DESIGNER GENES
FASHIONING MISSION PURPOSED MICROBES

PROFESSOR DR. RAHA HJ ABDUL RAHIM

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PROFESSOR DR. RAHA HJ ABDUL RAHIM
BSc Microbiology, Oklahoma State University, USA
MSc Microbial Genetics, University of Oklahoma, USA
PhD Molecular Biology, University of Strathclyde, Scotland, UK

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Contents

ABSTRACT

Genes are pieces of DNA that give mankind their genetic heritage. They are passed down through generations of living organisms copiously and diligently by mechanisms that have still not been fully deciphered. These genes are very stable and any changes will either be repaired or will cause malady to the cells. However, natural, `quirky' things do happen and changes to the genes that are beneficial to the host cells will be well accepted and embraced as part of life's progression. Nevertheless, it is now the beginning of the era of **direct genetic modification**. Whilst images of `Terminators' and `Transformers' would initially come to mind when the word `artificial intelligence' is mentioned, the first such `artificial being' is actually a simple single celled microorganism. In the area of biotechnology, microbes are usually used as the source of useful proteins, enzymes, metabolites and antibiotics. The first altered organisms emerged from recombinant DNA technology in the mid-70s. Microorganisms such as *Escherichia coli*, *Saccharomyces cereviciae* and *Bacillus subtilis* have been genetically modified and used to produce industrially and pharmaceutically important proteins and enzymes. More recently, several types of microbes including *Salmonella* spp, *Lactobacillus* spp and *Lactococcus* spp were characterized and are identified as potential vaccine carriers and producers. Whilst there are many success stories, the development of better systems and microbial hosts for the improved production of important biotechnology products is still being relentlessly pursued.

This publication contains examples of work carried out by our research group in the design and development of gene carriers and microbes aimed at advancing the production of homologous and heterologous proteins. It is hoped that the work described here will provide insights into the multiple approaches needed in the

development of mission purposed bacterial strains, specifically *L. lactis*, for use in the diverse areas of biotechnology. The identification of new plasmids and specific characterisation of cryptic lactococcal plasmids are presented, and the construction of new and useful plasmids is discussed. In addition, the development of new strains of microbes harbouring useful proteins is described.

INTRODUCTION

Microbes are ubiquitous and have been on earth for 2 billion years before any other living organism. They were first observed by Antony van Leeuwenhoek in the 17th century. Microbes are remarkably adaptable to diverse environmental conditions whereby they are found in the bodies of all living organisms and in all parts of the earth; from land terrains to ocean depths, in arctic ice and glaciers, in hot springs, and even in the stratosphere. Microbes have a practical significance for humans, where some cause diseases in humans and domestic animals, affecting health and the economy. Some bacteria are useful in industry, particularly in the food, petroleum, and agriculture industries, while others can be harmful. As in higher forms of life, each bacterial cell arises either by division or through a combination of elements from two such cells via a sexual process. There are more bacteria, existing as separate individuals, than any other type of organism where there can be as many as 1- 2.5 billion cells in one gram of fertile soil (Roesch *et al*., 2007). While most rely on carbon compounds as an energy source, different species vary widely in their exact metabolic processes. Factors such as change in temperature, nutrients and source of energy commonly play a big role in the diversity of living organisms.

The idea of exploiting microbial products is not new. The first biotechnologists have long enlisted bacteria and yeast to make bread, wine and cheese and to produce antibiotics that help fight diseases. The advent of molecular biology in the 1970s opened up new possibilities, extending the potential use of bacteria as bio-therapeutic agents from just a few natural strains to also include recombinant strains.

Back in 1944, Avery, Macleod and McCarty first demonstrated gene transfer with isolated DNA obtained from the bacterium *Streptococcus pneumonia* (Avery *et al*., 1944). This process

involved genetic alteration of a bacterial cell by uptake of DNA isolated from a genetically different bacterium and its recombination with the host-cell genome. Their experiments provided the first evidence that DNA is the genetic material. Subsequent studies showed that such genetic alteration of a recipient cell can result from the uptake of exogenous extrachromosomal DNAs (e.g. plasmids) that do not integrate into the host-cell chromosome. The term `transformation' is used to denote genetic alteration of a cell caused by the uptake and expression of foreign DNA regardless of the mechanism involved.

The bacterium *Escherichia coli*, whilst known to cause diseases, has been the flag bearer for the development of transgenic microbes, favored to generate many important products since it is very well characterised (Presser *et al*., 1999; Viera *et al*., 2001) and has minimal nutritional requirements. However, harmless and beneficial bacteria far outnumber the harmful varieties. These good bacteria, of which the Lactic Acid Bacteria is one, are ingested regularly and form part of our intestinal flora. They can be made useful and serve as vehicles for new types of vaccines and be developed as cell factories for the production of molecules of particular interest.

In order to manipulate the microbes, however, there must first be an understanding of the genetic makeup, gene sequences of interest and how these genes can be regulated. Our group is interested to study the fundamentals of lactococcal plasmid biology and using the knowledge to improve and develop specialized vectors. We have embarked on utilising the good bacteria *Lactococcus lactis* as well as the ubiquitous *E. coli* as models in our pursuit to enhance microbial production and delivery systems.

THE GENETIC CODE

In a living organism, the totality of the genetic information is called genome. Genome size is the total amount of DNA contained within one copy of a genome. It is typically measured in terms of mass in picograms (pg) (trillionths (10^{-12}) of a gram), or as the total number of nucleotide base pairs (bp) typically in megabases (millions of base pairs, abbreviated Mb or Mbp). One picogram equals 978 megabases. A single celled organism would contain chromosomes ranging from 0.6 Mbp to over 10 Mbp (Table 1). The genome information is written in such a way that it is understood by all living organisms, which is termed the universal code of life. In the coding system only four nucleotides are used to spell out all the instructions of how to make proteins. Nucleotides comprising of adenine (A), guanine (G), cytosine (C) and thymine (T) is arranged in 3-letter words which in turn code for any one of the 20 amino acids. A gene is a certain length of DNA, with specific number of nucleotides that commonly has information for the production of one specific protein (an open reading frame). The use of information carried by genes in organisms is plentiful. Signature genes have been useful in the identification of pathogenic microorganisms, whilst gene polymorphisms have been utilized to understand diseases. Full length or parts of genes can be used to elicit immunogenicity in new hosts and the tweaking of genes and their products can affect metabolic pathways.

Table 1 Comparison between different genome sizes (source; Wikipedia)

	Organism type	Organism	Genome (bp)
1.	Virus	SV40	5,224
2.	Bacterium	*Escherichia coli*	4,6000,000
3.	Plant	Arabidopsis thaliana	157,000,000
4.	Yeast	*Saccharomyces cereviceae* (bread yeast)	12,100,000
5.	Insect	*Drosophila melanogaster* (fruit fly)	130,000,000
6.	Mammal	*Homo sapiens* (human)	3,200,000,000
7.	Fish	*Protopterus aethiopicus* (marbled lung fish)	130,000,000,000

So, how does a cell know when and how much protein to produce? Each gene has a stretch of DNA that contains the regulatory elements for the specific gene, known as the **promoter**. It functions like the `ignition site' of a car, waiting for the right `key' to start its engine. An example is the production of insulin, when a message arrives in the form of a molecule that says more insulin, the ignition site will accept the message and the molecule will `lock in' and activates the `switch' to start the process of gene expression. The information is then immediately copied (**transcribed**) in threadlike molecules called RNA. RNA is very similar to DNA except that it is single stranded. This molecule acts as the messenger which distributes the information to little work units in the cell that **translates** the information into proteins (**Figure 1**). This whole process where DNA is transcribed and then translated into protein is the central dogma of molecular biology.

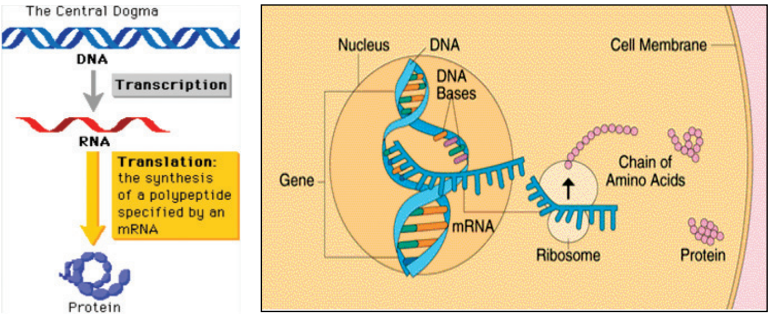


Figure 1 The central dogma of molecular biology
(Source; Google.com)

HOW TO TRANSFER GENES INTO OTHER CELLS

Genetic engineering is used to take genes and segments of DNA from one species and put them into another species, for example putting viral genes into bacteria, bacterial genes into plants etc. The DNA is cut, isolated, characterized, multiplied and stuck next to any other DNA of another cell or organism. The engineering of genes provides a set of techniques, such as **transformation,** biolistics, electroporation and transduction; frequently using vectors (either bacterial plasmids or viruses), to break through the species barrier and shuffle information intra and inter-species.

WHAT IS A PLASMID?

If the genome is a book with genes being the chapters or recipes, then plasmids are brochures, often containing information that can be exchanged rapidly. Plasmids can be found in many bacteria and are small rings of DNA with limited number of genes (**Figure 2**). The term *plasmid* was first introduced by the American molecular biologist Joshua Lederberg in 1952.

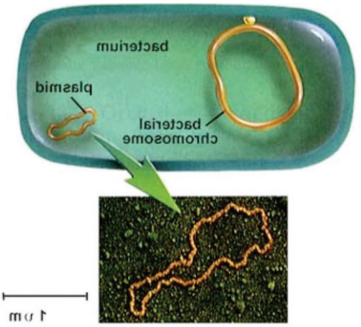


Figure 2 Plasmid in bacteria (Source; Google.com)

Plasmids are autonomously replicating double stranded DNA. They are part of the DNA material in microbes but are not dependent on the replicating mechanism of the host cells and can easily be moved around. A plasmid is typically smaller compared to the genome, commonly ranging between 1 - 400 kilobasepair in size. A single cell may contain a copy to hundreds of copies of a plasmid, or even thousands of copies, for certain artificial plasmids selected for high copy number. Plasmids often contain genes or gene cassettes that confer selective advantage to the bacterium harbouring them, such as the ability to make the bacterium antibiotic resistant.

Every plasmid contains at least one DNA sequence that serves as an *origin of replication*, or *ori* (a starting point for DNA replication), which enables the plasmid DNA to be duplicated independently from the chromosomal DNA. The plasmids of most bacteria are circular, but linear plasmids are also known, which superficially resemble the chromosomes of most eukaryotes. Many industrially important traits of microbes such as lactose fermentation, proteinase activity, citrate metabolism, bacteriophage resistance, and bacteriocin production are encoded by plasmids. On the other hand, most of the pathogenic and toxin producing microbial strains harbour virulence coding genes on their plasmids. In recombinant

DNA technology and genetic engineering, plasmids are important vectors used to clone and express genes of interests. These vectors allow foreign genes to be replicated and maintained in their new hosts. However, whilst vectors developed for the expression of genes in *E. coli* and yeast have been commercially available since the 1980s, there were no commercially available vectors for Gram positive bacteria until very recently.

LACTIC ACID BACTERIA

The last decade has seen enormous expansion of research on the molecular genetics of Lactic Acid Bacteria (LAB). The LAB are Gram positive, acid-tolerant, generally non-sporulating, non-respiring rod or cocci, has low GC content and are associated by their common metabolic and physiological characteristics. LABs are widely used in industrial food fermentation, contributing to the flavor, texture and preservation of fermented products. These bacteria are generally regarded as safe (GRAS), and certain strains can be used to treat human diseases. *Lactococcus* lactis is considered a prototype of the lactic acid bacteria. Intensive studies on the fundamental mechanisms of *Lactococcus* genetics have provided opportunities for the use of these bacteria in new applications such as the production of heterologous proteins in bio-reactors, in fermented food products or directly in the digestive tract of humans or animals. Most promising is their use as live vectors for delivery of biologically active molecules such as enzymes, antigens or therapeutic drugs. Thus, there is a lot of interest to study their plasmids and in developing vector systems and host strains to enable the expression of important genes in the lactococci.

LACTOCOCCUS LACTIS

Lactococcus lactis are non-sporulating, non-motile, A-T rich, Gram posititve cocci that group in pairs and short chains. Their typical size is 0.5 – 1.5 μm in length. They are routinely cultured in complex broth (M17 or MRS) supplemented with the appropriate carbohydrate and produce large quantities of lactic acid under anaerobic conditions. The optimal growth temperature is 30°C with a doubling time of 35 – 60 minutes. During exponential growth, the lactococci form chains of cells, like beads on a short string (**Figure 3**).

