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UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERISATION OF pAR141, A CRYPTIC *LACTOCOCCUS LACTIS* PLASMID, AND ITS DEVELOPMENT INTO AN EXPRESSION VECTOR

HOOI WEI YENG

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By

HOOI WEI YENG

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

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Chairman: Professor Raha Abdul Rahim, PhD

Faculty: Biotechnology and Biomolecular Sciences

Lactococcus lactis is one of the best characterised lactic acid bacteria (LAB). It is widely used in traditional biotechnology as dairy starter culture for the production of cheese, butter and buttermilk. Its applications have been further expanded in modern biotechnology. *L. lactis* has been used as an alternative to *Escherichia coli* as a cell factory for the production of chemicals, pharmaceuticals and neutraceuticals. It has also been engineered to be a live oral vaccine. To engineer the cells, plasmids serve as vectors for the introduction of foreign DNA into the hosts, and hence giving the hosts unique features for special purposes. The basic knowledge and understanding of the plasmid is significant for the development of a prominent cloning and expression system. However, there are limited information and no commercially available vectors for *L. lactis* in the market currently. In this study, the Gram-positive cocci isolated from cow's milk were identified by comparing the partial 16S ribosomal RNA (rRNA) gene sequences to Internet databases. Among these cocci,

eight strains were identified as *L. lactis* subspecies *lactis* and were further characterised by plasmid profiling, antibiotic resistance pattern and antimicrobial activity. Two of the strains, *L. lactis* M12 and M14, were found to carry multiple plasmids of various sizes, ranging from 1.6 kilo base pair (kb) to approximately 46 kb. However, they exhibited the same antibiotic resistance pattern as the plasmidless strain *L. lactis* MG1363. These two strains were able to inhibit the growth of other lactococcal strains isolated from the same source. The smallest plasmid from *L. lactis* M14, designated as pAR141, was chosen for further analysis. The restriction enzyme-digested fragments of the plasmid were cloned and sequenced. The sequence analysis of pAR141 indicated that it replicated via rolling circle (RC) mechanism. This 1,594-base pair (bp) cryptic plasmid carried essential genes required for its own replication and control, which included the transcriptional repressor *repA* and replication initiator *repB* genes, in a single operon. Other elements such as the putative coding region of a small countertranscribed RNA (ctRNA), the double strand origin (*dso*) and single strand origin (*sso*) of replication, were also identified. A constitutive expression vector, pAR1411, was constructed by cloning the erythromycin resistance marker (*ery*), P_{32} promoter and a multiple cloning site (MCS) into pAR141. The functionality of the new vector was verified by using the chloramphenicol acetyltransferase (*cat*) gene as the reporter gene. The *cat* gene was successfully cloned into pAR1411 and expressed in *L. lactis* MG1363. In conclusion, the small lactococcal cryptic RC replicating plasmid, pAR141, was isolated and characterised. The newly developed pAR1411 could be used as an expression vector for *L. lactis*.

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PEMENCILAN DAN PENCIRIAN pAR141, SUATU PLASMID KRIPTIK *LACTOCOCCUS LACTIS*, DAN PEMBENTUKANNYA KEPADA SATU VEKTOR PENZAHIRAN

Oleh

HOOI WEI YENG

September 2008

Pengerusi: Profesor Raha Abdul Rahim, PhD

Fakulti: Bioteknologi dan Sains Biomolekul

Lactococcus lactis adalah salah satu bakteria asid laktik (LAB) yang paling banyak dikaji ciri-cirinya. Ia banyak digunakan dalam bioteknologi tradisional sebagai kultur pemula tenusu untuk penghasilan keju, mentega dan susu mentega. Penggunaannya telah dikembangkan selanjutnya dalam bioteknologi moden. *L. lactis* telah digunakan sebagai "kilang sel" selain daripada *Escherichia coli* untuk penghasilan bahan kimia, farmaseutikal dan neutraseutikal. Ia juga telah dibina untuk menjadi vaksin oral. Untuk tujuan-tujuan tersebut, plasmid digunakan sebagai vektor bagi pengenalan DNA asing ke dalam perumahnya, dan oleh itu memberi perumahnya ciri-ciri unik bagi tujuan-tujuan tertentu. Pengetahuan asas dan pemahaman tentang plasmid adalah penting bagi pembinaan satu sistem pengklonan dan penzahiran yang menonjol. Akan tetapi, maklumat-maklumat tersebut adalah kekurangan dan vector bagi *L. lactis* tiada dalam pasaran pada masa kini. Dalam kajian ini, kokus Gram-positif yang dipencilkan dari susu lembu dikenalpasti

