ISOLATION AND CHARACTERIZATION OF A CRYPTIC PLASMID FROM *LACTOBACILLUS PLANTARUM*

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Short Title

MOLECULAR CHARACTERIZATION OF A *LACTOBACILLUS PLANTARUM* PLASMID

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Abstract

Lactic acid bacteria (LAB), a group of generally recognized as safe (GRAS) organisms that metabolize sugars into primarily lactic acid, have traditionally been used for the fermentation and preservation of various foods and beverages. There is increasing interest in the genetic manipulation of LAB to improve existing characteristics or introduce novel, industrially pertinent phenotypes. However, because these bacteria have food-related applications, their genetic modification requires the use of food-grade genetic engineering tools. LAB plasmids, self-replicating extrachromosomal DNA molecules, can be used to derive food-grade cloning vectors. The rationale of this research was to develop a food-grade cloning vector using a lactobacilli cryptic plasmid and to investigate its cloning and expression properties. The main objectives were to (i) screen *Lactobacillus* spp. for plasmids, (ii) isolate and characterize a plasmid, and (iii) use the plasmid replicon to construct a cloning vector and express heterologous genes in various hosts. This is the first step in the development of a new family of food-grade cloning vectors for the genetic modification of lactobacilli.

Résumé

Les bactéries lactiques sont des organismes qui métabolisent les sucres en acide lactique. Elles sont utilisées dans la fermentation et la conservation d'une variété d'aliments et de breuvages. La modification génétique des bactéries lactiques suscite un intérêt marqué, que ce soit pour améliorer les caractéristiques de souches industrielles ou pour introduire de nouveaux phénotypes et ainsi exprimer divers métabolites hétérologues. Par contre, comme les bactéries lactiques ont des applications alimentaires, toute modification génétique se doit d'être GRAS. Les vecteurs de clonage dérivés de plasmides cryptiques de bactéries lactiques dépourvus de gènes de résistance aux antibiotiques sont reconnus comme étant acceptables pour modifier génétiquement les bactéries lactiques. Ce projet de maîtrise avait comme objectifs (i) de cribler des souches de lactobacilles pour des plasmides cryptiques, (ii) d'isoler et de caractériser un plasmide, et (iii) d'utiliser son réplicon pour dériver un vecteur de clonage et exprimer des gènes hétérologues dans diverses souches de lactobacilles. Ceci constitue la première étape du développement d'une nouvelle famille de vecteurs de clonage GRAS qui serviront à modifier des souches de lactobacilles.

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General Introduction

Lactic acid bacteria (LAB) constitute a group of Gram-positive bacteria, including *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Bifidobacterium* species that share the ability to ferment sugars into primarily lactic acid. LAB have a long history of use in the traditional production of fermented foods, beverages and animal feed and are therefore generally recognized as safe (GRAS) (Davidson et al., 1996; Stiles & Holzapfel, 1997; Axelsson, 1998). In addition to fermenting characteristics, there is now mounting evidence that some LAB strains have probiotic properties and can provide health benefits to their human hosts (Reid et al., 2003).

Moreover, not only are LAB being studied for their potential uses in clinical practice, but also for their properties as host systems for the overexpression of various proteins and metabolites (de Vos, 1999a). Much research is focusing on the development of expression systems in LAB, because unlike *E. coli* host systems, LAB are GRAS and have the ability to secrete metabolites directly into the fermentation medium, thereby decreasing production and purification costs.

There is increasing interest in the genetic manipulation of LAB to improve existing characteristics or introduce novel, industrially pertinent phenotypes. However, because LAB have food-related applications, their genetic modification requires the use of food-grade genetic engineering tools that are composed solely of DNA from GRAS organisms and that do not rely on antibiotic resistance genes for selection. LAB engineered with such tools are deemed safe for human and/or animal consumption. Various methods are available to engineer food-grade recombinant LAB, one being to use cloning vectors derived from LAB plasmids that do not contain any antibiotic resistance genes as selective markers.

Many cryptic plasmids from lactic acid bacteria have been isolated, characterized and used to construct cloning vectors (reviewed by Shareck et al., 2004). Nonetheless, many aspects of recombinant DNA technology in LAB have yet to be studied thoroughly. The rationale for the development of cloning vectors derived from LAB plasmids is the

need for new food-grade genetic engineering tools that are tailored for specific applications, including enhancing the performance of industrial strains of LAB, developing expression systems in LAB for the effective production of various ingredients and creating novel probiotic foods that could permit the *in vivo* production of health-benefiting compounds.