Lactococci are found associated with plant material, mainly grasses, from which they are easily inoculated into milk. Hence, they are normally found in milk and may be a natural cause of souring. *L. lactis* has two subspecies, *lactis* and *cremoris*, both of which are essential in the manufacture of many varieties of cheese and other fermented milk products.

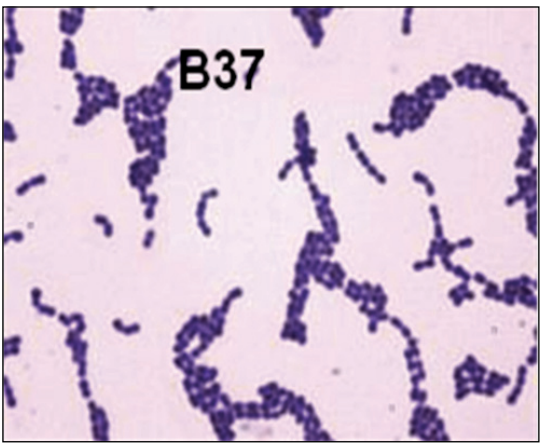


Figure 3 *Lactoccoccus lactis* – Gram positive cocci (lab isolate) 1000X magnification

L. lactis is also related to other lactic acid bacteria such as *Lactobacillus acidophilus* present in our intestinal tract and *Streptococcus salivarius* found in the mouth. However, *Lactococcus* does not normally colonize human tissues and differs from many other lactic acid bacteria in its pH, salt and temperature tolerances for growth, which are important characteristics relevant to its use as a starter culture in the cheesemaking industry. *L. lactis* is vital for manufacturing cheese such as the Cheddar, Colby, cottage cheese, cream cheese, Camembert, Roquefort and Brie, as well as other dairy products such as cultured butter, buttermilk, yogurt, sour cream and kefir. It can also be used for vegetable fermentations such as cucumber pickles and sauerkraut. The bacterium can be used in single strain starter cultures, or in mixed strain cultures with other lactic acid bacteria such as the *Lactobacillus* and *Streptococcus* species. When *L. lactis* is added to milk, the bacterium uses enzymes to produce energy molecules, called ATP, from lactose. The byproduct of ATP production is lactic acid. The lactic acid curdles the milk which then separates to form curds, which are used to produce cheese and whey.

The lactic acid produced by the bacterium lowers the pH of the product and prevents the growth of unwanted bacteria and molds while other metabolic products and enzymes produced by *L. lactis* contribute to the more subtle aromas and flavors that distinguish different types of cheese.

LACTOCOCCUS LACTIS – A FOOD GRADE GMO?

Several laboratory *Lactococcus* strains such as IL1403, NZ9000, NZ900 *clpP-htrA* and MG1363, which are derived from cheese starter cultures, are available for research (Nouaille *et al*., 2003, Cortez-Perez *et al*., 2006). Whilst *Lactococcus* spp have a GRAS status, many of the strains constructed at Universities and in

Research Institutions are mainly for `proof of concept' and are not food grade. A genetically modified *Lactococcus* would normally have a foreign DNA incorporated where antibiotic resistance markers are often used for selection. These strains, cannot however, be used in the food industry unless a number of safety criteria are fulfilled. In order for a genetically modified organism (GMO) to be used in food, whilst maintaining a GRAS definition, the food grade GMO must only contain DNA from the same species, or alternatively, genes from other GRAS food microorganisms. In either case, the use of antibiotic resistance markers is not allowed.

We undertook studies to isolate and characterize *Lactococcus* spp. for potential use as industrial strains and as hosts for hetelogous protein expression. Whilst most of the available isolates are milk based, more current studies have focused on LAB from plants. Traditional plating and identification methods were utilised to isolate new strains of *L. lactis* from various sources (Raha *et al.*, 2002, Raha *et al*., 2006). The isolates that tested positive via Gram staining, catalase and biochemical tests were subjected to 16S rRNA sequence analysis. Species-specific primers were designed according to published nucleotide sequences for the identification of *L. lactis* and the amplified regions sequenced. Part of the 16S rDNA was isolated by PCR amplification using a pair of primers P1 (5'-GCGGCGTGCCTAATACATGC-3') and P4 (5'-ATCTACGCATTTCACCGCTAC-3') designed by Klijn *et al*. (1991). These primers target the conserved regions of the gene that flank the variable regions which are useful in discriminating different species of *Lactococcus*. Then, the PCR products were cloned into TOPO cloning vectors (Invitrogen) prior to sequencing and analysed using the BLAST program (Altschul *et al.*, 1997).

Confirmed Lactococcal species, such as C1, C5, M4, M12, M14, K1 and K2 could be developed into industrial or pharmaceutical

strains of specific importance. Among our isolates, *Lactococcus lactis* M4 has been found to be devoid of low molecular weight plasmids and has the potential to be developed further as a new host for transformation of genes of interest. A preliminary transformation and growth study have indicated the ability of this strain to accept plasmid pMG36e and to maintain its stability to up to 100 generations. A number of food grade genetic modifications such as gene deletion, gene replacement, increasing gene copy number, and introducing new genes into the strain could then be carried out.

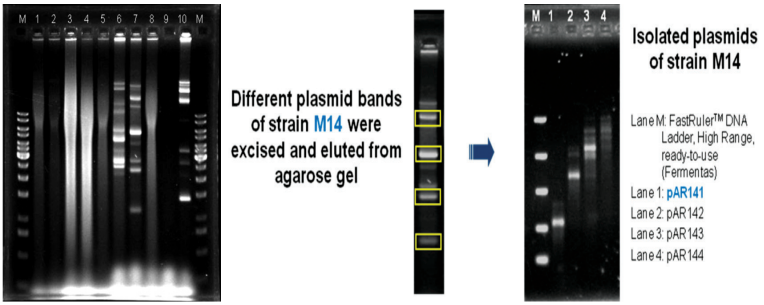
DEVELOPMENT OF *L. LACTIS* CLONING AND EXPRESSION SYSTEMS

Lactococcal Plasmids

Most lactococcal strains harbour between 4 to 7 plasmid molecules, with plasmid sizes ranging from 2 to 80 kb. The plasmids carrying several functions are relatively larger, whilst those that encode no recognizable phenotype other than their replicative functions are smaller and known as cryptic plasmids. Examples of plasmids harbouring genes expressing specific functions include the pLL10236 for lactose fermentation, pHP003 for proteinase activity and p0B4-6 for bacteriocin production and immunity. The cryptic plasmids include pWV01 (Leenhouts *et al.,* 1991) and pBM02 (Sanchez and Mayo, 2003). Both types of plasmids can be valuable tools for DNA cloning, gene expression and other biotechnological applications (Leenhouts and Venema, 1993). However, a limited number of lactococcal plasmids have been studied in detail and extensive molecular studies are required before they can be used in genetic manipulation.

We have isolated several small plasmids from chicken cecum-, milk- and plant-based *L. lactis* strains using the techniques of Anderson and McKay (1983) with some modifications. The bacterial isolates were subjected to antibiotic susceptibility and antagonistic activity tests and plasmid samples were analysed by agarose gel electrophoresis. Using the extraction method, two isolates (M12 and M14) from the milk sample were shown to possess several plasmids of different sizes whilst the rest of the isolates did not give good plasmid profiles. These isolates may carry some other low copy number plasmids which are hardly detectable in ethidium bromide-stained agarose gel. On the other hand, these lactococcal isolates may also contain huge plasmids which are difficult to extract and may be easily degraded, as *Lactococcus* are known to harbour huge indigenous plasmids which aid their growth in milk (Christensson *et al.*, 2001).

The smallest of these plasmids, such as pAJ01 (Raha *et al.,* 2002) and pAR141 (Raha *et al.,* 2006) were further characterised and sequenced. Gel electrophoresis analyses of how the small plasmid pAR141 was isolated and identified are shown in **Figure 4**.

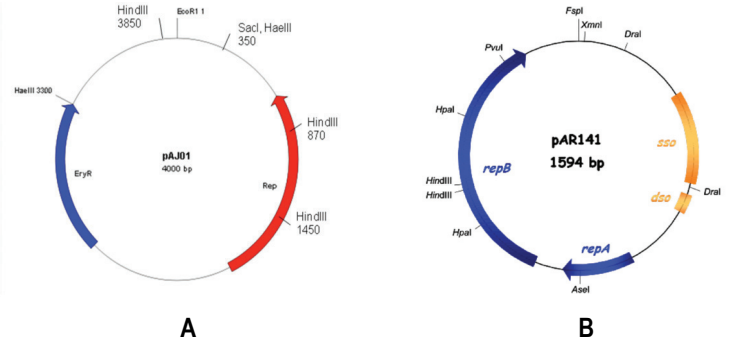


A **B**

Lane 1: Plasmids from M2
Lane 2: '' M4
Lane 3: '' M5
Lane 4: '' M6
Lane 5: '' M11
Lane 6: '' M12 – multiple plasmids
Lane 7: '' **M14 – multiple plasmids**
Lane 8: '' M16
Lane 9: '' *L. lactis* subsp. *cremoris* MG1363 (plasmidless)
Lane 10: '' *L. lactis* subsp. *lactis* ATCC 11454

Figure 4 Agarose gel electrophoresis plates of **A**, plasmids isolated from different milk isolates of *L. lactis* and **B**, isolated plasmids from M14 (Adapted from Hooi, MS thesis).

Figures 5A and 5B show the genetic maps of pAJ01 and pAR141. The smaller pAR141 is shown to encode only its replication sequences whilst pAJ01 carries the genes coding for erythromycin resistance in addition to its replication. The *rep* gene of pAJ01 and the complete sequence of pAR141 have been elucidated and deposited into the GenBank (accession no. AY057995 and DQ288662).



Figures 5 A, The genetic map of plasmids pAJ01 (From Raha *et al*., 2002) and **B**, pAR141 (From Raha *et al.*, 2006)

Two different mechanisms of replication are known to operate in *L. lactis* i.e. the rolling circle and theta replications. Theta type DNA synthesis is unidirectional and mostly found in the Gram-negative bacteria. Replication intermediates appeared as a typical theta (Θ) shaped molecules. RCR plasmids on the other hand are ubiquitous in the Gram-positive bacteria, although they have been reported in many Gram-negative bacteria and archea. These plasmids are usually small in size (less than 10 kb), have multiple copies and are tightly organized. Three important elements i.e. a gene encoding the initiator (Rep) protein, the double strand origin (*dso*) and the single strand origin (*sso*), are contained in all RCR plasmids (Khan, 2005). Rolling circle DNA replication is initiated by an initiator protein encoded by the plasmid DNA, which nicks one strand of the double-stranded, circular DNA molecule at a site called the double-strand origin, or DSO. The initiator protein remains bound to the 5' phosphate end of the nicked strand, and the free 3' hydroxyl end is released to serve as a primer for DNA synthesis by DNA polymerase III. Using the un-nicked strand as a template, replication proceeds around the circular DNA molecule,

displacing the nicked strand as single-stranded DNA. Displacement of the nicked strand is carried out by a host-encoded helicase in the presence of the plasmid replication initiation protein.

The initiator protein then makes another nick to terminate synthesis of the first (leading) strand. RNA polymerase and DNA polymerase III then replicate the single-stranded origin (SSO) DNA to make another double-stranded circle. DNA polymerase I removes the primer, replacing it with DNA, and the enzyme DNA ligase joins the ends to make another molecule of double-stranded circular DNA (**Figure 6**).

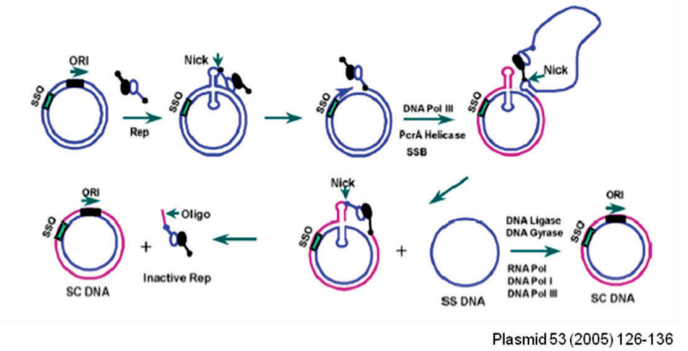


Figure 6 A model for rolling circle replication (From Khan, 2005)

Southern blot analysis on the S1 nuclease treated DNA indicated that plasmid pAR141 replicates via the rolling circle replicating (RCR) model. The RCR plasmids can be classified into at least five groups based on sequence similarity of their *dso* and *rep*B genes. Alignments of these sequences show that pAR141 belongs to the pMV158 plasmid family or the group II of the RCR plasmids on the RCR replicon database (http://www.essex.ac.uk/bs/staff/osborn/DPR/DPR_RCRIIalign.htm). A detailed nucleotide sequence of the plasmid pAR141 is shown in **Figure 7**.

