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CLONING OF EUKARYOTIC EXPRESSION CASSETTE INTO PMG36E LACTOCOCCAL VECTOR

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BACHELOR OF SCIENCE (HONS.) CELL AND MOLECULAR BIOLOGY

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CLONING OF EUKARYOTIC EXPRESSION CASSETTE INTO pMG36e LACTOCOCCAL VECTOR

By

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Thesis submitted to the Department of Cell and Molecular biology, Faculty of Biotechnology and Biomolecular Sciences, in Fulfilment of the requirement for Degree of Cell and Molecular Biology.

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Abstract of thesis presented to the Department of Cell and Molecular Biology in fulfillment of the requirement of the degree of Cell and Molecular Biology.

Cloning of Eukaryotic Expression Cassette into pMG36e Lactococcal Vector

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Lactococcuslactis(*L. lactis*) is a Gram-positive bacteria traditionally used in food industry for flavouring and fermenting processes. In recent years, *L. lactis* was found to be able to deliver plasmid DNA into eukaryotic cells and express heterologous protein, which makes it being developed as safer alternative for vaccination. The increasing demand of *L. lactis*beyond application in food industry raises the need to develop lactococcal bicistronic vector with target gene expression in the eukaryotic system. The objective of this study is to construct a lactococcal vector containing modified eukaryotic expression cassette, with internal ribosome entry site (IRES) inserted between CMV promoter and Poly A signal to allow transcription and translation of two proteins in a single cassette. IRES sequence was PCR-amplified from pRetroX-IRES-ZsGreen1 vector, digested with *Eco*RV and *Xho*I, and ligated into eukaryotic expression cassette from pcDNA 3.1 HisA. Constructed vector was transformed into *E. coli* TOP 10 and the orientation of IRES fragment in the modified eukaryotic expression cassette was confirmed by DNA sequencing. The modified eukaryotic expression cassette was PCR-amplified from pcDNA3.1HisA/IRES before sub-cloning into lactococcal vector pMG36e.pMG36eharbouringmodified eukaryotic expression cassette was successfully transformed into *E. coli* TOP 10 but not *L. lactis* NZ9000. Optimization of transformation protocol for *L. lactis* NZ9000 using electroporation is needed to enhance transformation efficiency. Segragational instability of constructed vector in *E. coli* host was detected, which may have been caused by production of single-stranded intermediates and high-molecular weight DNA due to rolling-cycle replication of the pMG36e vector.

Keywords: eukaryotic expression cassette, IRES, *Lactococcuslactis*, pMG36e

Abstrak thesis yang dikemukakan kepada Jabatan Biologi Sel dan Molekul sebagai memenuhi keperluan untuk ijazah Sel dan Molekula Biologi.