Generally, vector construction starts with the molecular characterization of plasmids, which permits to gain insight on the genes and potential functions encoded by a plasmid, and to target the regions that will be used to derive cloning vectors. The objectives of this research were to (i) collate and review data concerning cryptic plasmids isolated from lactic acid bacteria, (ii) screen strains of *Lactobacillus* spp. for plasmids, (iii) isolate a suitable plasmid and characterize it, and (iv) use the plasmid replicon to derive a cloning vector and express heterologous genes in various hosts.

In this research, a small cryptic plasmid was isolated and characterized, and used to derive a cloning vector that uses the green fluorescent protein (*gfp*) gene as a reporter gene and erythromycin resistance (Em^r) as a selective marker. The first step in vector construction, i.e. the molecular characterization of a plasmid, has been completed and should permit the further development of a new family of food-grade cloning vectors for the genetic modification of lactobacilli.

Chapter 1. Literature review

Cryptic plasmids in lactic acid bacteria: their characteristics and potential applications in food-grade recombinant DNA technology

This chapter is an excerpt of a publication entitled *"Cloning vectors based on cryptic plasmids isolated from lactic acid bacteria: their characteristics and potential applications in biotechnology"*, a review written by Shareck, J., and supervised by Choi, Y., Lee, B. and Miguez, C. B. It appeared in Critical Reviews in Biotechnology, 24(4): 155-208 (2004). This review serves as an introduction to lactic acid bacteria plasmids and their characteristics. Cloning vectors derived from *Lactobacillus* cryptic plasmids are collated and discussed. Food-grade recombinant DNA technology is reviewed, with regards to the development of food-grade cloning vectors and inducible expression systems.

1.1 Abstract

Lactic acid bacteria (LAB) are Gram-positive bacteria, widely distributed in nature, and industrially important as they are used in a variety of industrial food fermentations. The use of genetic engineering techniques is an effective means of enhancing the industrial applicability of LAB. However, when using genetic engineering technology, safety becomes an essential factor for the application of improved LAB to the food industry. Cloning and expression systems should be derived preferably from LAB cryptic plasmids which generally encode genes for which functions can be proposed, but

no phenotypes can be observed. This review presents information concerning LAB cryptic plasmids, their structures, functions and applications. A total of 139 cryptic plasmids collated are discussed.

1.2 Introduction

Lactic acid bacteria (LAB), a group of generally recognized as safe (GRAS) organisms that metabolize sugars into primarily lactic acid, are well known for their health-benefiting properties (Salminen et al., 1998). Several species of LAB have a long history of use in the traditional production of fermented foods, beverages and animal feed and are therefore generally recognized as safe (GRAS) (Davidson et al., 1996; Stiles & Holzapfel, 1997; Axelsson, 1998). LAB have also been exploited as host systems for the production of heterologous proteins, a promising avenue for the pharmaceutical, medical and food technology fields (de Vos, 1999a). There is increasing interest in the genetic manipulation of LAB to improve existing characteristics or introduce novel, industrially pertinent phenotypes.

While genetic engineering of LAB could have a great positive impact on the pharmaceutical and food industries, its progress could be impeded by legal issues related to the controversy surrounding this technology. The safe use of genetically modified LAB requires the development of food-grade cloning systems composed solely of DNA from the homologous host or GRAS organisms and that do not rely on antibiotic markers.

The rationale for the development of cloning vectors derived from LAB cryptic plasmids is the need for new food-grade genetic engineering tools. Cryptic plasmids are extrachromosomal DNA elements that encode no recognizable phenotype besides their replication functions (von Wright & Sibakov, 1998). Their replicons can be used to construct cloning vectors.

In this review, cryptic plasmids isolated from various LAB including *Bifidobacterium* will be compiled. Cloning vectors based on lactobacilli plasmids will be investigated and compared on the basis of size, mode of replication, selection markers, segregational and structural stability, and host-range.