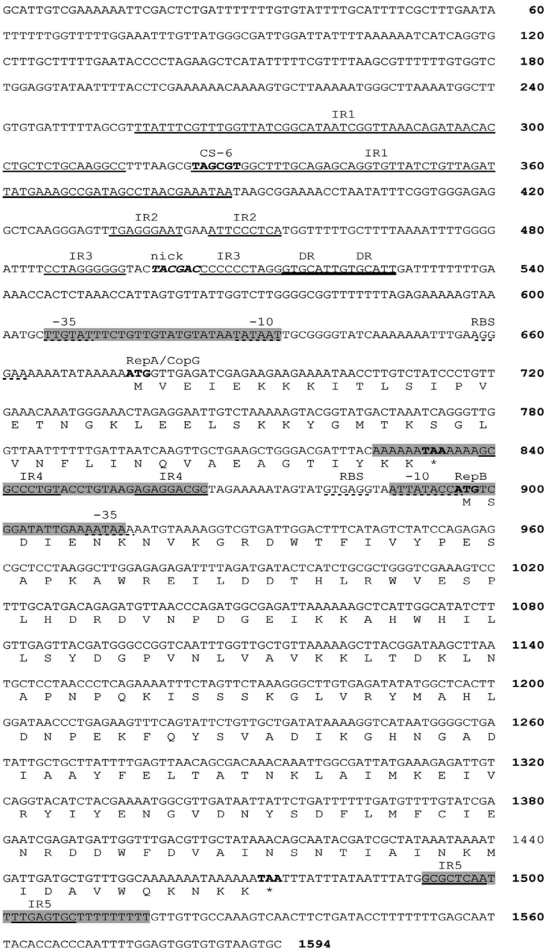


Figure 7 Detailed DNA sequence of plasmid pAR141. The inverted repeats (IRs) are underlined while the direct repeats (DRs) are double underlined. The nicking sequence of the *dso* is depicted in bold and italics. The deduced amino acid sequences of CopG and RepB are shown. The putative promoter and terminator sequences are shaded, while the ribosome binding sites (*rbs*) and the -35 and -10 sequences are highlighted by dotted lines. The start and stop codons are bold, the ctRNA sequence is deduced to be between 880 and 832 nucleotides. (From Raha *et al.,* 2006).

The G+C content of 36.1% of pAR141 falls within the normal range of lactococcal RCR plasmids. At the DNA level, only small regions of pAR141 gave significant homology to sequences from other small lactococcal plasmids. The *dso* and *sso*, which are the start sites of the leading- and lagging-strand synthesis of RCR plasmid can be detected on the non-coding region of pAR141. The 214 nucleotide *sso* region is located a short distance upstream of the *dso* and so is not exposed to a single strand form until the leading strand replication is almost completed. Two inverted repeats (IRs) were identified in this region. IR1 is an imperfect 132 bp, flanking a 6 bp consensus sequence, CS-6 (5'-TAGCGT-3'), within the terminal loop of the IR1. A shorter IR (IR2) is located downstream of IR1. Sequence alignment analysis indicated that the *sso* of pAR141 belongs to the *sso*W family and has been identified in some small *Lactococcus* and *Lactobacillus* plasmids (Kramer *et al*., 1999). However, the *sso*A and *sso*W type origins were found to function efficiently only in their native host (del Solar *et al*., 1987; Khan, 2005).

Downstream of *sso*W is another IR (IR3) and two direct repeats (DRs) or iterons. All these sequences could form the *dso* which participates in the leading strand replication of RCR plasmids. At position 486, a 29 nucleotide IR3 is present. The 9 bp sequence 5'-TACTACGAC-3', which is only found conserved among RCR plasmids of the pMV158 family (del Solar *et al*., 1998), is located at the loop of the stem. This sequence is predicted to be the *nic* region, where the nicking site of this plasmid is located between the G and A residues as has been shown in the case of pMV158 (de la Campa *et al*., 1990).

Analysis of the sequence predicted two putative open reading frames (ORFs)-encoding products larger than 50 amino acids, downstream of the *dso*. These two putative ORFs cover 46% of

the plasmid pAR141 and are located in the same orientation. The DNA and protein sequence were searched for homology and both putative ORFs showed highest similarity to the rolling circle replicating plasmids of the pMV158 family. Transcriptional repressor CopG was assumed to be involved in copy-number control, while replication initiator RepB is involved in plasmid replication. Genes-encoding other functions were not detected in this cryptic plasmid. Further analysis of the nucleotide sequence suggested that the *copG* and *repB* genes were organized as a single operon, with typical ribosomal binding sites located upstream of each gene. A putative promoter was detected upstream of the *cop*G (nt 580 – 629). Downstream of *repB*, an IR (IR5) followed by a stretch of TS was found, forming a possible transcriptional terminator for the operon.

Nucleotide sequence analysis also revealed that plasmid pAR141 could encode a small counter-transcribed RNA (ctRNA) on the complementary strand, located between the *cop*G and *rep*B genes. A putative promoter P_{ct} consisting of a less conserved -35 region (5'-TTTATT- 3') and an extended consensus -10 region (5'-TGNTATAAT- 3') can be observed on the complementary strand overlapping the 5'-end of the *repB* coding sequence. A U-tract (from 838 to 826) that follows IR4 may be a rho-independent site involved in the instrinsic termination of transcription of the ctRNA. Similar genetic organisation of the ctRNA is common in plasmids of the pMV158 family.

Constitutive and Inducible Expression Vectors

L. lactis has potential to be a vector for delivery of useful proteins especially in the pharmaceutical and medical fields. This bacterium is of particular interest for oral delivery of functional proteins since it is a non-commensal food bacterium that cannot survive in

the gastrointestinal tracts of humans and animals. A great deal of interest has been shown to genetically modify these organisms to improve their traits by introducing specific genes through cloning techniques. Development of cloning vectors based on lactococcal plasmids and the isolation of promoters and signal sequences have made it possible to introduce genes from non-lactococcal sources into *Lactococcus*. A plasmid vector characteristically should be of low molecular weight, non-conjugative, possess an origin of replication, has restriction enzyme sites, multiple cloning sites, promoters, ribosomal binding sites and a selective marker.

Several available vectors such as pMG36e have a constitutive promoter (P_{32}), which does not allow for controlled expression of recombinant proteins. These promoters are not controlled by any regulator or growth conditions and those used in the laboratory such as P21, P23, P32, P44 and P59 are randomly isolated from the *L. lactis* genomic library and distinguished by the chloramphenicol acetyl transferase activity levels.

On the other hand, inducible promoters drive the expression of genes involved in cell adaptation to its environment, by stress conditions such as phage attack and temperature or pH shift. A number of well-studied inducible promoters in *L. lactis* include the P_{170} promoter - self inducible via lactic acid accumulation at pH<6, the *dnaJ* promoter - induced by heat shock, the P_{BusA} promoter - controlled by salt levels (Siren, *et al*., 2009), and the P_{zn}*zit*R - represses expression by competing with RNA polymerase binding (Lull and Poquet, 2004). The most commonly used inducible promoter in *L. lactis* is the nisin-controlled promoter system.

The nisin inducible gene expression system is based on the autoregulatory properties of the *L. lactis* nisin gene cluster. Nisin is a small cationic, hydrophobic peptide of 32 amino acids that belongs to the lantibiotic class of bacteriocins (de Ruyter *et al*.,

1996). It is a natural antimicrobial agent with activity against a wide variety of Gram-positive bacteria, including food-borne pathogens such as *Listeria, Staphylococcus* and *Clostridium*. The primary target of nisin is believed to be the cell membrane. Unlike some other antimicrobial peptides, nisin does not need a receptor for interaction with the cell membrane; however, the presence of a membrane potential is required. Nisin is a natural preservative present in cheese made with *L. lactis* spp. *lactis*, but it is also used as a preservative in heat processed and low pH foods. Since nisin cannot be synthesized chemically, the nisin-producing *L. lactis* strains are used for its industrial synthesis (Kuipfers *et al*., 1995). Two genes in the cluster, *nisA* and *nisF*, are induced by nisin via a two-component signal transduction pathway containing a histidine protein kinase, NisK, and a response regulator, NisR. Expression of both *nisR* and *nisK* is driven by the constitutive promoter *nisR*.

Expression vectors will allow the researcher to express the genes of interest directly from their recombinant DNAs. A typical expression vector will have a promoter upstream of the DNA containing the sequence to be expressed. An inducible expression vector was constructed in our lab (Varma *et al*., 2004). The vector pRnis, contains the *gfp* gene cloned under the control of the nisA promoter. Green fluorescence protein (GFP) is a protein of 238 amino acids, isolated from the Pacific jelly fish (*Aequoriea Victoria*), which spontaneously emits green light at 508 nm when excited with blue light at 395 nm in the presence of oxygen (Chalfie, 1995). GFP has the advantage of being an auto-fluorescent protein that does not require a substrate, allowing its detection in living cells in real time. This plasmid vector allows the regulation of the transcription from P_{nis} promoter depending on the presence of galactose in the growth medium. Genomic DNA of *L. lactis* ATCC 11454 was isolated using the method of Engelke *et al.* (1992) with minor modifications.

Polymerase chain reaction technique was used to amplify the promoter region of the *nis A* gene using primers designed from published sequences (de Ruyter *et al*., 1996). This is a 215 bp fragment from positions -156 to +3 with respect to the *nis A* gene promoter transcription start site, which includes the -35 and -10 sequences and ribosomal binding site of *Lactococcus*. Primers were designed to include *EcoR*I and *Xma*I restriction enzyme recognition sites (**Figure 8A**). The *gfp* gene was then amplified and cloned downstream of the *nis A* promoter where a positive expression indicated a functional inducible promoter (**Figure 8B**).

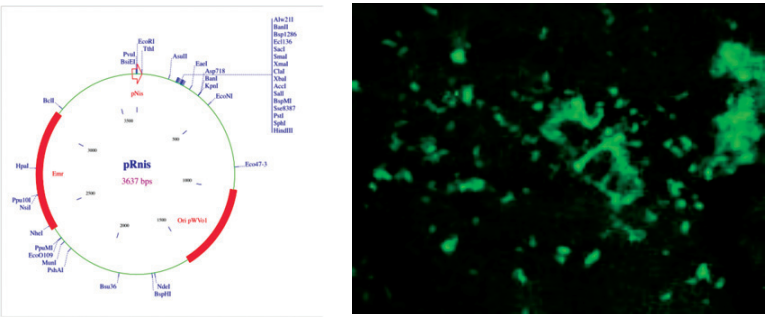


Figure 8 **A**, Map of pRnis and **B**, a fluorescence micrograph of *L. lactis* (pRnisGFP) expressing GFP (From *Varma et al.,* 2004)

In this study, the P_{Nis} promoter was shown to be able to be induced by galactose in a host system that is without the *nis K* and *nis R* operon as part of its chromosome or included in the plasmid construct. This simple inducible expression system could contribute to the pool of available expression systems for *Lactococcus* spp. and thus could be a useful tool in expressing genes of interest. In addition, we showed that *gfp* could be used as a reporter gene for the analysis of gene expression in *L. lactis.*

A second expression vector was also constructed in our laboratory from the indigenous cryptic plasmid pAR141 utilizing the constitutive promoter P_{32} (Hooi *et al.*, 2009). This vector, named pAR1411, incorporates multiple cloning sites, terminator and gene coding for an erythromycin resistance and has been shown to express the chloramphenicol reporter gene constitutively in *L. lactis*. Interestingly, although pAR141 and its derivatives were expected to be functional in a wide range of bacteria due to sequence similarity to pWV01, pAR1411 was not able to replicate in *E. coli*. This could be explained by the detection of the ssoW type origin replication in pAR141 that allows the plasmid to function efficiently only in its native host (Raha *et al*., 2006).

Shuttle Vectors

Vectors that can replicate in two or more hosts are called shuttle vectors. **Shuttle vectors** allow DNA to be transferred between two different species. The shuttle vector has *two origins of replication*, allowing replication to occur in either system/host where it "shuttles" between two different species. Typically, one host is bacterial (e.g. *E. coli*) and the other host is a eukaryotic organism (e.g. yeast). The bacterial host is used for all of the cloning steps and the eukaryotic host can be used to study the expression from that cloned gene or can be used to synthesize a product from the gene. A shuttle vector can also be designed to allow "shuttling" between two different groups of bacteria, or between the same group and different strains (Kleerebezem *et al*., 1997). Shuttle vectors can also be used to perform what is called reverse genetics. It is possible to replace or alter the sequence of regulatory elements that control expression of a given gene, and then put the gene back into their normal host cells to see how gene behavior has changed. This provides information

on how the regulatory element might function and which are the important sequences within the regulatory element itself.