Pengklonan Kaset Pengekspresan Eukariot ke dalam Vektor Lactococcal pMG36e

Oleh

Lim Yi Yi

June 2015

Dr. Nurulfiza Mat Isa, PhD
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Lactococcus lactis (*L. lactis*) merupakan bakteria Gram-positif yang digunakan untuk proses penambahan perisa and fermentasi dalam industry makanan. Kebelakangan ini, kebolehan *L. lactis* untuk menyalurkan DNA plasmid ke dalam sel eukariot dan ekspres protein "heterologous" telah dijumpai, seterusnya menjadikan *L. lactis* sebagai alternatif yang selama tuntuk vaksinasi. Pemintaan tinggi *L. lactis* dalam pelbagai bidang selain daripada industry pemakanan telah merangsang pembanguan vector lactococcal "bicistronic" yang mensasarkan ekspresi gen di dalam system eukariot. Objektif kajian ini adalah untuk membina vector lactococcal yang mempunyai kaset pengekspresan eukariot yang diubahsuai dengan pengisian tapak kemasukan ribosomd alaman (IRES) di antara promoter CMV dan isyarat Poly A untuk transkripsi dan pengekspresan dua jenis protein dalam satu kaset. Jujukan IRES telah diamplifikasi dengan saringan PCR dari vektor pRetroX-IRES-ZsGreen1, dipotong dengan *Eco*RV and *Xho*I, dan ditampalkan ke dalam kaset pengekspresan eukariot dari pcDNA 3.1 HisA.Vektor yang telah dibina ditransform ke dalam bacteria *E.coli* dan orientasi jujukan IRES dalam kaset pengekspresan eukariot yang diubahsuai telah disahkan dengan penjujukan DNA. Kaset pengekspresan eukariot yang telah diubahsuai diamplifikasi dengan saringan PCR dari pcDNA3.1HisA/IRES sebelum pengklonan semula ke dalam pMG36e. Rekombinan pMG36e telah berjaya ditransform ke dalam bacteria *E. coli* tetapi tidak berjaya pada *L. lactis*. Pengubahsuaian protocol transformasi *L. lactis* perlu dioptimumkan. Ketidakstabilan "Segregational" oleh rekombinan pMG36e telah dikesan dalam perumah bacteria *E. coli* yang mungkin disebabkan produksi bebenang tunggal pengantara dan juga berat DNA molekul yang tinggi akibat replikasi gulungan kitaran pMG36e.

Keywords: kasetpengekspresaneukariot, IRES, *Lactococcuslactis*, pMG36e

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Declaration

Declaration by undergraduate student

I hereby confirm that:

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Name of Chairman of Supervisory Committee: Dr.Nurulfiza Mat Isa

Approval

This thesis was submitted to the Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences accepted as fulfillment of the requirement for the degree of Cell and Molecular Biology. The member of the Supervisory Committee as follows:

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LIST OF ABBREVIATIONS

bp	Base pairs
DNA	Deoxyribonucleic acid
EDTA	EthylenediamineTetraacetic Acid
E. coli	*Escherichia coli*
GM17	M17 supplemented by 0.5% (w/v) glucose
HCl	Hydrochloric acid
IRES	Internal ribosome entry site
L. lactis	*Lactococcuslactis*
LB	Luria Bertani
ng	Nanogram
OD	Optical density
PCI	Phenol:chloroform: isoamyl alcohol
PCR	Polymerase Chain Reaction
PolyA	Polyadenylation
RNA	Ribonucleic acid
SGM17	M17 supplemented by 0.5% glucose and 0.5M sucrose
TE	Tris-EDTA (EthylenediamineTetraacetic Acid)
Tris	Tris(hydroxymethyl) aminomethane
µL	Micoliter
µM	Micromolar

CHAPTER 1: INTRODUCTION

Lactococcus lactis (*L. lactis*) is a Gram-positive coccus from the lactic acid bacteria group with novel properties for application in the preservation and flavor production of food. Due to their contribution in the food industry, *L. lactis* has been designated as generally recognized as safe (GRAS) organism (Pontes *et al.*, 2011). The non-pathogenic characteristic coupled with the ability to express heterologous proteins rendered *L. lactis*the ideal choice for safe production of antigens and medically important proteins (De Ruyter *et al.*, 1996). In a more recent development, *L. lactis* was found to be able to invade and deliver plasmid DNA encoding proteins into the eukaryotic cells. Thus, there is growing interest in developing *L. lactis* as bacterial delivery system for antigens as part of safer alternatives of vaccination.

The wider application of *Lactococcus* bacteria beyond the food industry mediates the need for development of lactococcal vectors. There are less commercially available vectors for *L. lactis* compared to vectors for *E. coli*. Besides, a lactococcal vector usually consists of transcriptional and translational structures recognized by only prokaryotic host. For it to be expressible in eukaryotic host, the existing lactococcal vector must be modified to carry eukaryotic transcription and translation features.