1.3 Lactic acid bacteria

Originally, the term lactic acid bacterium, dating back to the late nineteenth century, was a synonym of "milk-souring organisms". The first pure culture of LAB obtained in 1873 by J. Lister was *Bacterium lactis* (i.e. *Lactococcus lactis*) (Axelsson, 1998). LAB are Gram-positive, catalase negative, nonsporing, fastidious and acid tolerant cocci or rods. They ferment carbohydrates and yield mainly lactic acid as an end product. While this general rule applies to homofermentative LAB, heterofermentative LAB produce lactic acid and other compounds such as acetic acid, CO_2 and ethanol. Some LAB are also responsible for producing flavour compounds characteristic of fermented products, e.g. diacetyl in cultured butter. Moreover, organic acids and additional metabolites produced during fermentation play important roles in the preservation of foods; lower pH and bacteriocins inhibit growth of spoilage and pathogenic organisms, extending the shelf-life of fermented foods.

LAB have a long history of use in the fermentation of traditional foods, namely dairy products (yogurt, butter, cheese, kefir, kumiss), meat (salami, sausages), vegetables (sauerkraut, pickles, olives), wine and silage (Geis, 2003). There are references to the leavening of (sourdough) bread in the Bible (e.g. Matthew, 13, 33). Fermented dairy products (cheese, yogurt, butter) are mentioned in ancient texts from Uruk/Warka (Irak), dated around 3,200 B.C. (Nissen et al., 1991).

LAB strains used in food fermentations are generally associated with habitats rich in nutrients such as various food products. Other LAB are members of the normal flora of the mouth, intestine and vagina of mammals, namely bifidobacteria. The genus *Bifidobacterium* shares some of the phenotypic traits of genuine LAB, but bifidobacteria are phylogenetically unrelated, belonging to the *Actinomyces* branch of bacteria (Schleifer & Ludwig, 1995) and have a unique mode of sugar fermentation. Traditional LAB are further differentiated from *Bifidobacterium* species based on mol% G + C content in the DNA. All LAB have less than 55 mol% G + C content in their DNA (low G + C content), while bifidobacteria have more than 55 mol% G + C (high G + C content).

Bifidobacteria are gaining much deserved attention as growing scientific evidence shows that some strains of *Lactobacillus* and *Bifidobacterium* have probiotic properties,

i.e. have the capacity to modulate and maintain a healthy intestinal microflora by competing with potentially harmful bacteria and by stimulating the growth of preferred microorganisms (Salminen et al., 1998). As the market for probiotic products expands, *Bifidobacterium* species are being used for the production of fermented milks, yogurts and cheeses (Sanders & Huis in't Veld, 1999). Interest in improving characteristics of *Bifidobacterium* strains by genetic manipulation is driving the development of cloning vectors, which is why bifidobacteria are to be included in this review with the more traditional LAB.

1.4 Cryptic plasmids in lactic acid bacteria

Present in prokaryotes and in some lower eukaryotes, plasmids are extrachromosomal DNA elements, usually double-stranded molecules which are autonomous and self-replicating. Plasmids are extremely diverse in terms of size (1.5 kb to more than 600 kb), copy number (1 to several hundreds per cell) and phenotypes conferred to their hosts (Osborn et al., 2000). Plasmids are inherited independently from the bacterial chromosome, but some do rely on proteins encoded by the host for their replication and transcription. While plasmids are not normally essential for the growth of bacteria, specific phenotypes such as (i) hydrolysis of proteins, (ii) metabolism of carbohydrates, amino acids and citrate, (iii) production of bacteriocins, exopolysaccharides, pigments and (iv) resistance to antibiotics, heavy metals and phages have been found to be plasmid-encoded (Wang & Lee, 1997). Nevertheless, many of the plasmids that occur in LAB are cryptic, a term used to describe plasmids that do not have any apparent function.

Usually small and abundant, cryptic plasmids have no known effect on the host's phenotype, range in size from 1 to >100 kb and have been reported in many groups of LAB (von Wright & Sibakov, 1998). The minimum requirement for a plasmid is its ability to replicate and hence, the replicon is considered the most important feature of a cryptic plasmid. The replicon is the region on a plasmid that encodes *rep* genes, essential for plasmid replication. Rarely exceeding 3 kb, the replicon consists of (i) an origin of replication (*ori*); (ii) *cop/inc* gene(s) involved in the control of the initiation of replication

and (iii) *rep* gene(s) encoding Rep proteins required for replication (Espinosa et al., 2000). A replicon mainly allows plasmid replication and its maintenance in a host cell. The degree of plasmid autonomy, reflected in plasmid promiscuity, depends on the functionality of a replicon in different hosts. Narrow host-range plasmids only replicate in a few closely related hosts, while broad host-range plasmids have a replicon that is functional in a wide range of hosts.