The high copy number, small molecular weight and the presence of an erythromycin resistance gene suggest the potential use of the plasmid pAJ01 as a cloning vehicle for *L. lactis*. A shuttle-cloning vector, pAJ02, for replication in both *E. coli* and *L. lactis* was constructed by cloning the plasmid into pUC19 (Raha *et al.*, 2002) as shown in **Figure 9**. The erythromycin resistant gene carried by the plasmid pAJ02 was shown to express in both *L. lactis* and *E. coli* through the induction of sub-inhibitory levels of 5 and 150 µg/ml erythromycin. This is important because other antibiotic resistant genes such as kanamycin and ampicillin, which are easily selectable markers in *E. coli* and *B. subtilis*, are problematic markers for selection in *L. lactis*.

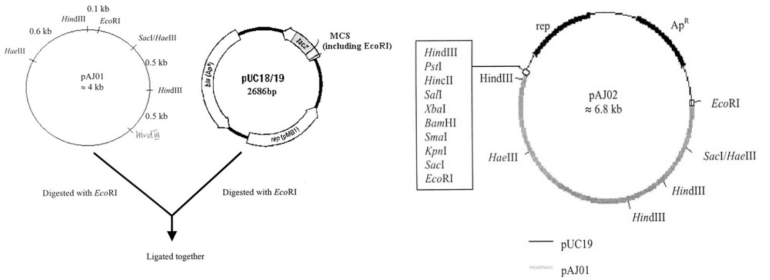


Figure 9 Construction of a *L. lactis* - *E. coli* shuttle vector (Adapted from Raha *et al*., 2002)

Plasmid stability is one of the major concerns in vector construction. Apart from size and harbouring genetic markers, it is important for a vector to be highly stable so that the desired gene that has been cloned will be stably maintained in the new host. The maintenance of a plasmid often induces a stress response especially when a target protein is highly expressed. Such stress

responses resemble environmental stress situations such as heat shock, amino acid depletion or starvation (Hoffmann *et al*., 2002). Stress induced by plasmid maintenance is often related to plasmid copy number (Bailey, 1993), while the main perturbation can be attributed to genes encoded by the plasmid and even constitutively expressed genes such as antibiotic resistance genes. In all our work with vector development, the stability test for plasmids will often be carried out in their hosts for up to 100 generations in non-selective growth conditions. In the case of pRnis, pAJ102 and pAR1411, we found that our new constructs were all stably maintained, indicating a functional replication mechanism.

Antibiotic Resistance Free Vectors?

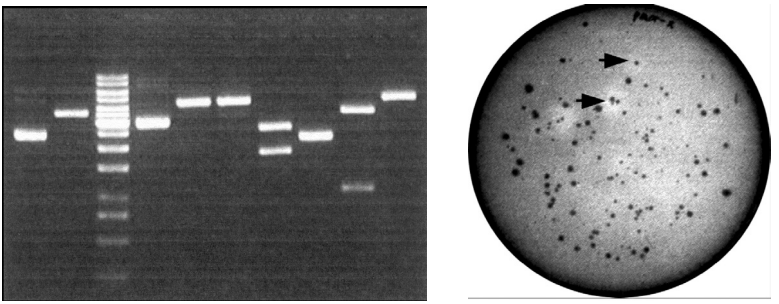
A plasmid vector would contain a drug-resistance gene for selective amplification. Once the vector enters the host cells, it will proliferate and divide with the host. However, most transformation efficiency is very low and transformed host cells will have to compete with non transformed cells. Typically, antibiotics will be used to kill host cells that do not contain the vectors carrying the drug-resistance gene whilst transformed cells will be protected by the resistance gene residing within the plasmids that had successfully entered the cells. Unfortunately, there are inhibitions of using the drug-resistance genes as markers for the host carrying the genes of interest. This is due to the worry of possible horizontal transfer of the antibiotic resistance genes, and the presence of which is discouraged in clinical applications. Many attempts have been made to produce vectors that are free of antibiotic resistance, among them the *Listeria monocytogenes-Escherichia coli* shuttle plasmid that is retained by complementation of D-alanine racemase-deficient mutant strains both *in vitro* and *in vivo* (Verch *et al*., 2004) and the eukaryotic amber suppressor t-RNA encoding pFAR plasmids

(Marie *et al*., 2010). The choice of the alternative markers and the selection mechanism of transformants are of utmost importance when designing these types of vectors.

Xylanases catalyse the hydrolysis of xylan, the major constituent of hemicelluloses. The use of these enzymes could greatly improve the overall economics of processing lignocellulosic materials for the generation of liquid fuels and chemicals. In the pulp and paper industries, as well as the food industry, xylanases have the potential to replace the chlorine-based paper bleaching process in paper production and for bioconversion of lignocelluloses material to fermentative products (Lubeck *et al.*, 1997).

Xylanases are found in a variety of microorganisms and the genes encoding them have been cloned in homologous and heterologous hosts with the objective of overproducing the enzyme and altering its properties to suit commercial applications (Baba *et al.*, 1994). We sub-cloned and expressed the xylanase gene from *Bacillus coagulans* ST-6 in *L. lactis* using the expression vector pMG36e (a gift from Leenhouts, K., the Netherlands). This work was carried out to gauge the potential of this enzyme to be used as an alternative selective marker for cloning and expression studies of recombinant proteins in *Lactococcus* (Raha *et al.*, 2006). The ability to express xylanase and the ease of detection were two criteria that were looked into. The xylanase gene was amplified from a chimeric plasmid pBNXy, containing a fragment of the xynalolytic genomic DNA of *B. coagulans* ST-6. The 750 bp PCR product was then subcloned into the unique *Nhe*I site of *E. coli - L. lactis* shuttle vector pMG36e and subsequently transformed into competent *E. coli* XL1-blue MRF' cells and *L. lactis* MG1363 cells. Growth temperature of the recombinant *E. coli* harbouring pMG36e-Xy at 37^{o}C was seen to cause considerable loss of recombinant plasmids (after 10 generations) as opposed to growing the cells at 30 - 32^{o}C.

Whilst it took only 24 hours for the production of clear zones to be formed around the *E. coli* transformants, it took more than 48 hours before the same could be detected around the *L. lactis* transformants (**Figure 10**).



A B

Figure 10 **A**, Restriction enzyme digestion analysis of pMG36e-Xy isolated from *Escherichia coli* XL1-Blue MRF; **B**, *Lactococcus lactis* MG1363 containing pMG36e-Xy on SGM17-RBB-Xylan agar. The clear halo zones surrounding the colonies are due to the diffusion of Remazol Brilliant Blue upon the breakdown of xylan by the secreted xylanase (From Raha *et al.*, 2006).

Bacterial culture containing pMG36e-Xy was shown to have an enzyme activity of 390 μg xylose ml $^{-1}$ culture min $^{-1}$ respectively when compared with only 40 μg xylose ml^{-1} culture 30 min $^{-1}$ for the control (plasmidless strain).

There were significant differences in the optimum growth temperature and plasmid stability in the transformants. Plasmid instability is frequently observed with recDNA especially in Gram positive bacteria. One of the reasons is because of segregational instability which refers to the loss of the plasmid population among the cells. Amongst the factors important for the maintenance of plasmids in bacterial cells is their replication and accurate

partitioning during cell division that can be disrupted by structural instability because of DNA deletions or rearrangements. From this study, we concluded that whilst the thermostable xylanase from *B. coagulans* ST-6 could be expressed in *L. lactis*, the slow release of the enzyme and the instability of the plasmids harbouring the gene indicate that xylanase is not an advantageous selection material to be used in *Lactococcus*. However, the use of *Lactococcus* as a host to produce xylanase can be looked into and developed further.

Surface Display

Bacterial surface displays have received considerable attention in the fields of vaccine delivery, whole cell adsorbents, biocatalysts and diagnostic tools. Many studies have been carried out to evaluate the effect of antigen presenting schemes by Gram positive bacteria on the immune system. In some schemes, the antigen is secreted or expressed intracellularly in the host cytoplasm (Wells *et al*., 1993). In others, due to the advantages related to having a direct contact between the antigen and immune system, the development of schemes to display the foreign antigens on the surface of bacterial cells is pursued (Ribeiro *et al*., 2002). The food-grade *L. lactis* is a potential vector to be used as live vaccine.

Several bacterial and viral antigens and cytokines have been produced efficiently in *L. lactis* (Ribeiro *et al*., 2002; Bermudez *et al.*, 2003). Improvement in the expression systems for *L. lactis* includes the use of inducible and constitutive promoters, secretory and intracellular expression of heterologous antigen and use of `GEM particles' to present the antigenic epitope on the cell surface (Bosma *et al*., 2006: van Roosmalen *et al.*, 2006).

N-Acetylmuraminidase AcmA is an autolysin of *L. lactis* with a molecular weight of 46 kDa that is required for cell separation and is responsible for cell lysis during the stationary phase (Buist *et al*.,

1997; Steen *et al.,* 2003). The major peptidoglycan hydrolase of *L. lactis* comprises of three domains including the N-terminal signal sequence, an active domain and a C-terminal membrane anchor. Three repeated regions comprising 44 amino acids are present in the C-terminal domain of the protein. Whilst all three repeats are involved in bacterial cell wall binding, binding to cell wall is possible with only one of the repeats.

Using the knowledge gathered from literature, and due to lack of commercially available lactococcal plasmids at that time, our group came up with a hypothesis that the repeats in the *acm*A sequence could be expressed as a fusion protein, expressed in another simple host, purified and re-anchored to the surface of *L. lactis* (**Figure 11**). Several other cell wall proteins of *L. lactis*, such as nisP (Raha *et al*., 2004, 2006), were also cloned to facilitate future work on surface display.

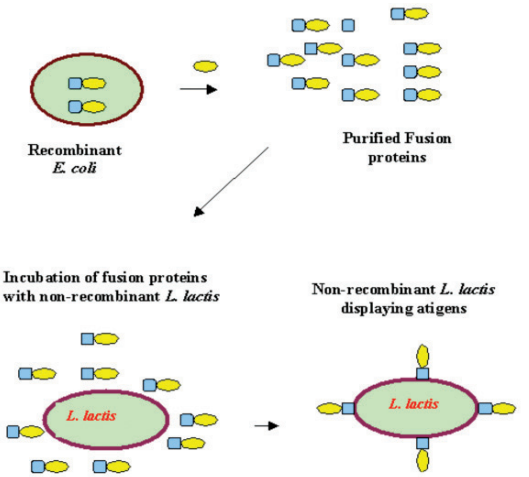
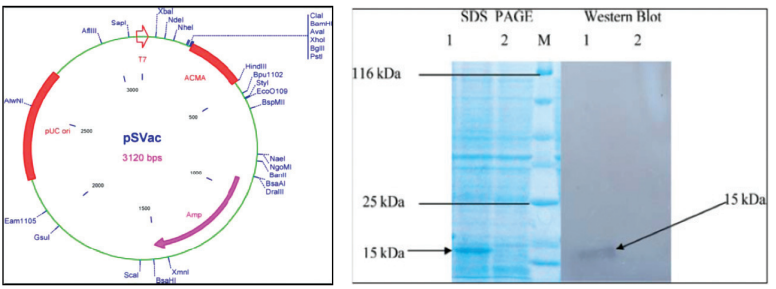


Figure 11 Model of the *L. lactis* docking system

We constructed a plasmid vector pSVac based on an *E. coli* expression vector that harbours a 255 bp single repeat sequence of the cell wall binding region of the AcmA protein (**Figure 12**). The pRSET vectors are pUC-derived vectors designed for high-level protein expression in *E. coli*. The plasmid includes an ATG translation initiation codon, a poly-His tag, a transcript-stabilizing sequence, a strong T7 promoter, an anti-express epitope and the enterokinase cleavage recognition sequence. Double digestion with the restriction enzymes *Pst*I and *Hind*III showed that the 255 bp repeat sequence of the C-terminal region of the cell wall binding protein AcmA was successfully cloned in-frame with the T7 promoter downstream of the His tag sequence.



A **B**

Figure 12 **A**. Map of pSVac construct, vector contains T7 promoter, ribosomal binding site, the start of an open reading frame, His-tag, MCS, pUC origin of replication, and ampicillin resistance gene. **B**. SDS-PAGE and Western blot analyses of the over-expressed AcmA recombinant protein. Lane 1, Total protein of BL21 (DE3) pLysS (pSVac), lane 2, total protein of BL21(DE3) pLysS pRSETC as negative control, lane M, protein marker (From Raha *et al*., 2005).