melalui perbandingan sebahagian jujukan gen RNA ribosom (rRNA) 16S dengan jujukan-jujukan yang terdapat dalam databes Internet. Di antaranya, lapan strain telah dikenalpasti sebagai *L. lactis* subspecies *lactis* dan dicirikan selanjutnya melalui profil plasmid, perintangan kepada antibiotik dan aktiviti antimikrob. Dua strain, *L. lactis* M12 dan M14, didapati mempunyai beberapa plasmid yang berlainan saiz, dari 1.6 kilo pasangan bes (kb) ke 46 kb. Akan teetapi, mereka menunjukkan corak rintangan antibiotik yang serupa dengan strain yang tiada plasmid *L. lactis* MG1363. Dua strain ini juga berupaya merencat pertumbuhan strain lactococci lain yang dipencil dari sumber yang sama. Plasmid terkecil dari *L. lactis* M14 yang dinamakan pAR141 telah dipilih bagi analisis selanjut. Serpihan-serpihan plasmid cernaan enzim pembatas telah diklon dan jujukannya ditentukan. Analisis jujukan pAR141 menunjukkan bahawa plasmid ini bereplika melalui mekanisma replikasi bulatan bergulung (rolling circle; RC). Plasmid kriptik yang bersaiz 1,594 pasangan bes (bp) ini membawa gen-gen yang diperlukan bagi replikasi dan pengawalannya sendiri, yang termasuk gen penindas transkripsi *repA* dan gen pemula replikasi *repB*, dalam satu operon tunggal. Unsur-unsur lain seperti tapak pengekodan RNA transkrip-bertentangan (countertranscribed RNA; ctRNA), "double strand origin" (*dso*) dan "single strand origin" (*sso*) replikasi, juga dikenalpasti. Satu vektor penzahiran berterusan, pAR1411, telah dibina dengan pengklonan penanda kerintangan terhadap eritromisin (*ery*), promoter P_{32} dan tapak pengklonan berganda (MCS) ke dalam pAR141. Keberfungsian vektor baru ini telah disahkan dengan menggunakan gen asetiltransferase kloramfenikol (*cat*) sebagai gen pelapor. Gen *cat* telah berjaya diklonkan ke dalam pAR1411 dan dizahirkan dalam *L. lactis* MG1363. Sebagai kesimpulan, satu plasmid replikasi RC *Lactococcus* kecil,

pAR141, telah dipencil dan dicirikan. pAR1411 yang baru dibina ini boleh dijadikan sebagai vektor penzahiran bagi *L. lactis*.

I certify that an Examination Committee has met on 5 September 2008 to conduct the final examination of Hooi Wei Yeng on her degree of Master of Science thesis entitled "Isolation and Characterisation of pAR141, a Small *Lactococcus lactis* plasmid, and Its Development into an Expression Vector" 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 degree of Master of Science.

Members of the Examination Committee were as follows:

Tan Wen Siang, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Khatijah Mohd Yusoff, PhD
Professor Datin Paduka
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

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

Zulkeflie Zamrod, PhD
Vice President
Research and Development
Inno Biologics Sdn. Bhd.
(External Examiner)

_____

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

Date: 27 November 2008

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)

Son Radu, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

Mariana Nor Shamsudin, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

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

Date: 19 December 2008

DECLARATION

I 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.

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HOOI WEI YENG

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LIST OF ABBREVIATIONS

~	approximately
A_{260}	Absorbance at 260 nm
a.a.	amino acids
ABC	ATP-binding cassette
Amp^r	ampicillin resistant
AT	adenosine and thymine
ATCC	America Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
$CaCl_2$	calcium chloride
CAMHB	cation-adjusted Mueller-Hinton broth
CDD	Conserved Domain Database
Chl^r	chloramphenicol resistant
CLSI/NCCLS	Clinical and Laboratory Standard Institute/NCCLS
ctRNA	countertranscribed RNA
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DDBJ	DNA Data Bank of Japan
DIG	digoxigenin
DR	direct repeat
dsDNA	double-stranded DNA
dso	double strand origin
EDTA	ethylene diamine tetraacetic acid
EMBL	European Molecular Biology Laboratory
Erm^r	erythromycin resistant
FDA	Food and Drug Administration
G+C	guanine plus cytosine
g	gravity force
GM17	M17 supplemented with 0.5% (w/v) glucose
GRAS	generally regarded as safe
HTH	αhelix-turn-αhelix
IR	inverted repeat
IS	insertion sequence
kb	kilo base pair
kDa	kilo Dalton
LAB	lactic acid bacteria
LB	Luria Bertani
LSM	LAB susceptibility test medium
LZ	leucine zipper
M	Molar
MCS	multiple cloning sites
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MIC	minimum inhibition concentration
Mol%	mole percent
mRNA	messenger RNA