1.4.1 Plasmid replication mechanisms

The mode of replication of plasmids has an important impact on some characteristics of plasmid-derived vectors, namely host-range, stability and copy number. In LAB, the most common replication mechanisms are the sigma and theta modes of replication.

1.4.1.1 Sigma-replicating plasmids (rolling-circle mechanism)

The sigma mode of replication, also known as rolling-circle replication (RCR), seems to be restricted to relatively small cryptic plasmids (Khan, 1997). RCR plasmids constitute a group of small, multicopy replicons that are widely spread among bacteria. Genetic elements that are involved in RC replication are the *rep* gene which encodes the replication initiation protein (Rep) controlled by a repressor and its target site, the plus origin of replication or double-stranded origin (*dso*). Additionally, most RCR plasmids have a minus origin of replication or single strand origin (*sso*), a specific sequence that enables the conversion of single-stranded DNA (ssDNA) intermediates into double-stranded DNA (dsDNA) molecules (Gruss & Ehrlich, 1989).

The Rep protein is a site-specific nuclease, which produces a single-stranded nick at the plus origin (*dso*), initiating positive strand replication and terminating it when a leading strand (ssDNA) is synthesized (Gruss & Ehrlich, 1989). The leading-strand replication generates: (i) a dsDNA molecule constituted by the parental [-] strand and the newly synthesized [+] strand and (ii) a ssDNA intermediate which corresponds to the

parental [+] strand. Generation of ssDNA is the trademark characteristic of RC replication. Finally, lagging-strand synthesis occurs and ssDNA intermediates are converted to dsDNA at the minus origin (*sso*). The last step involves supercoiling of the replicated DNAs by the host DNA gyrase (Espinosa et al., 2000).

Cloning vectors based on RC replicons usually have low segregational stability due to accumulation of ssDNA intermediates (Posno et al., 1991a; Vujcic & Topisirovic, 1993) and insertion of foreign DNA may further reduce their stability (Gruss & Ehrlich, 1989). Accumulation of ssDNA is generally due to the absence of a *sso*, consequently hindering the conversion of ssDNA to dsDNA molecules (del Solar et al., 1998).

Based on similarities in the structure of Rep proteins and *dso*, RC replicons from Gram-positive bacteria have been grouped into five families: pMV158/pE194, pC194, pT181, pSN2 and pTX14-3 (Khan, 1997). Lactococcal RC replicons belong to the pMV158/pE194 family, except for pWC1, which was included in the pC194 family (Pillidge et al., 1996). *Lactobacillus* RCR plasmids are divided between the pE194 and pC194 families while *S. thermophilus* replicons mostly belong to the pC194 group. *Leuconostoc* RCR plasmids seem to be associated with several different groups: pCI411 belongs to the pE194 family (Coffey et al., 1994); pLo13 is included in the pC194 group (Fremaux et al., 1993a) while pRS1, pOg32 and pFR18 are grouped with the pT181 family (Alegre et al., 1999; Brito et al., 1996; Biet et al., 1999). RC replicons have yet to be detected in *Pediococcus*. As for *Bifidobacterium* plasmids, most follow the RC mechanism of replication, except pMB1 (Matteuzzi et al., 1990).

1.4.1.2 Theta-replicating plasmids

Although it was assumed that rolling-circle plasmids were the most widespread in Gram-positive bacteria, a large number of theta-replicating plasmids recently have been isolated and characterized, many originating from LAB. Theta-replicating plasmids tend to be medium and large size plasmids that encode important metabolic functions, enzymes such as lactase (pSK11L, Horng et al., 1991), lactase-protease (pUCL22, Frere et al., 1993) and citrate permease (pSL2, Jahns et al., 1991), bacteriophage resistance (pCI528, Lucey et al., 1993), exopolysaccharide production (pNZ4000, van Kranenburg

& de Vos, 1998) and pediocin production (pSMB74, Motlagh et al., 1994; pMD136, Kantor et al., 1997).