Traditionally, co-transfection of two different vectors; one carrying reporter protein and another carrying protein of interest,is needed for most delivery of vectors into mammalian cells. The transfection of the protein of interest which is presumed to be successful with the successful transfection of the reporter protein is unreliable and unpredictable (Hunt, 2005). Internal ribosome entry site (IRES) elements are able to internally initiate the translation of RNA and facilitate the expression of two or more proteins. When modified into expression vectors, these bicistronic expression vectors are able to translate the first gene in a cap-dependent manner and the second in IRES dependent manner (Vagner *et al.*, 2001). Using IRES-incorporated bicistronic expression vector, the selection efficiency during transgenesis improved, as over 90% of cells expressing the selectable marker also express the gene of interest.

Therefore, the objective of this study was to construct lactococcal vector pMG36e containing eukaryotic expression cassette. The eukaryotic expression cassette was first created by amplification of IRES fragment using polymerase chain reaction before cloning into the expression cassette of pcDNA 3.1 HisA. The expression cassette is then sub-cloned into pMG36e using the same cloning strategy and transformed into *L. lactis*. The added features enabled the expression of heterologous proteins in both eukaryotic and prokaryotic host while the prokaryotic backbone ensured continuous propagation of the vector in prokaryotic host. IRES incorporated enable the expression of multiple proteins in the host.

References

Abdul Mutalib, N.E., Isa, N.M., Alitheen, N.B., Song, A.A.L. and Rahim, R.A. (2014).IRES-incorporated *lactococcal* bicistronic vector for target gene expression in a eukaryotic system. *Plasmid* 73: 26-33.

Bradrick, S.S., Dobrikova, E.Y., Kaiser, C., Shveygert, M. and Gromeier, M. (2007). Poly (A)-binding protein is differentially required for translation mediated by viral internal ribosome entry sites. *RNA* 13: 1582-1593.

de Quinto, S.L., Sáiz, M., de la Morena, D., Sobrino, F. and Martínez-Salas, E. (2002). IRES-driven translation is stimulated separately by the FMDV 3′-NCR and poly (A) sequences. *Nucleic Acids Research* 30: 4398-4405.

De Ruyter, P.G., Kuipers, O.P. and De Vos, W.M. (1996).Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin.*Applied and Environmental Microbiology* 62: 3662-3667.

Dechering, K. J., Konings, R. N., Cuelenaere, K. and Leunissen, J. A. (1998).Distinct frequency-distributions of homopolymeric DNA tracts in different genomes. *Nucleic acids research*, *26*(17), 4056-4062.

Elroy-Stein, O., Fuerst, T.R. and Moss, B. (1989). Cap-independent translation of mRNA conferred by encephalomyocarditis virus 5' sequence improves the performance of the vaccinia virus/bacteriophage T7 hybrid expression system. *Proceedings of the National Academy of Sciences* 86: 6126-6130.

Foley, S., Bron, S., Venema, G., Daly, C. and Fitzgerald, G.F. (1996).Molecular Analysis of the Replication Origin of the*Lactococcus lactis*Plasmid pCI305. *Plasmid*36:125-141.

França, L.T., Carrilho, E. and Kist, T.B. (2002).A review of DNA sequencing techniques. *Quarterly Reviews of Biophysics*35: 169-200.

Glenting, J. and Wessels, S. (2005). Ensuring safety of DNA vaccines. *Microbial Cell Factories* 4: 26.

Gram, G. J., Fomsgaard, A., Thorn, M., Madsen, S.M. and Glenting, J. (2007).Immunological analysis of a *Lactococcus lactis*-based DNA vaccine expressing HIV gp120. *Genetic Vaccines and Theraphy* 5.

Gromak, N., West, S. and Proudfoot, N.J. (2006). Pause sites promote transcriptional termination of mammalian RNA polymerase II. *Molecular and Cellular Biology* 26: 3986-3996.

Gruss, A. and Ehrlich, S.D. (1988). Insertion of foreign DNA into plasmids from gram-positive bacteria induces formation of high-molecular-weight plasmid multimers. *Journal of bacteriology*170:1183-1190.