N	Normality
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
NICE	nisin-controlled gene expression
nt	nucleotide
OD_{600}	optical density at 600 nm
ORF	open reading frame
PCI	phenol-chloroform-isoamyl alcohol
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
RAPD	randomly amplified polymorphic DNA
RBS	ribosome binding site
RC	rolling circle
RCR	rolling circle replicating
rDNA	rRNA gene
RDP-II	Ribosomal Database Project
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
R/M	restriction/modification
RNA	ribonucleic acid
rpm	revolution per minute
rRNA	ribosomal RNA
sdH_2O	sterile distilled water
SDS	sodium dodecyl sulphate
SGM17	GM17 containing 0.5 M sucrose
SGM17MC	SGM17 containing 20 mM $MgCl_2$ and 2 mM $CaCl_2$
SSB	single-stranded DNA binding protein
SSC	NaCl-sodium citrate
ssDNA	single-stranded DNA
ssi	single-stranded initiation
sso	single strand origin
subsp.	subspecies
TAE	Tris-acetate-EDTA
T_m	melting temperature
U	unit
V	Volt
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1

INTRODUCTION

As a member of the lactic acid bacteria (LAB) which has a long history of safe use in food fermentation, *Lactococcus lactis* was given the generally regarded as safe (GRAS) status by U.S. Food and Drug Administration (FDA). It has been widely used in the manufacture of dairy products such as cheese and buttermilk. However, the traits of lactose metabolism and protein degradation frequently experienced spontaneous loss during consecutive subcultures (McKay *et al.*, 1972). In order to stabilise and enhance these industrially important traits, extensive biochemical, physiological and genetic studies have been carried out on this valuable Gram-positive bacterium.

As information accumulates, researchers start to exploit the potential of *L. lactis* other than in traditional dairy fermentation. The capability of this bacterium to be used as production host of various proteins, chemicals, pharmaceuticals and neutraceuticals, either homologous or heterologous, has been examined (Hugenholtz and Smid, 2002). Currently, the application of *L. lactis* as vaccine delivery vector is being widely explored (Mercenier *et al.*, 2000).

Genetic modification techniques are used in the strain improvements of *L. lactis* as dairy starter, as well as in the engineering of this bacterium for modern applications. Generally, plasmids are used as vectors for these purposes. Plasmids are extrachromosomal DNA elements which replicate autonomously. These plasmids have to be characterised thoroughly to reveal their functions and interactions with

their host which could allow better control and enhanced performance. Although many studies on lactococcal plasmids have been reported, only a limited number have been analysed in detail. From these studies, several cloning and expression vectors have been developed. These vectors have shown to produce recombinant proteins successfully. The application of these vectors can be further improved by the addition of certain elements such as tags and signal peptides which could serve under different conditions and for various purposes. Unfortunately, at present, none of these vectors are commercially available, and thus, restrict strain improvements.

As the potential of advanced biotechnology applications of this Gram-positive host is growing, this project is aimed to contribute to the fundamental knowledge of lactococcal plasmid biology, as well as to develop a constitutive expression vector. The main objectives of this study are:

- To identify and characterise *Lactococcus lactis* isolates
- To isolate and characterise a small lactococcal plasmid
- To construct an expression vector for *Lactococcus lactis*

The lactococcal strains identified from the milk isolates (Chapter 3) were used as the source of new lactococcal plasmids. Selected plasmid was then further studied (Chapter 4) before an expression vector was constructed based on it (Chapter 5).

CHAPTER 2

LITERATURE REVIEW

2.1 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are a heterologous group of bacteria widespread in nature and are commonly found in milk and dairy products, plant material, silage, and intestinal tracts and mucous membranes of humans and animals (Aguirre and Collins, 1993). They are fastidious organisms and require carbohydrates, amino acids, peptides, nucleic acid derivatives and vitamins for growth. As delineated by their name, LAB are referred to a group of Gram-positive non-sporeforming bacteria that produce lactic acid as the major end-product from carbohydrate fermentation. They are either cocci or rod shape and generally have a DNA G+C content of less than 50 mol% (Stackebrandt and Teuber, 1988).