Some cryptic plasmids have also been shown to follow theta replication: pCI305 (Hayes et al., 1991), pVS40 (von Wright & Raty, 1993), and pWV02 (Kiewiet et al., 1993). Moreover, theta replication is no longer exclusively reserved to large plasmids, as some very small plasmids replicate via the theta mode, namely p4028 (4.4 kb, Zuniga et al., 1996), pTXL1 (2.7 kb, Biet et al., 2002) and pMB1 (1.9 kb, Matteuzzi et al., 1990; Corneau et al., 2004).

The main difference between sigma and theta replication is that theta-replicating plasmids do not produce ssDNA intermediates. This results in greater structural and segregational stability, making theta-replicating plasmids better candidates for vector construction as they can stably maintain large heterologous DNA inserts (up to 16.8 kb) (Kiewiet et al., 1993; de Vos & Simons, 1994).

Theta replication has three key components (i) an initiator protein (Rep) necessary for strand opening, (ii) an origin of replication (*ori*) with specific DNA structural organization for strand opening and initiator-protein binding and (iii) a host-encoded polymerase I for nascent strand DNA synthesis (del Solar et al., 1998; Alpert et al., 2003). In the theta mode, sites for priming of leading- and lagging-strand synthesis are located close to one another within the replication origin. During replication, both DNA strands remain covalently closed, except during the resolution of daughter molecules (de Vos & Simons, 1994).

1.4.2 Lactococcus cryptic plasmids

Members of the *Lactococcus* (*L.*) genus are mesophilic LAB used extensively in milk fermentation to produce a number of different products. The genus includes *L. garviae*, *L. plantarum*, *L. piscium* and *L. raffinolactis*, but *L. lactis* remains the best characterized *Lactococcus* species with regards to physiology and molecular genetics. Plasmids in *L. lactis* are a common component: they vary in number from 2 to 11 per cell, but most strains usually possess 4 to 7 plasmids, ranging in size from 3 kb to more than 130 kb (Davidson et al., 1996). *L. lactis* subsp. *cremoris* strain Wg2 harbours 5 plasmids:

pWV01 (2.2 kb), pWV02 (3.8 kb), pWV03 (7 kb), pWV04 (19 kb) and pWV05 (27kb) (Kok et al., 1984; Kiewiet et al., 1993; Seegers et al., 1994) which all follow theta replication, except for RCR plasmid pWV01. This supports the theory that many theta-replicating plasmids can be maintained in the same cell, but only one RCR plasmid can stably co-exist (Seegers et al., 1994).

Lactococcal plasmids are of great importance as some industrially relevant characteristics are plasmid-encoded, such as sugar metabolism (Gasson, 1990), proteolysis (Kok, 1990), phage resistance, conjugal transfer (de Vos & Davies, 1984; McKay & Baldwin, 1990) and bacteriocin production (Klaenhammer, 1993). *L. lactis* also harbours cryptic plasmids that do not encode any known metabolic functions. These plasmids have replicons that are homologous to RCR replicon pWV01 or to theta-type replicons pWV02/pCI305.

Most RCR lactococcal cryptic plasmids, namely pWV01 (Kok et al., 1984; Leenhouts et al., 1991), pSH71 (de Vos et al., 1987), pD125 (Xu et al., 1990), pCL2.1 (Chang et al., 1995) and pBM02 (Sanchez & Mayo, 2003) belong to the pE194 family of RCR plasmids, the exception being pWC1 (Pillidge et al., 1996), which is a pC194-type of replicon (Table 1.1). Recently, pSRQ700, a theta-replicating plasmid encoding an anti-phage resistance mechanism was found to contain a second replication module, a RC replicon (Boucher et al., 2001).

The advantage of RC lactococcal replicons is that most are functional in Gram-positive bacteria and in *E. coli*. Wide host-range vectors have been derived from these replicons (i.e. pGK-, pNZ-, pFX-series) and proven to be very useful to transform LAB and other hosts (de Vos & Simons, 1994). However, these vectors tend to suffer from segregational instability due to accumulation of ssDNA intermediates.

To solve vector stability problems, the focus has been on developing vectors based on theta-replicating plasmids. Although such vectors have a narrow host-range, they have the advantages of (i) being compatible with endogenous RCR plasmids (Hayes et al., 1991), (ii) having greater segregational stability (Kiewiet et al., 1993) and (iii) having the capacity to carry large fragments of heterologous DNA. Theta-replicating plasmids are widespread, the lactococcal prototype being pCI305 (Hayes et al., 1991). It was suggested that pCI305 followed theta replication as no homology was found between

repA, *repB* and replication regions of well-known RCR plasmids. In addition, ssDNA intermediates could not be detected.

