of genes encoding the antigens to be

delivered, into non-pathogenic carrier for expression (Liljeqvist and Ståhl, 1999). Among the Gram-positive bacteria, *Lactococcus* are designated GRAS or 'generally recognised as safe' organisms, which are traditionally used in the preservation of foods and the development of flavour and texture in the final product. Furthermore, they are non-pathogenic, non-invasive, non-colonising and they bare no threat to human and animal health. They also have the capacity to secrete proteins allowing surface expression or extracellular production of heterologous enzymes or proteins. These features render them to be the ideal choice for the safe production of commercially significant foreign proteins and medical applications (van de Guchte *et al.*, 1992; Leenhouts and Venema, 1992; 1993). Developments in genetic manipulation have given these Gram-positive lactic acid bacteria (LAB) the advantage to be used as a host expression system for antigen delivery to induce immune response (Wells *et al.*, 1993b; Robinson *et al.*, 1997).

The type of selectable marker present on vaccine delivery vector system is a matter of great concern. Vectors require a selective property usually antibiotic resistance gene such as ampicillin, streptomycin and erythromycin resistance gene. This type of vector system may carry the risk of anaphylactic reaction in the recipient and the possible transfer of the resistance gene into the natural environment often resulting in the emergence of new drug resistant pathogens (Dertzbaugh, 1998). Therefore, development of the LAB for use as a vaccine delivery vehicle to be devoid of antibiotic resistance genes can be seen as the safer alternative. Suitable chromogenic genes may replace antibiotic resistance genes as the selectable marker in vector systems. An example of a suitable chromogenic marker is the xylanase gene of *Bacillus coagulans* ST-6, which has the ability to hydrolyse glycosidic linkages of

the xylan polysaccharide. The activity of this enzyme can be detected on a suitable substrate such as Remazol Brilliant Blue-Xylan (RBB-Xylan) as a clear halo zone against a dark blue background (Biely *et al.*, 1985). Hence, this characteristic gives xylanase the potential to be used as a selectable marker on any vector system.

On the other hand, the matrix or membrane (M) protein of Newcastle disease virus (NDV), a negative-strand RNA virus belonging to the *Paramyxoviridae* family (Faaberg and Peebles, 1988) can be useful as an antigen to generate antibody response towards NDV infection in chickens. NDV causes severe respiratory infection in poultry requiring control by vaccination or quarantine with slaughter of all birds in confirmed outbreaks. Successful cloning of the immunogenic M gene in a food-grade vector system without antibiotic resistance selectable marker and followed by the expression of the fusion protein in a *Lactococcus* may be an interesting approach in the development of vaccine delivery systems.

Objectives

The objectives of this study are:

1. to clone the xylanase gene from *B. coagulans* ST-6 in the lactococcal expression vector pMG36e and to express it in *L. lactis* MG1363,
2. to clone the M gene from NDV strain AF 2240 in the newly constructed plasmid, pMG36e-X, and
3. to analyse the expression of the fused M gene in *L. lactis* MG1363 at the transcriptional level.

CHAPTER II

LITERATURE REVIEW

Viral Vaccine

Introduction

Defense against infectious agents relies on the ability of the host immune system to recognise and neutralise the invading organism. To prevent virus infection, vaccination has been practiced for over two centuries since the time of Edward Jenner, as it is the most important and cost-effective means to control and prevent infectious diseases such as viral diseases in poultry and pigs industries apart from quarantine and other strategies to eradicate diseases. Outbreak of viral diseases can cause substantial barriers to international trade in animal and animal products. The successful development of vaccines has kept many major diseases under control. Yet, there is a growing need for improvements of existing vaccines in terms of increased efficacy and improved safety (Yong Kang, 1989; Liljeqvist and Ståhl, 1999). Better technological possibilities, combined with increased knowledge in related fields, such as immunology and molecular biology, allow new vaccination strategies. However, relatively little changes have been made and the immunogens widely used today are killed or live attenuated viruses, for example measles, rubella and polio vaccine (Plotkin, 1993). Live virus vaccines carry the risk that they may revert to a virulent state, which may then lead to disease when using virus especially in immunocompromised hosts. Likewise, killed or inactivated virus vaccines also

have potential risks; such as inadequate inactivation could lead to disease outbreaks caused by residual active wild-type virus in the vaccine. Moreover, mutation of the wild-type virus could result in the existence of a highly resistance virus (Yong Kang, 1989).