Gu, X.X., Tan, J.X., Tian, H.T., Zhang, Y.L., Luo, Y.B. and Guo, X.H. (2014). Construction of a food-grade expression vector based on pMG36e by using an α-galactosidase gene as a selectable marker. *Journal of Integrative Agriculture*13: 1802-1808.

Guimarães, V., Innocentin, S., Chatel, J.M., Lefèvre, F., Langella, P., Azevedo, V. and Miyoshi, A. (2009).A new plasmid vector for DNA delivery using lactococci. *Genetic Vaccines and Theraphy* 7.

Guimarães, V.D., Gabriel, J.E., Lefèvre, F., Cabanes, D., Gruss, A., Cossart, P., Azevedo, V. and Langella, P. (2005). Internalin-expressing *Lactococcus lactis* is able to invade small intestine of guinea pigs and deliver DNA into mammalian epithelial cells. *Microbes and Infection* 7: 836-844.

Guimarães, V.D., Innocentin, S., Lefèvre, F., Azevedo, V., Wal, J.M., Langella, P. and Chatel, J.M. (2006). Use of native lactococci as vehicles for delivery of DNA into mammalian epithelial cells. *Applied and Environmental Microbiology* 72: 7091-7097.

Ha, S.H., Liang, Y.S., Jung, H., Ahn, M.J., Suh, S.C., Kweon, S.J., Kim, D.H., Kim, Y.M. and Kim, J.K. (2010). Application of two bicistronic systems involving 2A and IRES sequences to the biosynthesis of carotenoids in rice endosperm. *Plant Biotechnology Journal* 8: 928-938.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In *Nucleic Acids Symposium Series*,Vol 41. pp 95-98.

Hellen, C.U. and Sarnow, P. (2001). Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes and Development* 15: 1593-1612.

Hunt, I. (2005). From gene to protein: a review of new and enabling technologies for multi-parallel protein expression. *Protein Expression and Purification* 40: 1-22.

Kibbe, W.A. (2007). OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Research* 35: W43-W46.

Kim, K.J., Kim, H.E., Lee, K.H., Han, W., Yi, M.J., Jeong, J. and Oh, B.H. (2004). Two-promoter vector is highly efficient for overproduction of protein complexes. *Protein Science* 13: 1698-1703.

Kim, W.S., Ren, J. and Dunn, N.W. (1999). Differentiation of *Lactococcus lactis* subspecies *lactis* and subspecies *cremoris* strains by their adaptive response to stresses. *FEMS Microbiology Letters*171: 57-65.

Kok, J., van der Vossen, J.M. and Venema, G. (1984).Construction of plasmid cloning vectors for *lactic streptococci* which also replicate in *Bacillus subtilis* and *Escherichia coli*. *Applied and Environmental Microbiology* 48: 726-731.

Kuipers, O.P., Beerthuyzen, M.M., Siezen, R.J. and Vos, W.M. (1993).Characterization of the nisin gene cluster nisABTCIPR of *Lactococcus lactis*. *European Journal of Biochemistry* 216: 281-291.

Kunji, E.R., Slotboom, D.J. and Poolman, B. (2003).*Lactococcus lactis* as host for overproduction of functional membrane proteins. *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1610: 97-108.

Lewis, P.J. and Babiuk, L.A. (1999). DNA vaccines: a review. *Advances in Virus Research* 54: 129-188.

Liljeqvist, S. and Ståhl, S. (1999). Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines. *Journal of Biotechnology* 73: 1-33.

Liu, M.A. (2003). DNA vaccines: a review. *Journal of Internal Medicine*253: 402-410.

Macias, M.P., Huang, L., Lashmit, P.E. and Stinski, M.F. (1996). Cellular or viral protein binding to a cytomegalovirus promoter transcription initiation site: effects on transcription. *Journal of Virology* 70: 3628-3635.

Malecki, M., Przybyszewska, M. and Janik, P. (2003).Construction of a bicistronic proangiogenic expression vector and its application in the experimental angiogenesis in vivo. *Acta Biochimica Polonica 50*: 875-882.