Theta-replicating cryptic lactococcal plasmids are members of a family of highly related, compatible replicons, as first identified for plasmid pCI305. The group is comprised of several plasmids, including pWV02 (Kiewiet et al., 1993), a 28 kb plasmid used to construct nisin-resistant pVS40 (von Wright et al., 1990; von Wright & Raty, 1993), pWV04, pWV05, pIL7 (Seegers et al., 1994), pCIS3 (Seegers et al., 2000), pJW563 (Gravesen et al., 1995), pND324 (Duan et al., 1999) and *L. lactis* strain FG2 plasmid replicon (Liu et al., 1997a).

Several theta-replicating cryptic plasmids found in *Lactococcus* are interesting for the development of vectors, as they naturally encode selectable markers: pVS40 encodes nisin resistance, pND302, cadmium resistance (Liu et al., 1996; 1997b) and pND324 has a thermosensitive replication region (Table 1.2).

1.4.3 Lactobacillus cryptic plasmids

The genus *Lactobacillus* (*Lb.*) is comprised of many relatively diverse species, which include *Lb. plantarum*, *Lb. pentosus*, *Lb. fermentum*, *Lb. reuteri*, *Lb. acidophilus*, *Lb. casei*, *Lb. helveticus*, *Lb. hilgardii*, *Lb. curvatus*, *Lb. delbrueckii bulgaricus*, *Lb. delbrueckii lactis* and *Lb. sakei*. Lactobacilli are widespread in nature and many have been used in food fermentation processes, including milk, meat and plant material (Stiles & Holzapfel, 1997). In addition, some species of *Lactobacillus* are used as active agents in probiotic preparations (Molin, 2001).

First discovered by Chassy et al. (1976), plasmids are found in most, but not all *Lactobacillus* species (reviewed by Pouwels & Leer, 1993; Klaenhammer, 1995; Wang & Lee, 1997). *Lb. delbrueckii bulgaricus* is known to harbour very few plasmids: it was recently found that out of 48 strains, only one contained a plasmid, cryptic pLBB1 (Azcarate-Peril & Raya, 2002). It is generally thought that most *Lactobacillus* strains harbour one or more plasmids, the size of which can vary from 1.2 kb to 150 kb.

To this date, many lactobacilli plasmids have been found, but most remain cryptic (Wang & Lee, 1997). However, some functions have been found to be plasmid-encoded

which relate to lactose metabolism, antibiotic resistance, bacteriocin production and immunity, DNA restriction or modification (R-M), exopolysaccharide production, N-acetyl glucosamine fermentation and certain amino acid (cysteine) transport (reviewed by Pouwels & Leer, 1993; Arihara & Luchansky, 1994).

Cryptic plasmids in lactobacilli are numerous and quite diversified with regards to their size, mode of replication and family of replicons. The larger sized plasmids tend to follow a theta mode of replication, while the smaller plasmids replicate via RC. Although this is generally true, two theta-replicating plasmids appear to be very small, 3.3 kb pLJ1 from *Lb. helveticus* (Takiguchi et al., 1989) and 4.4 kb pKC5b from *Lb. fermentum* (Pavlova et al., 2002), while 9.3 kb pLP9000 from *Lb. plantarum* (Daming et al., 2003) and 9.8 kb pGT633 from *Lb. reuteri* (Tannock et al., 1994) are RCR plasmids, despite their fairly large sizes (Table 1.3).

Based on Rep protein structure and *dso* homologies, completely or partially sequenced RCR plasmids either belong to the pE194 or to the pC194 general classes of RC replicons. pA1, pLB4, a 7 kb plasmid (*Lb. plantarum*), pLF1311 (*Lb. fermentum*), pLC2 (*Lb. curvatus*) and pLA106 (*Lb. acidophilus*) have so far been determined as members of the pE194 family of replicons. RCR plasmids of the pC194 type are pC30il, pLP1, p8014-2, pLP2000 (*Lb. plantarum*), p353-2 (*Lb. pentosus*), pLEM3 (*Lb. fermentum*), pLAB1000 (*Lb. hilgardii*), pGT232 and pTC82 (*Lb. reuteri*). *Lb. plantarum* plasmid pLP9000 (Daming et al., 2003) also replicates via RC, but has not been assigned to any replicon family, as no homologies could be found with any known RC replicons. Some RC plasmids are also in the same situation (Fortina et al., 1993; Tannock et al., 1994; Pridmore et al., 1994).