Recent attention has focused upon the design and production of 'subunit vaccines' consisting of non-viable or non-replicating and non-infectious portions of the pathogenic agent that are still capable of eliciting a protective immune response. This type of vaccine is attractive because it circumvents some of the concerns associated with intact viral immunogens, such as incomplete inactivation or unsatisfactory attenuation of the virus stock and possible biological contamination of the vaccine (Yong Kang, 1989).

Advances in biotechnology especially molecular biology and protein chemistry technologies, offer prospects in the development of safer, cheaper and more effective vaccines and the possibility of the production of highly purified biologically active protein subunits directed towards the preparation of protein subunit vaccines. In addition, recombinant DNA technology are now dominating in the strive for ideal vaccines against infectious agents which cannot be cultivated (Yong Kang, 1989).

Conventional Viral Vaccines

Conventional viral vaccines for disease control in animals relied upon two approaches; inactivated vaccines and live attenuated vaccines. The former requires large-scale cultivation of the virus followed by specific inactivation and delivery

with an appropriate adjuvant to boost the animal's immune response. Immunity generated by this inactivated vaccines tends to be short lived; consequently frequent revaccination is required to maintain individual and herd immunity (Dertzbaugh, 1998).

As for the live attenuated vaccines, attenuation is achieved by multiple passages of the virus in a suitable laboratory host sometimes under aberrant culture conditions (e.g. cold temperature) or by the use of a naturally attenuated virus strain or a related virus from a similar host species (Dertzbaugh, 1998).

New Generation Viral Vaccines

Molecular biology has opened up entirely new approaches to the development of vaccines. Advanced strategies are employed for the production of genetically engineered subunit viral vaccines.

Ribosomal DNA and expression systems allow the large-scale production of viral antigens from suitable procaryotes and eukaryotes to be used as effective and protective vaccines. The power of recombinant DNA techniques allows portions of viral antigens to be expressed alone or as fusions (Yong Kang, 1989). Effective vaccine antigens have been produced with the expressed viral antigen, such as the hepatitis B surface antigen expressed in yeast (McAleer *et al.*, 1984).

In general, determinants of surface antigens (mainly proteins) are responsible for the induction of neutralising antibodies and hence induce immunity. Virus neutralisation

is mediated mainly by the binding of antibody to one or more portions of a viral surface protein required for some initial steps in virus infection. Thus, the primary target for antiviral vaccine development is an antigenic surface protein(s) of a virus (Yong Kang, 1989). However, it has been reported that neutralising antibodies alone is not sufficient for protection (Boere *et al.*, 1983; Meloen *et al.*, 1983). Internal antigens may act as a secondary target and it is also possible that an antigen that fails to induce neutralising antibodies can still induce an antibody that provides protection against disease. Despite the fact that the mechanism of such protection is not well documented but it may well be advantageous to use an internal core antigen or other antigens that do not elicit a neutralising antibody response when preparing subunit vaccine using recombinant DNA techniques. These antigens which induce non-neutralising antibodies should be considered as useful targets for vaccine development.

LAB as an Antigen and Enzyme Delivery System

Bacterial-based expression systems are the most convenient to use and they can express antigen(s) at very high levels. Although various studies on the development of recombinant bacteria for antigen delivery have been reported, they are often focused on the use of pathogenic bacteria (Aldovini and Young, 1991; Chatfield *et al.*, 1992; Stover *et al.*, 1993). On the contrary, the LAB offer a useful expression system for the production of large quantities of desired gene products for the induction of immune response, as they bear no threat to animal and human health. Moreover, the LAB is non-pathogenic, non-invasive and non-colonising and they have been used for centuries in the fermentation of foods and are generally