Martínez-Salas, E. (1999). Internal ribosome entry site biology and its use in expression vectors. *Current Opinion in Biotechnology* 10: 458-464.

McClure, W.R. (1985). Mechanism and control of transcription initiation in prokaryotes. *Annual Review of Biochemistry*54: 171-204.

Mierau, I. and Kleerebezem, M. (2005).10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. *Applied Microbiology and Biotechnology* 68: 705-717.

Ngoi, S.M., Chien, A.C. and Lee, C.G.L. (2004).Exploiting internal ribosome entry sites in gene therapy vector design. *Current Gene Therapy* 4: 15-31.

Nouaille, S., Ribeiro, L.A., Miyoshi, A., Pontes, D., Le Loir, Y., Oliveira, S.C., Langella, P. and Azevedo, V. (2003).Heterologous protein production and delivery systems for *Lactococcus lactis*. *Genetic and Molecular Research* 2: 102-111.

Papadakis, E.D., Nicklin, S.A., Baker, A.H. and White, S.J. (2004). Promoters and control elements: designing expression cassettes for gene therapy. *Current Gene Therapy* 4: 89-113.

Pereira, V.B., Azevedo, M.S.P., Saraiva, T.D.L., Souza, B.M., Turk, M.Z., De Castro, C.P., Agresti, P.M., Lima, F.A., Azevedo, V., Pontes, D.S. and Miyoshi, A. (2013).Use of bacteria in DNA vaccine delivery. *Vaccine* 12:15.

Pontes, D.S., De Azevedo, M.S.P., Chatel, J.M., Langella, P., Azevedo, V. and Miyoshi, A. (2011).*Lactococcus lactis* as a live vector: heterologous protein production and DNA delivery systems. *Protein Expression and Purification*79: 165-175.

Powell, I.B., Achen, M.G., Hillier, A.J. and Davidson, B.E. (1988).A simple and rapid method for genetic transformation of lactic streptococci by electroporation. *Applied and Environmental Microbiology*54:655-660.

Proudfoot, N.J. (2011). Ending the message: poly (A) signals then and now.*Genes and Development* 25: 1770-1782.

Que, Y.A., François, P., Haefliger, J.A., Entenza, J.M., Vaudaux, P. and Moreillon, P. (2001).Reassessing the role of *Staphylococcus aureus* clumping factor and fibronectin-binding protein by expression in *Lactococcus lactis*.*Infection and Immunity* 69: 6296-6302.

Raha, A.R., Chang, L.Y., Sipat, A., Yusoff, K. and Haryanti, T. (2006).Expression of a thermostable xylanase gene from *Bacillus* coagulans ST-6 in *Lactococcus lactis*. *Letters in Applied Microbiology* 42: 210-214.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular Cloning* ,Vol 1. No. 7.58. New York: Cold spring harbor laboratory press.

Sanders, J.W., Venema, G. and Kok, J. (1999).Environmental stress responses in *Lactococcus lactis*. *FEMS Microbiology Reviews* 23: 483-501.

Tao, L., Pavlova, S.I., Ji, X., Jin, L. and Spear, G. (2011).A novel plasmid for delivering genes into mammalian cells with noninvasive food and commensal lactic acid bacteria. *Plasmid* 65: 8-14.

Vagner, S., Galy, B. and Pyronnet, S. (2001). Irresistible IRES. *EMBO Reports*2: 893-898.

van de Guchte, M., van der Vossen, J.M., Kok, J. and Venema, G. (1989). Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. *lactis*. *Applied and Environmental Microbiology* 55: 224-228.

Wells, J.M. and Mercenier, A. (2008).Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. *Nature Reviews Microbiology*6: 349-362.

Wilkinson, G.W. and Akrigg, A. (1992).Constitutive and enhanced expression from the CMV major IE promoter in a defective adenovirus vector. *Nucleic Acids Research*20: 2233-2239.

APPENDICES