Much interest is focused on lactobacilli theta-replicating plasmids, the intention being to construct narrow host-range cloning vectors that have increased stability. Several theta-replicating plasmids have been identified as such because they do not exhibit typical RCR traits (Table 1.3): pKC5b (*Lb. fermentum*), pLJ1 and pLH1 (*Lb. helveticus*), pRV500, pLKS (*Lb. plantarum*) and pSAK1 (*Lb. sakei*). Other plasmids such as pLA103 and pLA105 (*Lb. acidophilus*) and pLBB1 (*Lb. delbrueckii bulgaricus*) are believed to replicate by theta mechanism, but confirmation is still pending.

More recently, three new *Lb. plantarum* plasmids have been isolated and characterized: p256, and pWCFS103 (Sorvig et al., 2005; van Kranenburg et al., 2005) were shown to replicate by theta mechanism, while pPB1, pWCFS101 and pWCFS102 (de las Rivas et al., 2004; van Kranenburg et al., 2005) were deemed RCR plasmids. Interestingly, pPB1 was shown to be mobilizable, as it carried *mob* genes and *oriT*, elements that are typical of mobilizable plasmids, while pWCFS103 was shown to be conjugative. To date, this is the first conjugative plasmid to be reported in *Lb. plantarum*.

1.4.4 Streptococcus thermophilus cryptic plasmids

Streptococcus (S.) thermophilus is the only streptococcal species used in food fermentations: its ability to grow at high temperatures (52°C) combined with its limited spectra of fermentable sugars distinguish it from other streptococci. Phylogenetically, it is the most closely related species to *L. lactis*. *S. thermophilus* is mainly used as a starter culture to manufacture yogurt and some cheeses such as mozzarella and Swiss, its main function being the production of lactic acid.

Like *Lb. delbrueckii bulgaricus*, *S. thermophilus* strains carry very few plasmids (Mercenier et al., 1990). While it has been reported that less than 20% of *S. thermophilus* strains carry one or two small plasmids (Herman & McKay, 1985; Somkuti & Steinberg, 1986; Girard et al., 1987; Janzen et al., 1992; Geis et al., 2003), one group recently found that 60% of the *S. thermophilus* strains screened harboured plasmids (Turgeon & Moineau, 2001).

Several small *S. thermophilus* plasmids have been sequenced and analyzed, and most have remained cryptic, as no apparent phenotypic traits seem to be associated to their presence (Table 1.4). Several plasmids have been found to encode small heat shock proteins (*hsp*): pER341 (Somkuti et al., 1998), pCI65st (O'Sullivan et al., 1999), pND103 (Su et al., 2002), pST04 and pER1-1 (Geis et al., 2003), pt38 (Petrova et al., 2003) in addition to pER7, pER16, pER26, pER35, pER36 and pER41 (Somkuti & Steinberg, 1999; Solow & Somkuti, 2000). It was initially thought that the presence of *hsp* influenced plasmid maintenance during fermentation at elevated temperatures (O'Sullivan et al., 1999). According to Geis et al. (2003), expression of these proteins is induced by

elevated temperatures and low pH, increasing thermo- and acid resistance of the strains that carry *hsp*. Moreover, the promoter of *hsp16.4* of pER341 is under investigation for potential use in temperature-controlled expression of heterologous genes in LAB (Somkuti et al., 1998). Genes for restriction-modification (R/M) systems have also been identified on plasmids, as reported for pCI65st (O'Sullivan et al., 1999), pER35 (Solow & Somkuti, 2001), pSt08 and pSt0 (Geis et al., 2003).