We then fused the *a1* and *a3* regions of the virus EV71 VP1 gene upstream to the *acm*A-225 and expressed the construct in *E. coli* BL21(DE3)pLysS. SDS-PAGE and Western blot analyses

showed the presence of fusion protein bands of the expected size. Although the expression of foreign genes in *E. coli* has been widely documented, this was the first report on the expression of a functionally active cell wall fragment domain of the *L. lactis* for surface display (Raha *et al*., 2005). Interestingly the recombinant protein was found to have maintained its capability to anchor onto the cell surface of *L. lactis*, as well as those of other LABs such as *Lactobacillus* and *Bacillus* (**Figure 13**). The stability assay carried out indicated that the fusion proteins were also stably docked onto the cell surface of *L. lactis* for at least five days.

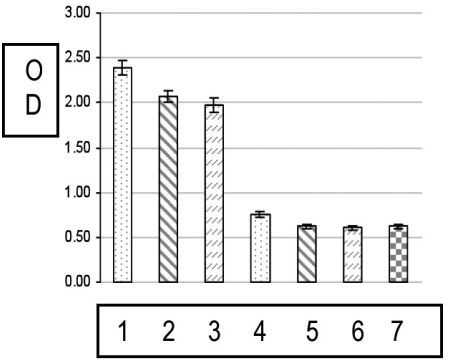


Figure 13 1, *L. lactis* incubated with AcmA protein; 2, *B. subtilis* incubated with AcmA protein; 3, *B. sphaericus* incubated with AcmA protein; 4, *L. lactis* not incubated with AcmA protein; 5, *B. subtilis* not incubated with AcmA protein; 6, *B. sphaericus* not incubated with AcmA protein; 7, PBS and substrate.

Through the work reported here, we have developed a strategy for display of antigenic determinants on the cell surface of *L. lactis* (Malaysia Patent filed - PI20044832). This method could also be used to display peptides or epitopes on to the cell surface of *L. lactis*

and other LABs for other useful applications such as biosensor and delivery of pharmaceutical and nutraceutical products.

Other Vectors

We have also constructed several other types of specialized vectors (submitted for publication). pSTag is a vector that has the His- and S-Tag cloned under the control of the nisin promoter. Its function would be to enhance the ability to detect and purify recombinant proteins expressed in *L. lactis*. We are also working on new surface display and integrative vectors for *L. lactis* as well as secretion-specific vectors for both *L. lactis* and *E. coli*.

Table 2 Summary of several Lactococcal plasmids and vectors constructed in our laboratory

	Name	Origin of plasmid	Genotype	Characteristics	Publications
1.	pAR141	Lactococcal Milk strain M4	1.6 Kb , rep ori	Rolling circle replication, full sequence	Plasmid (2005) 53. 126-136
2.	pAR1411	pAR141	P_{32}, camR, MCS	Constitutive	African Journal of Biotechnology (2009). 8(21): 5621-5626.
3.	pRnis	pMG36e	P_{nis}, eryR	Inducible	Current Science (2004) 87 (9). 1185-87.
4.	pAJ01	Lactococcal chicken intestine strain	4Kb, eryR	Has its own antibiotic resistance gene, RE map	Journal of Biochemistry, Molecular Biology and Biophysics (2002) 6 (1). 7-11
5.	pAJ02	pAJ01	6.8Kb, ampR, eryR, mcs	Partial sequence, Shuttle vector	Journal of Biochemistry, Molecular Biology and Biophysics (2002) 6 (1). 7-11
6.	pSVac	pRSET	T7 promoter, ampR, *acm*A	Surface display	Applied Microbiology and Biotechnology (2006) 6:875-8
7.	pMGxy	pMG36e	P_{32}, eryR, xylnase gene	Shuttle vector, expression of xylanase	Letters of Applied Microbiology (2006). 42: 210-214

THE MICROBIAL FACTORY

An important aspect of the research on microbes adopts the concept of using them as biofactories, which is their use in the production of molecules important to the pharmaceuticals and industrial sectors. The elementary nature of bacteria and the increased understanding of industry scale culture make them attractive bioproducers. In these instances, a close working relationship should be established between researchers in the different areas of biotechnology such as molecular biology, fermentation technology and enzyme technology. We have utilized available plasmids and bacterial hosts to express important enzymes and proteins. Below are some examples of the work carried out in our laboratory;

E. coli Hosts and Vectors

The production of proteins is one of the main applications of genetic engineering in biotechnology. Even though standard cloning procedures are now routine and a large variety of host-vector systems for gene expression are available, difficulties are encountered when theoretical strategies are put into practice, so gene expression is still quite empirical. *E. coli* remains an important host system for industrial production of proteins from cloned genes, and considerable experience has been accumulated since the ground-breaking gene expression experiments. The extensive knowledge on *E. coli*'s physiology and genetics accounts for its preferential use as a host for gene expression **(Figure 14)**.

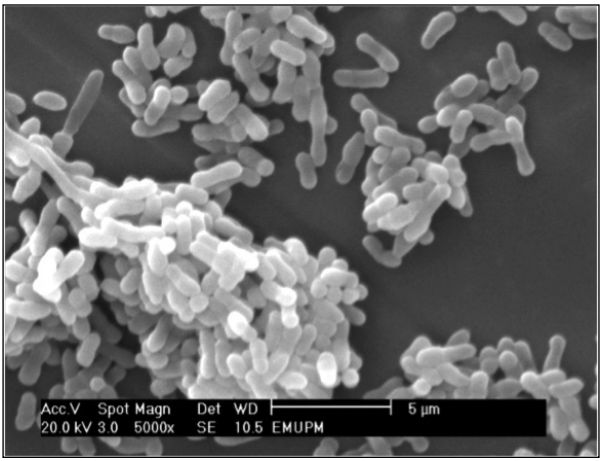


Figure 14 A scanning electron microscope of *E. coli* (lab isolate)

The use of *E. coli* confers several advantages to the users i.e. rapid and high level expression as a result of high speed growth to high density, low complexity and low cost of growth media and the ability to target proteins to the desired subcellular locations (Fernandes *et al*., 1999). Further, a number of mutant host strains and vectors that can improve the expression of recombinant proteins are available where host strains with mutations in the cytoplasmic protease genes reduce protein degradation. Host strains such as the TOP system allows for easy cloning and transformation, whilst the BL21, Origami and OrigamiB series are deficient in certain reductases and promote the formation of disulfide bonds (Sone *et al*., 1997). Vector such as the pET32 series increase the yield of soluble proteins in the cytoplasm (La Valie *et al*., 1993) while the IMPACT system (New England Biorad) offers a tag that combines the benefits of the chitin-binding domain with a self splicing protein element. Several commercially available host strains and vectors for *E. coli* are shown in **Tables 3** and **4**.

Table 3 List of several commercially available *E. coli* strains (Adapted)

Host	Genotype	Reference
TOP10	*F– mcrA Δ(mrr-hsdRMS-mcrBC) ψ80lacZ ΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG*	Kang *et al*., 2002
BL21	(*F' ompT hsdSB (rB– mB–) gal dcm*)	Kang *et al*., 2002
BL21(DE3)	[*F–, ompT, hsdSB (rB–, mB–), gal, dcm, Δ(srl-recA)306::tn10 (tetr) (DE3)*]	Toksoy *et al.*, 2002
BL21(DE3) pLysS	F-ompT hsdSB (rb- mB-) gal dcm (DE3) pLysS (CmR)	Hao *et al*., 2007
XL1 strain	[*F':tn10 (tetr), Δ(lacproAB), lacIq, recA1, endA1, gyrA96 (Nalr), thi-1, hsdR17(rk–,mk +), supE44, relA1, Δ(lac), λ–*]	Toksoy *et al*., 2002
W3110	(*F– mcrA mcrB IN(rrnDrrnE) 1 λ–*)	Kang *et al*., 2002
JM103	(r-, m +, *Alac-pro, thi, strA, supE, endA, sbcB15, hsdR4* [F', *traD36, proAB, lacI q, lacZAM15]*	Hellman *et al*., 1992

Table 4 List of several commercially available *E. coli* plasmid vectors (Adapted)

Vectors	Characteristics	Reference
TOPO Blunt	pUC ori, Kn^r, lacZα	Invitrogen
TOPO TA	pUC ori, amp^r, lacZα	Invitrogen
pET-32a-c (+)	pBR322 ori, amp^r	Novagen
pET-21a-d(+)	pBR322 ori, amp^r	Novagen
pET-22b(+)	pBR322 ori, ampr	Novagen
pBAD/His A,B,C	pBR322 ori, amp^r, AraC	Invitrogen
pFLAG-CTC	pBR322 ori, amp^r	sigma-aldrich
pTAC	pBR322 ori, amp^r	sigma-aldrich
pRSFduet-1	RSF ori, kn^r	Novagen

Researchers working with *E. coli* hosts are faced with a wealth of vector options when designing recombinant proteins. Every year new host strains with characteristic advantages and plasmid vectors are made available commercially. Additionally, since the types of proteins are intrinsically unique, researchers must know which system to choose and modify so that it can be used to optimize gene expression. Therefore, some of the pertinent questions to ask when deciding on *E. coli* systems would be which type of promoter and gene marker is required and whether a fusion protein is necessary to facilitate its localization, detection, enhanced solubility and purification.

a) Expression of the *ctxB* Gene from *Vibrio cholera*

Vibrio cholera is a well known human pathogen that has caused a worldwide cholera epidemic. Many different techniques have been utilized to detect and differentiate the *Vibrio* species, such as conventional bacteriological methods and immunological and molecular methods (Neela *et al.*, 2000; Somarny *et al.*, 2002; Tracz *et al.*, 2007). The symptoms of cholera are caused by the cholera toxin (CT), an 85 kDa composed of A (ctxA) and B (ctxB) subunits combined to form halotoxin. Although CT cannot be used in humans as a mucosal adjuvant, the nontoxic ctxB subunits have been described as a potent immunogen in the intestinal and nasal mucosal sites (Rudin *et al.*, 1999).

The *E. coli* BL21(DE3)pLysS and vector pBAD/HisB were used in our aim to carry out a preliminary optimization study for the production of the ctxB protein in *E. coli* under time course and different concentration conditions using SDS-PAGE and western blot analysis (Haryanti *et al*, 2008). The pBAD vector offers very tight regulation of gene expression, inducible in the presence of arabinose. The system was chosen because it has been demonstrated to be an attractive choice for expression of toxic proteins in *E. coli* due to its stringent control under uninduced conditions. The 450 bp CTB encoding gene was PCR amplified, cloned into pBAD/HisB, and transformed into *E. coli* BL21(DE3) pLysS strain. Transformants grown to 0.6 OD_{600} were induced at 37°C for 3 hours with the addition of arabinose at concentrations of 0 – 0.2% (w/v). Results showed that the optimum concentration of ctxB as detected by immunoblot against His-tag was produced at 0.2% arabinose concentration after 2 hours induction at 37°C. In this study, the expression of ctxB in *E. coli* under the inducible control of the arabinose promoter was demonstrated. The araBAD promoter provides a distinct advantage over some of the more

commonly used over-expression systems in bacteria (example the T7lac promoter of the pET system), as it provides a much tighter control over basal expression.

b) Expression and Production of L-ldh from *Enterococcus faecalis* KK1

Lactate is used in various industries such as food, pharmaceutical, medical and agriculture, where its production is approximately 15 million metric tons per year globally (Tsuji, 2002). The demand for lactic acid is rapidly expanding with the introduction of polylactate (PLA), a renewable, biodegradable plastic in the market place (Tsuji, 2002). Lactic acid is the main fermentation product excreted by the gram positive lactic acid bacteria (LABs) and can either be in the form of L-lactate or D-lactate. L-isomers are more abundant compared to the D-isomers for most uses of bioplastics. Engineering the L-lactate gene in *E. coli* offers advantages such as producing optically pure L-lactic acid with trace amounts of other fermentation products (Chang *et al*., 1999; Dien *et al*., 2001), and the capability of this host to utilize a wide variety of sugars.

An *E. coli* strain over-expressing the L-ldh gene was constructed. The 954 bp *L-ldh* gene amplified from the genome of *Enterococcus faecalis* KK1 was cloned into pBAD and transformed into a knockout *E. coli* strain SZ85. Comparison of nucleotide and amino acid sequences with the *L-ldh* gene of Lactococcal strains revealed a moderate degree of homology (68-70% amino acid). Genera *Lactococcus* and *Enterococcus* were once grouped under the same genus *Streptococcus* and this was reflected in the moderately high homology between the two in L-lactate dehydrogenase amino acid sequences. The presence of a 40 kDa band on SDS-PAGE confirmed the expression of the L-LDH enzyme and its maximum activity was achieved at 170 U/ml. Production of L-lactic acid by

the recombinant *E. coli* was initially studied in batch fermentation using fructose as a carbon source.