Traditionally, LAB consisted of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* (Aguirre and Collins, 1993). Due to the development in nucleic acid hybridisation and sequencing techniques, LAB have undergone major changes in their taxonomy and nomenclature. For example, streptococci were divided into three genetically distinct genera: *Streptococcus*, *Enterococcus* and *Lactococcus* (Schleifer *et al.*, 1985; Schleifer, 1987). New genera *Carnobacterium*, *Tetragenococcus* and *Vagococcus* have been established from previously acid-sensitive lactobacilli, *Pediococcus halophilus* and motile streptococci, respectively, while *Weissella* and *Oenococcus* were mainly derived from *Leuconostoc* (Stiles and Holzapfel, 1997).

Most of the LAB are involved in food fermentation and preservation. Some of them are used as probiotics which are live microbial supplements that beneficially affect the host by improving its intestinal microbial balance (Fuller, 1989). On the other hand, some *Pediococcus*, *Leuconostoc* and *Lactobacillus* are implicated in food spoilage (Stiles and Holzapfel, 1997). The association of some of the LAB such as *Enterococcus* sp. and *Lactococcus garvieae* with human and animals infections has also been reported (Salminen *et al.*, 1998; Fihman *et al.*, 2006).

2.2 The Genus *Lactococcus*

The genus *Lactococcus* was established in 1985 and encompasses most but not all of the Lancefield group N lactic streptococci (Schleifer *et al.*, 1985). They are spherical or ovoid in shape (0.5-1.2 × 0.5-1.5 μm), without capsules and endospores, nonmotile and occur in pairs or short chains in liquid media. They grow at 10°C but not at 45°C, with an optimum temperature at 30°C. They are facultative anaerobes, with an absence of growth at the upper-most level in broth culture. However, they are able to aerobically grow on agar media containing fermentable carbohydrate and supplemented with yeast extract, with very small and discrete surface colonies. These chemoorganotrophs have complex nutritional requirements and cannot survive with 0.5% NaCl. They are commonly found in dairy and plant products. Lactococci are homofermentative bacteria that ferment a number of carbohydrates and produce L(+)-lactic acid without gas production. The final pH in glucose broth is 4.0-4.5. They are catalase and oxidase negative (Holt *et al.*, 1994). The genus includes *L. lactis*, *L. garvieae*, *L. piscium*, *L. plantarum* and *L. raffinolactis*. *L. lactis* have been divided into three subspecies: *lactis*, *cremoris* and *hordniae* (Schleifer *et al.*, 1985; van Hylckama Vlieg *et al.*, 2006). Lactococci can be identified by 16S

rRNA gene sequence analysis. Polymerase chain reaction (PCR) detection of specific targets such as the *pepT* tripeptidase gene, *pepV* dipeptidase gene, histidine operon and dihydropteroate synthase gene has been developed for fast screening and identification of lactococci (Aoki *et al.*, 2000; Mori *et al.*, 2004; Ouzari *et al.*, 2006). Other than that, randomly amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE) of restriction enzyme (RE)-digested genomic DNA, electrophoretic esterase profile or whole cell protein profile have been applied for the typing of the strains (Kelly and Ward, 2002; de la Plaza *et al.*, 2006; Ouzari *et al.*, 2006).

2.3 *Lactococcus lactis*

L. lactis is among the immensely studied LAB, with respect to its genetics, molecular biology, physiology and metabolism. It was the first bacterial pure culture obtained by Joseph Lister in 1878, which was known as *Bacterium lactis* at that time (Stackebrandt and Teuber, 1988). It was previously grouped under the genus *Streptococcus* as *S. lactis*, and was then transferred to the genus *Lactococcus* based on the results of 16S rRNA analyses and extensive DNA-rRNA hybridisation studies (Schleifer *et al.*, 1985).

L. lactis can be isolated from milk and plant surfaces (Nomura *et al.*, 2006). It is widely used in the fermentation of dairy products, especially the subspecies *lactis* and *cremoris*. Traditionally, they are used as starter culture for the production of cheeses and buttermilk. These two commercially important subspecies could be differentiated based on the properties of subsp. *lactis* to metabolise arginine, to grow at temperatures above 37°C, and to tolerate salt, in contrast to subsp. *cremoris*