The effect of temperature and pH on the production of lactic acid using *E. coli* BAD85 was also investigated using batch cultivation in shake flask and 2L bioreactor. The final lactic acid yield was 62% (g/g) of the net theoretical fructose concentration from 1 g/L of fructose; meaning 0.62 g of lactic acid and 0.68 g of cell mass were produced from 1 g of fructose by the recombinant strain *E. coli* BAD85 at pH 7 and 37ºC. The production of L-lactic acid by *E. coli* BAD85 was further studied in a 2L bioreactor system at a controlled pH of 7.0. Our recombinant *E. coli* BAD85 (Tengku Elida *et al.*, 2009) was shown to have produced lactic acid 1.7 fold higher than the control *E. coli* SZ85 strain. The chirality of L-lactic acid was observed at 98%. The maximum level of L-ldh activity in *E. coli* BAD85 at 170 U/ml was 1.72 fold higher than that in the *E. coli* SZ85.

c) Expression of the SOD Gene from *L. lactis* M4

Oxygen (O_2) is an important element in many living organisms. However, there are several disadvantages related to the utilization of oxygen linked to its potential toxicities. During partial reduction of O_2, reactive oxygen species (ROS), such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) are formed (Kreig and Hoffman, 1986). These ROS impose oxidative stress which can cause oxidative damage to the cells, including deoxyribonucleic acid (DNA) strand breakage, protein inactivation and membrane lipid peroxidation (Burdon *et al*., 1996).

Living organisms have developed defense mechanisms against the toxic effects of ROS in order to protect themselves. Superoxide dismutase (SOD, EC 1.15.1.1) is a metalloenzyme which acts as a defense mechanism against oxidative stress by catalyzing the

formation of H_2O_2 and O_2 from O_2^- (Fridovich, 1995). The produced H_2O_2 is then detoxified by catalase or peroxidase. SOD can be found in almost all aerobic and some anaerobic organisms. SOD can be classified into 3 groups according to their metal cofactor: manganese (MnSOD), iron (FeSOD) and copper-zinc (CuZn-SOD). MnSOD, encoded by *sodA*, is found in prokaryotes and in mitochondria of eukaryotes. All previously tested streptococci (including *Lactococcus lactis* subsp. *lactis*) appear to carry a Mn-SOD (Sanders *et al*., 1995). The purpose of this work was to clone and express the *sod* gene from the *L. lactis* in *E. coli* BL21(DE3) pLysS, prior to investigating the potential of mass production of the *E. coli* BL21(DE3)pLysS harbouring the superoxide dismutase form *L. lactis*. *L. lactis* was chosen as the source for SOD because of its GRAS status and its use to produce many bioprocessed foods, especially fermented dairy and meat products.

A full-length *sod* gene amplified from a locally isolated *Lactococcus lactis* M4 was cloned into vector pRSET A and transformed into *E. coli* strain BL21(DE3)pLysS. Deoxyribonucleic Acid (DNA) sequencing results showed 96% homology to the published nucleotide sequence of *L. lactis* subsp. *lactis* IL1403 *sod* gene.

Expression of the recombinant protein was analyzed by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and confirmed with Western Blot (**Figure 15**).

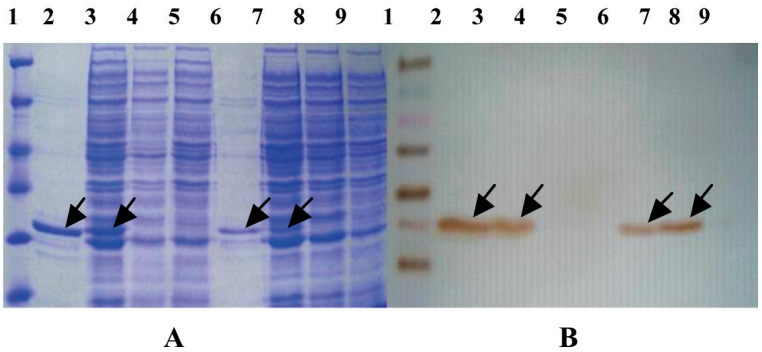


Figure 15 **A**, SDS-PAGE analysis of the denatured recombinant protein. **B** Western Blot analysis of the denatured recombinant protein. **A** Lane 1, Protein Molecular Weight Marker (Fermentas); lane 2, induced pRSOD with purification; lane 3, induced pRSOD; lane 4, induced pRSET A; lane 5, induced *E. coli* BL21(DE3)pLysS; lane 6, non-induced pRSOD with purification; lane 7, non-induced pRSOD; lane 8, non-induced pRSET A; lane 9, non-induced *E.coli* BL21(DE3) pLysS; **B**, Lane 1, Mid-Range Prestained Protein Marker (Mbiotech); lanes 2-9 as A. Arrows indicate the presence of recombinant SOD ~ 27 kDa.

The activity of the recombinant SOD was confirmed through nitroblue tetrazolium (NBT) negative staining system. Assay based on the NBT negative staining system detected achromatic bands on the nondenatured polyacrylamide gel after exposure to light. During subsequent illumination, the photochemically reduced riboflavin reduces dioxygen to O_2^-, which in turn reduces the NBT^{2+} to its purple formazan. SOD intercepts the photochemical flux of O_2^-, creating achromatic bands against the purple background of formazan. Apart from the expressed recombinant protein, three other bands were visualized on the gel. We suspect (Tan, MS thesis 2009 – submitted for publication) that those additional bands are the isoforms of MnSOD. MnSOD may appear in the form of homodimer and heterodimer. Western blot analysis

was carried out to confirm the presence of the isoforms of the recombinant protein. The result indicated that all of the four bands were detected by monoclonal anti-His antibody. This suggests that the additional protein may be isoforms of the recombinant SOD. Further investigation needs to be carried out to confirm the presence of isoforms such as by performing isoelectric focusing and two-dimensional gel electrophoretic analyses.

Experimental studies in molecular biology with transformed *E. coli* assume that the recombinant plasmids are stable. However, different growth and storage conditions can evoke changes in the transformed population (Smith and Bidochka, 2004). Shiloach and Fass (2005), reviewed the methods of growing recombinant *E. coli* strains and the development of the microbes to achieve high cell density cultivation. They stated that the overall productivity of the recombinant protein depends on numerous factors such as plasmid stability, promoter response to inducer, post transcriptional inhibition events, and post translation inhibition caused by proteolysis and improper folding. Our study (Wan Omar *et al*., 2008) showed that the recombinant plasmid carrying the *sod* gene (pRSOD) was stable up to 200 generations in the *E. coli* BL21(DE3) pLysS strain. The addition of IPTG induced the expression of SOD at the early exponential phase. In shake flask cultivation, LB and TB were used as the growth media and the highest dry cell weight of the recombinant *E. coli* was obtained at 3 g/L in the TB medium with 20 g/L of glucose.

Working with non-*E. coli* Bacterial Strains

Whilst *E. coli* remains the most attractive host for heterologous protein expression, in some cases a microbe will need to have its metabolic pathway engineered in order to improve production

of its useful proteins. When dealing with strains that do not have commercially available mutant hosts and vectors, the researcher must find ways to either develop their own vectors and hosts or solicit the help of others working with the same microbe.

a) Overexpression of the Hydrogenase Gene in *Clostridium butyricum* EB6

Hydrogen gas has been identified as the ideal clean energy of the future. Gaseous hydrogen is widely produced by microorganisms, but virtually absent in higher organisms. Anaerobic bacteria, facultative bacteria and photosynthetic bacteria have been reported to produce hydrogen efficiently either from synthetic medium or waste from various industries (Zhu *et al.*, 2008; Tang *et al.*, 2008). These microorganisms which generally have the ability to produce hydrogen gas during catabolism of carbohydrates and hydrogenases (EC 1.12.7.2) are known to release hydrogen gas from the reduced form of ferredoxin.

Clostridia species were identified as efficient hydrogen producers from wastewater. They are found in most hydrogen producing sludge and are able to ferment biomass polymers such as polysaccharides and protein to obtain energy and reducing powers such as proton/electron and reduced compounds in cells. A gram positive anaerobic bacterium, *Clostridium butyricum* EB6, was isolated from POME sludge. It was characterized as hydrogen producing bacteria that is capable of utilizing organic material in palm oil mill effluent (POME). Hydrogen production by *C. butyricum* EB6 was optimized to produce 2.2 mol H_2/mol glucose and the co-production of butyric acid and acetic acid (Chong *et al*., 2009).

In this work, the [Fe]-hydrogenase (*hyd*A) gene of *C. butyricum* EB6 was amplified and cloned into plasmid vector pTZ57R/T

(Fermentas, CA). A set of primer was designed based on the published sequence of *hydA*: 5' CATATCAATTCTTTGGCGCTTT 3' and 5' ATATATATTAGCATCTGTA 3'. Each PCR mixture (total volume, 25 μL) consisted of 2.5 μL of 10x PCR buffer, 2.5 μL of $MgCl_2$ (25 mM), 0.5 μL of dNTPs (10 mM for each), 0.5 μL of each primer (10 μM), 0.2 μL of Taq polymerase (5 U/μL), 0.5 μL of the above template and adequate amount of distilled water added to reach the desired volume. Sequencing results of the *hyd*A gene identified an open reading frame of 1725 bp which encodes 574 amino acids of approximately 64 kDaltons. The ribosomal binding site of GGAGG was located at -15 upstream of the *hydA* open reading frame. A -35 and -10 region (AAAAAA and AATTTA) with spacing of 18 bp were also located. Homology searches in NCBI carried out with the BLAST function based on similar sequence program (blastn) found that the *hydA* gene sequence of *C. butyricum* EB6 was 80.5% similar to the *hydA* of *C. acetobutylicum* P262 and closely similar to the *Clostridia* hydrogenase.

For overexpression of *hydA* in *C. butyricum* EB6, the hydA gene was subcloned into *E.coli-C. perfringers* shuttle vector, pJIR418 (ATTC) and a modified method of electroporation on *C. butyricum* EB6 was established for transformation (Chong, PhD thesis 2009).

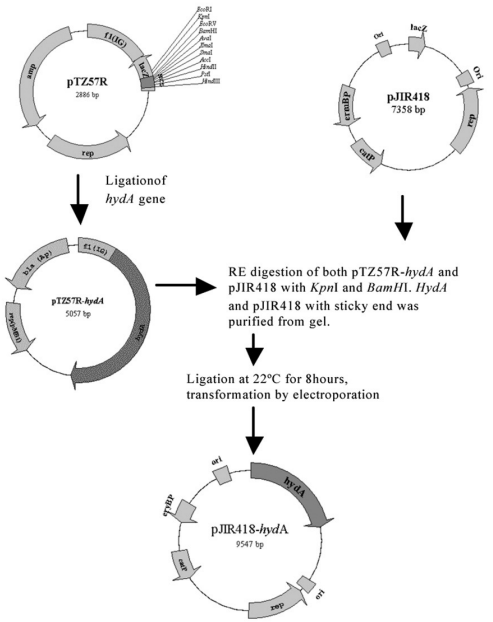


Figure 16 Overview of the construction of expression vector pJIR418-*hydA (*Adapted from Chong M.L., PhD thesis 2009).

When *C. butyricum* EB6 was cultivated in 1L of synthetic medium with 15.7 g/L of glucose and 0.39 g/L of $FeSO_4$, hydrogen production was achieved at 4.1L H_2/L medium or yield of hydrogen at 2.2 mol H_2/mol glucose (**Figure 17**). In conclusion, an increase of hydrogen gas percentage and total gas was obtained from the recombinant strain where the gas percentage increased from 55 to 75%, total hydrogen gas production from 4.2L to 4.8L and the yield of hydrogen from 2.2 to 2.5 mol H_2/mol glucose. Hydrogen production was shown to have increased 1.14 fold compared to the wild type (**Figure 18**). These results show that an increase in the copy number of the hydrogenase gene played a role in the increase

of hydrogen production in *C. butyricum* EB6. However, as observed in most recombinant strains, the growth of the recombinant *C. butyricum* was found to be lower than the wild type.

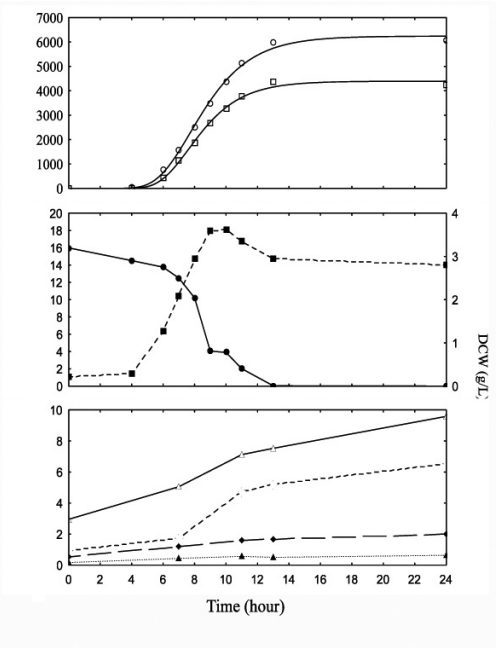


Figure 17 Batch biohydrogen fermentation of *C. butyricum* EB6 at pH 5.7, 37ºC, 15.7g/L of glucose and 0.39 g/L $FeSO_4$. (a) Profile of biogas and biohydrogen production (ml), (b) Profile of dry cell weight (g/L) and glucose utilization (g/L), (c) Profile of acid accumulation (g/L). ○: Total accumulated biogas (ml), □: Total accumulated hydrogen gas (ml), ■: Dry cell weight (g/L), ●: Glucose utilization (g/L), Δ: Total accumulated acid (g/L), ♦: Butyric acid (g/L), ◊: Acetic acid (g/L), ▲: Formic acid (g/L). (Adapted from Chong *et al*., 2009)

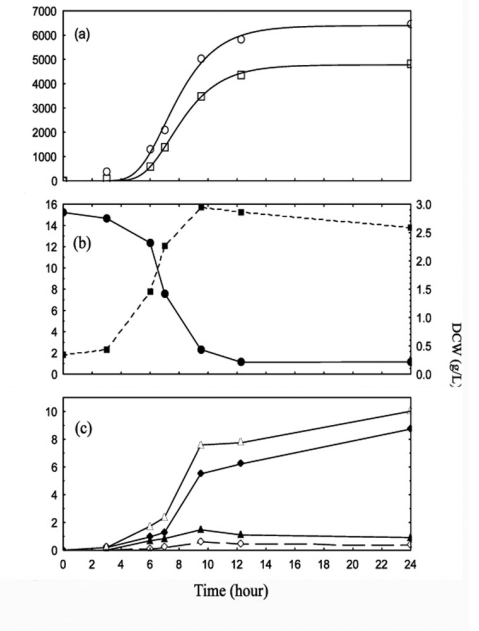


Figure 18 Batch biohydrogen fermentation of recombinant *C. butyricum* EB6 with overexpression of hydrogenase gene at pH 5.7, 37ºC, 15.7g/L of glucose and 0.39 g/L $FeSO_4$. (a) Profile of biogas and biohydrogen production (ml), (b) Profile of dry cell weight (g/L) and glucose utilization (g/L), (c) Profile of acid accumulation (g/L). ○: Total accumulated biogas (ml), □: Total accumulated hydrogen gas (ml), ■: Dry cell weight (g/L), ●: Glucose utilization (g/L), Δ: Total accumulated acid (g/L), ♦: Butyric acid (g/L), ◊: Acetic acid (g/L), ▲: Formic acid (g/L). (Adapted from Chong *et al*., 2009)

b) Expression of the VP Genes of Chicken Anemia Virus in *L. lactis*

Chicken anemia virus is a ubiquitous pathogen which clinically affects chicks aged 1 day to 2 weeks old. Clinical signs of the disease include anemia and thymic depletion (Yuasa *et al*., 1976). A horizontal transmission of the virus to chickens older than two

weeks results in subclinical infection which contributes to between 10% to 30% mortality rates leading to economic loss. CAV has three immunogenic major proteins. The viral protein 1 (VP1) is the capsid protein, viral protein 2 (VP2) is a scaffold protein, while viral protein 3 (VP3) has been identified as an apoptin, which causes apoptosis in infected cells (Noteborn *et al*., 1994). Polymerase chain reaction (PCR) was used to amplify the VP1 and VP2 genes of chicken anemia virus (CAV) CUX-1 and VP3 gene fragment of a CAV local isolate. The PCR products were cloned into vector pCR2.1-TOPO, transformed into *E. coli* TOP 10F' and positive transformants were selected by blue-white screening. Analysis of the recombinant plasmids showed the presence of the respective fragments which were then subcloned into pMG36e and electrotransformed into *L. lactis* MG1363. Northern blot and RT-PCT analyses confirmed the presence of the recombinant transcripts. SDS-PAGE and Western blot results showed the presence of the translational product of the VP3 gene (Raha *et al*., 2000).

DELIVERY OF LIVE VACCINES

The use of microbial vectors has provided a vaccine technology with broad applicability that could have significant impact on vaccine development worldwide. Over the past 20 years, experimental bacterial vaccine vectors that elicit immune responses against bacterial, viral, protozoan and metazoan pathogens in laboratory animals have been produced. The application of bacterial vector vaccines to human diseases has been studied most extensively in attenuated strains of *Salmonella*. Live, attenuated strains of *Shigella, Listeria monocytogenes, Mycobacterium bovis-BCG* and *Vibrio cholerae* provide unique alternatives in terms of antigen delivery and immune presentation, and also show promise as potentially useful bacterial vectors. The lactic acid bacteria, which we ingest

and which forms part of our intestinal flora, may serve as a vehicle for new types of oral vaccines.

In the last decade, the potential of live recombinant lactococci to deliver such proteins to the mucosal immune system has been investigated (Dieye *et al*., 2003; *Robinson et al*., 2003; Bermudez-Humaran, L.G., *et al*, 2005; Le Loir *et al*, 2005; Hanniffy *et al*, 2007, Yuvaraj *et al*., 2008). The usage of *L. lactis* as a vaccine vector is becoming increasingly important, due to its classification as a GRAS organism that is nonpathogenic and noncolonizing (Wells *et al*., 1996). Studies on the feeding of live lactococcus to human volunteers have shown that the passage of this bacterium through the intestinal tract is transitory (Drouault *et al*., 1999). Despite its lack of invasiveness, *L. lactis* has been shown to deliver heterologous antigens to the systemic and mucosal immune systems via mucosal routes (Steidler *et al*., 2000; Xin *et al*., 2004; Ramasamy *et al*., 2006).

Whilst the focus elsewhere is to develop *L. lactis* as potential vaccines against human pathogens, our approach is to utilise *L. lactis* as live vaccine carrier for the agriculture industry. We have thus far used the available vectors to clone and transform the genes coding for immunogenic epitopes of poultry and fish pathogens. The approach of oral delivery using *L. lactis* offers several advantages over traditional methods of vaccination of animals, i.e. easy administration and the ability to elicit both systemic and mucosal immune responses.

Live Oral Vaccine for Fish

Although fish farming has great development potential in Asia, fish diseases such as those caused by the bacteria *Aeromonas hydrophila* still remain a major constraint to its successful expansion. Aeromonads are opportunistic bacteria that can cause

infection which, depending on the species and the virulence of the strains encountered, may have life-threatening consequences. *A. hydrophila* has specifically been associated with several diseases of fish, including tail rot, fin rot, and hemorrhagic septicemias (Miyazake and Kaige, 1985; *Nielsen et al., 2001; Ronaldi et al., 2004).* A number of virulence factors may contribute to the overall virulence of *A. hydrophila* (*Radu et al*., 1997), including the extracellular products (ECPs). The characterization of antigens present in the ECPs and cell wall of motile *Aeromonas* sp. isolated from rainbow trout culture systems showed heterogeneity not only among the different serogroups but also within the same serotype (Santos *et al*. 1996).

Most bacterial vaccines are inactivated products and the recombinant vaccine technology has so far been used only to a very limited extent. Fish are usually immunized with multivalent vaccines by intraperitoneal injection. In marine fish species vaccination is generally performed by immersion. Oral vaccination has the advantage that it is a very easy vaccine administration method with no stress to the fish.

In our work we developed a live recombinant vaccine using *L. lactis* as the carrier for protection of Tilapia against *A. hydrophila* (*Journal of Applied Microbiology*, accepted 2010). The polymerase chain reaction (PCR)-amplified 250 bp and 750 bp sequences coding for domains D1 and D4 of aerolysin were individually cloned into pNZ8048 (a gift from Kees Leenhouts, the Netherlands) and electrotransformed into *L. lactis* NZ9000 (a gift from Kees Leenhouts, the Netherlands). The recombinant vaccine candidates were then either orally fed or injected intraperitoneally into tilapia.

Table 3 Antibody production in tilapia vaccinated orally with different immunogenic epitopes of *A. hydrophila* expressed in *L. lactis*

Week	A_{450nm}						
	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**
0	0.24 ± 0.00	0.27 ± 0.01	0.27 ± 0.01	0.27 ± 0.01	0.27 ± 0.01	0.25 ± 0.01	0.27 ± 0.01
1	0.73 ± 0.01	1.06 ± 0.03	1.30 ± 0.06	1.84 ± 0.05	0.41 ± 0.02	0.27 ± 0.04	0.32 ± 0.03
2	2.08 ± 0.04	1.73 ± 0.06	1.99 ± 0.02	2.07 ± 0.05	0.76 ± 0.02	0.54 ± 0.08	0.52 ± 0.05
3	1.27 ± 0.03	1.21 ± 0.07	1.26 ± 0.02	1.63 ± 0.07	0.34 ± 0.00	0.31 ± 0.04	0.37 ± 0.02
4	1.32 ± 0.03	0.93 ± 0.04	1.18 ± 0.03	0.95 ± 0.03	0.31 ± 0.03	0.26 ± 0.02	0.25 ± 0.03

T1, commercial diet + recombinant *L. lactis* with D1 (106 CFU/g); T2, commercial diet + recombinant *L. lactis* with D1 (108 CFU/g); T3, commercial diet + recombinant *L. lactis* with D4 (106 CFU/g); T4, commercial diet + recombinant *L. lactis* with D4 (108 CFU/g); T5, commercial diet + *L. lactis* NZ9000; T6, commercial diet + *L. lactis* NZ8048; T7, commercial diet. (± indicates standard error).

Table 3 shows the development of antibodies in sampled fish given oral vaccine compared to control groups indicating that the recombinant epitopes expressed in *L. lactis* were able to elicit an

immunogenic response in tilapia. Interestingly, the lower doses of both Lac-D1ae and Lac-D4ae gave higher antibody levels over the study period. Fish immunized with both Lac-D1ae and Lac-D4ae together showed the highest level of protection and significant reduction in the mortality rate compared to control strains in both modes of vaccination (data not shown).

We have shown that the recombinant *L. lactis* strain expressing D1 and D4 produced aerolysin specific serum IgM in tilapia. Both D1 and D4 promoted 55 to 82 % relative percentage survival against *Aeromonas* infection through intraperitoneal injection, whereas the relative percentage survival following oral feeding of the vaccine was 70 to 100 %. This was the first report of such work for the use of an oral vaccine using recombinant *L. lactis* in aquaculture, and we hope that it will be the beginning of many other improved vaccine systems.

CONCLUSION

Microbes offer great opportunities and also challenges. The development of mission purposed microbes by designing specialized vectors and insertion of genes was presented. The task of genetically engineering microbes to fulfill the needs of humankind more often than not outweighs fear of the unknown. Microorganisms such as *L. lactis* have potential to be utilised as safe vehicles to deliver therapeutic agents and useful proteins. The work towards developing *L. lactis* and other microbes as potential producers and carriers of useful proteins is far from over. UNDERSTANDING THE CHALLENGES AHEAD, we are now focusing our efforts on further improving our plasmid vectors and using new technologies like functional genomics to develop our *Lactococcal* and *E. coli* hosts for industrial and pharmaceutical purposes to support the

biotechnology agenda of the country and the world.
I end with these quotes…….

"I feel that the greatest reward for doing is the opportunity to do more."
Jonas Salk

"Nature has evolved to do what it does, and to get it to do something different is a nontrivial task."
Anonymous

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BIOGRAPHY

Raha Abdul Rahim was born in Johor Bahru, Johor. She received her early education at Convent Primary, Muar, Johor and at the Tunku Kurshiah College, Seremban. She graduated with a Degree in Microbiology in 1987 and was an active member of the Golden Key Society (American Chapter). Raha then proceeded to do her MS degree at the University of Oklahoma and completed her MS thesis on 'Transposon Mutagenesis of *Haemophilus influenzae*' in 1990. She returned to Malaysia and joined Universiti Putra Malaysia (UPM) as a tutor at the Department of Biotechnology in June 1991, and 4 months later was on her way to Glasgow, Scotland to pursue her PhD at the University of Strathclyde. Under the supervision of Prof. Roy H. Burdon, she completed her PhD thesis on 'Molecular Characterization of the Heat Shock Genes in Cot Death Incidences' in 1995 and published two papers in international journals.

Raha's academic career thus began in 1996 as a lecturer in UPM. Apart from teaching, she was indeed lucky as during that time there was ample opportunity for a new lecturer like herself to apply for research grants from the University and the Government. She applied and received short term grants to work on two small projects from which one student graduated with an MS in Molecular Biology. In 1997, together with several other senior researchers in UPM, she applied, received and became the Project leader of a research grant under the Top Down (Intensified Research Priority Area) program for a project entitled 'Recombinant Bacteria for Vaccine Delivery'. She then went on three month attachment with the University of Groningen, the Netherlands. This allowed her to develop her research on molecular genetics of *Lactococcus lactis* and the recombinant bacterial delivery systems.

Over the next 14 years, the group obtained additional grants and published many papers in journals and proceedings as well

as presenting their work at various international and national conferences. Raha was the main supervisor for 6 PhD and 9 MS students, who have since graduated, and the grants received have also supported the final year projects of many BS Biotechnology students. Additionally, 4 patents have been filed and a spin-off product from the research is in the process of being commercialized. The Microbial Molecular Biology laboratory has been graced by undergraduate students doing their practical from other local Institutions of higher learning, such as Universiti Malaya (UM), Universiti Malaysia Sabah (UMS), Universiti Tun Razak (UTAR), Sedaya College, Universiti Selangor Malaysia (UNISEL) and the Malaysian Universiti of Science and Technology (MUST). Post-graduate students and research officers from Vietnam, Indonesia, Thailand and Iran had also spent up to a few months in the laboratory learning about recombinant DNA and gene expression techniques.

Apart from being the project leader and managing her own grants, Raha is also actively involved in other research projects in her capacity as a molecular geneticist/molecular biologist. Her other research interests include molecular characterisation and identification of human and food borne pathogens, engineering of metabolic pathways in Gram positive microbes, identification of genetic markers and authentication of halal products by molecular methods. Together with her colleagues, she has supervised and co-supervised more than 40 post-graduate students and published more than 150 papers in journals and proceedings. She is also an external examiner of MS and PhD theses of students from USM, UM, UKM and UTM and has refereed several Professorial applicants for UTM and UKM.

As a lecturer, she teaches courses related to her area of expertise such as Cell and Molecular Biology, Genetic Engineering,

Introduction to Microbial Biotechnology, Techniques in Molecular Biology and Industrial Microbiology at the undergraduate level, and Research Methodology at the Post-graduate level. She received the award of `Most Outstanding Lecturer in the Faculty of Food Science and Biotechnology' in the year 2003. Raha has been on the committee involved in designing and developing the curriculum for BS Biotechnology and BS Cell and Molecular Biology in UPM and a member of the panel evaluating the Biotechnology and Bioscience BS and MS Programs for UTM and UM.

In addition to lecturing and research, Raha is active in extension work. Whilst at the former Faculty of Food Science and Biotechnology, she served on several committees such as curriculum, social club and also safety, among others. Her term as advisor of the undergraduate student society in 1997 – 98 permitted her to be involved in many student activities. She represented the Faculty at several National meetings on genetically modified organisms and food pathogens at the Health and Science Ministries. She was presented with the `Anugerah Khidmat Cemerlang' by the Faculty and University in 2003 and 2004. Commencing August 2004, in the new Faculty of Biotechnology and Biomolecular Sciences, she was one of the Editors of the Biotech Communication (Faculty of Biotech's research bulletin), and a member of the research, curriculum, ISO quality assurance committee, and internal auditor for ISO.

Raha has been consultant to several Government bodies and Biotechnology-related companies; the most prominent being the Malaysian Royal Police (PDRM) and Malaysian Islamic Council (JAKIM/IKIM) on topics related to DNA fingerprinting and genetically modified organisms and food. Examples of biotechnology companies include Ecocillus and Malaysian Agri-High Tech. Raha has also been interviewed several times by the

local media on Biotechnology and Genetic Engineering issues. She served on the technical panel for assessment of grant applications for UPM, MARDI, UKM and UiTM as well as a reviewer for many national and international journals. She has also served on the National Science Fellowship panel in interviewing selected candidates pursuing their MSc and PhD and was a member of the National Technical Advisory Committee for Food Safety Procedures 1985. She is a panel member of the Malaysian Qualification Agency. In recognition of her expertise, Raha often serves as a panel member for the monitoring of research progress of recipients of e-science and Institutional initiative grants under MOSTI. She sits as a jury member for recipients of the Young Scientist award for Merck, a multinational drug company. She has also mentored the winning Tunku Kurshiah College's English debating as well as Science Project Teams.

Raha served as the Deputy Director of UPM's Research Management Center from 2005-2007, and is currently the Head of the Department of Cell and Molecular Biology at the Faculty of Biotechnology and Biomolecular Sciences. She is the Coordinator of the Culture Collection Unit and a member of the Biosafety committee at the Institute of Biosciences, UPM. Raha is also a member of UPM's Biosafety committee and is a member of the University's research exhibition selection committee. At the national level, Raha is the past President of the Malaysian Society for Microbiology and is a continuing EXCO member. She is also affiliated to several other professional societies such as the Malaysian Society for Molecular Biology and Biotechnology, where she held the post of Auditor for two terms, and the Malaysian Genetics Society. At the international stage, she is a member of the American Society for Microbiology and an EXCO member to the Asian Federation of Societies for Lactic Acid Bacteria.

Last but most importantly, she is happily married to Assoc. Prof. Dr. Abu Bakar, who is the Director of the Graduate Institute of Management, MMU and blessed with two lovely daughters, Adeela and Adreena.

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I am also indebted to UPM, JPA, MOSTI, MOHE, JICA, JSPS for all the grants and scholarships that had enabled me to pursue knowledge and carry out research work in this area. I would like to acknowledge the Lactococcal group at the University of Groningen, the Netherlands, especially Kees Leenhouts for their tremendous help when I first embarked on the Lactococcus project.

Finally, to my husband, Abu Bakar, and children Adeela and Adreena, thank you for all the support, sacrifices, massages, hugs and kisses …… I love you all.

"If I have seen further it is by standing on the shoulders of giants."
Isaac Newton

LIST OF INAUGURAL LECTURES

1. Prof. Dr. Sulaiman M. Yassin
   The Challenge to Communication Research in Extension
   22 July 1989

2. Prof. Ir. Abang Abdullah Abang Ali
   Indigenous Materials and Technology for Low Cost Housing
   30 August 1990

3. Prof. Dr. Abdul Rahman Abdul Razak
   Plant Parasitic Nematodes, Lesser Known Pests of Agricultural Crops
   30 January 1993

4. Prof. Dr. Mohamed Suleiman
   Numerical Solution of Ordinary Differential Equations: A Historical Perspective
   11 December 1993

5. Prof. Dr. Mohd. Ariff Hussein
   Changing Roles of Agricultural Economics
   5 March 1994

6. Prof. Dr. Mohd. Ismail Ahmad
   Marketing Management: Prospects and Challenges for Agriculture
   6 April 1994

7. Prof. Dr. Mohamed Mahyuddin Mohd. Dahan
   The Changing Demand for Livestock Products
   20 April 1994

8. Prof. Dr. Ruth Kiew
   Plant Taxonomy, Biodiversity and Conservation
   11 May 1994

9. Prof. Ir. Dr. Mohd. Zohadie Bardaie
   Engineering Technological Developments Propelling Agriculture into the 21st Century
   28 May 1994

10. Prof. Dr. Shamsuddin Jusop
    Rock, Mineral and Soil
    18 June 1994

11. Prof. Dr. Abdul Salam Abdullah
Natural Toxicants Affecting Animal Health and Production
29 June 1994

12. Prof. Dr. Mohd. Yusof Hussein
Pest Control: A Challenge in Applied Ecology
9 July 1994

13. Prof. Dr. Kapt. Mohd. Ibrahim Haji Mohamed
Managing Challenges in Fisheries Development through Science and Technology
23 July 1994

14. Prof. Dr. Hj. Amat Juhari Moain
Sejarah Keagungan Bahasa Melayu
6 Ogos 1994

15. Prof. Dr. Law Ah Theem
Oil Pollution in the Malaysian Seas
24 September 1994

16. Prof. Dr. Md. Nordin Hj. Lajis
Fine Chemicals from Biological Resources: The Wealth from Nature
21 January 1995

17. Prof. Dr. Sheikh Omar Abdul Rahman
Health, Disease and Death in Creatures Great and Small
25 February 1995

GFP

18. Prof. Dr. Mohamed Shariff Mohamed Din
Fish Health: An Odyssey through the Asia - Pacific Region
25 March 1995

19. Prof. Dr. Tengku Azmi Tengku Ibrahim
Chromosome Distribution and Production Performance of Water Buffaloes
6 May 1995

20. Prof. Dr. Abdul Hamid Mahmood
Bahasa Melayu sebagai Bahasa Ilmu- Cabaran dan Harapan
10 Jun 1995

21. Prof. Dr. Rahim Md. Sail
Extension Education for Industrialising Malaysia: Trends, Priorities and Emerging Issues
22 July 1995

22. Prof. Dr. Nik Muhammad Nik Abd. Majid
The Diminishing Tropical Rain Forest: Causes, Symptoms and Cure
19 August 1995

23. Prof. Dr. Ang Kok Jee
The Evolution of an Environmentally Friendly Hatchery Technology for Udang Galah, the King of Freshwater Prawns and a Glimpse into the Future of Aquaculture in the 21st Century
14 October 1995

24. Prof. Dr. Sharifuddin Haji Abdul Hamid
Management of Highly Weathered Acid Soils for Sustainable Crop Production
28 October 1995

25. Prof. Dr. Yu Swee Yean
Fish Processing and Preservation: Recent Advances and Future Directions
9 December 1995

26. Prof. Dr. Rosli Mohamad
Pesticide Usage: Concern and Options
10 February 1996

27. Prof. Dr. Mohamed Ismail Abdul Karim
Microbial Fermentation and Utilization of Agricultural Bioresources and Wastes in Malaysia
2 March 1996

28. Prof. Dr. Wan Sulaiman Wan Harun
Soil Physics: From Glass Beads to Precision Agriculture
16 March 1996

29. Prof. Dr. Abdul Aziz Abdul Rahman
Sustained Growth and Sustainable Development: Is there a Trade-Off 1 or Malaysia
13 April 1996

30. Prof. Dr. Chew Tek Ann
Sharecropping in Perfectly Competitive Markets: A Contradiction in Terms
27 April 1996

31. Prof. Dr. Mohd. Yusuf Sulaiman
Back to the Future with the Sun
18 May 1996

32. Prof. Dr. Abu Bakar Salleh
Enzyme Technology: The Basis for Biotechnological Development
8 June 1996

33. Prof. Dr. Kamel Ariffin Mohd. Atan
The Fascinating Numbers
29 June 1996

34. Prof. Dr. Ho Yin Wan
Fungi: Friends or Foes
27 July 1996

35. Prof. Dr. Tan Soon Guan
Genetic Diversity of Some Southeast Asian Animals: Of Buffaloes and Goats and Fishes Too
10 August 1996

36. Prof. Dr. Nazaruddin Mohd. Jali
Will Rural Sociology Remain Relevant in the 21st Century?
21 September 1996

37. Prof. Dr. Abdul Rani Bahaman
Leptospirosis-A Model for Epidemiology, Diagnosis and Control of Infectious Diseases
16 November 1996

38. Prof. Dr. Marziah Mahmood
Plant Biotechnology - Strategies for Commercialization
21 December 1996

39. Prof. Dr. Ishak Hj. Omar
Market Relationships in the Malaysian Fish Trade: Theory and Application
22 March 1997

40. Prof. Dr. Suhaila Mohamad
Food and Its Healing Power
12 April 1997

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17 June 1998

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15 May 1999

43. Prof. Dr. Aini Ideris
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10 July 1999

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45. Prof. Dr. Azizah Hashim
The Endomycorrhiza: A Futile Investment?
23 Oktober 1999

46. Prof. Dr. Noraini Abdul Samad
Molecular Plant Virology: The Way Forward
2 February 2000

47. Prof. Dr. Muhamad Awang
Do We Have Enough Clean Air to Breathe?
7 April 2000

48. Prof. Dr. Lee Chnoong Kheng
Green Environment, Clean Power
24 June 2000

49. Prof. Dr. Mohd. Ghazali Mohayidin
Managing Change in the Agriculture Sector: The Need for Innovative Educational Initiatives
12 January 2002

50. Prof. Dr. Fatimah Mohd. Arshad
Analisis Pemasaran Pertanian di Malaysia: Keperluan Agenda Pembaharuan
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