S. thermophilus plasmids can be grouped according to their sizes (Janzen et al., 1992): group I consists of small plasmids ranging in size from 2.1 to 3.5 kb; group II, 4.2 kb; group III, 5.2 kb; group IV, 6.8 kb; and group V, 7.4 kb. Based on mechanism of replication, *S. thermophilus* plasmids isolated replicate via RC, and belong to the pC194 family of replicons (Somkuti & Steinberg, 1986; Janzen et al., 1992; Hashiba et al., 1993; Solaiman & Somkuti, 1998; Somkuti et al., 1998; O'Sullivan et al., 1999; Su et al., 2002; Petrova et al., 2003; Geis et al., 2003), except pSMQ172 (Turgeon & Moineau, 2001), which has been assigned to the pMV158/pE194 family of replicons. Although the presence of ssDNA intermediates has never been demonstrated in any of the plasmids besides in pSMQ172 and pt38, it is still assumed that they replicate via RC, as suggested by homologies to known RCR plasmids.

1.4.5 Leuconostoc and Oenococcus oeni cryptic plasmids

Strains of *Leuconostoc* (*Ln.*) can be found in many natural and man-made habitats, such as grass, herbage and silage. The genus *Leuconostoc* is comprised of heterofermentative organisms that play an important role in the fermentation of vegetables (cabbage, cucumbers) by initiating spontaneous lactic acid fermentations (Davidson et al., 1996). The ability of some species to ferment citric acid in milk to the flavour compound diacetyl, the "butter flavour" in dairy products, has led to their use as industrial dairy starters. *Leuconostoc* species contribute to both flavour development and preservation of food (Johansen & Kibenich, 1992). Although *Ln. oenos*, primarily used in malolactic fermentations, has been reclassified as *Oenococcus oeni* (Dicks et al., 1995), it is more convenient to include it in this section. *O. oeni* is an acid and alcohol tolerant LAB that is found naturally in fruit mashes and related habitats. Used in winemaking, it

converts malate to lactate and reduces wine acidity, thereby improving the organoleptic properties and the stability of the final product (Davis et al., 1985).

Few studies report the presence of plasmids in *Leuconostoc* spp. (Table 1.5). Janse et al. (1987) reported that after screening 42 strains of *Leuconostoc*, only 11 plasmids could be recovered from 8 strains of *Ln. oenos*. These plasmids were small, between 2.47 kb and 4.61 kb and had a low copy number, factors supporting the statement that in this genus, little genetic information is plasmid-encoded. However, some *Leuconostoc* plasmids have been shown to encode metabolic functions such as lactose utilization and citrate permease activity (David et al., 1992) as well as bacteriocin production and immunity (Hastings et al., 1991).

Nonetheless, most *Leuconostoc* plasmids characterized to date remain cryptic (Coffey et al., 1994), replicate via RCR and belong to various replicon families, including pT181, pE194 and pC194. Among LAB RCR plasmids, the only plasmids assigned to the pT181 class of RC replicons have been found in *Leuconostoc* species (pOg32, pRS1 and pFR18).

Two plasmids, p4028 from *Ln. oenos* and pTXL1 from *Ln. mesenteroides*, reportedly follow theta replication, a surprising discovery considering the size of the plasmids, 4.4 kb and 2.7 kb, respectively (Zuniga et al., 1996; Biet et al., 2002).

1.4.6 Pediococcus and Tetragenococcus halophila cryptic plasmids

Pediococci are a group of homofermentative LAB that are ecologically, morphologically and physiologically similar to the lactococci (Gonzalez & Kunka, 1983). Comparison of 16S rRNA sequences confirmed the heterogeneous phylogeny of the *Pediococcus* (*P.*) genus and *P. halophilus* was reclassified as *Tetragenococcus (T.) halophilus* (Benachour et al., 1997). Pediococci are naturally found on plant material, in fermented vegetables and in beer. They form a group of economically important microorganisms as they are used as starter cultures for sausage making, fermentation of vegetables and soy milk, and for silage inoculation (Davidson et al., 1996). Moreover, *T. halophilus* is salt tolerant and is used in the brewing of soy sauce (Uchida, 1982) and in the curing of salted anchovies (Villar et al., 1985).

Pediococci, mainly *P. pentosaceus* and *P. acidilactici*, harbour many different plasmids that encode a variety of traits, namely utilization of raffinose, melibiose and sucrose (Gonzalez & Kunká, 1986) as well as bacteriocin production (Klaenhammer, 1993). Pediocin, an anti-listerial bacteriocin, is produced by several pediococcal strains (Marugg et al., 1992; Motlagh et al., 1994). A semi-pure form of pediocin is produced commercially (Alta^{TM} 2341) and is used as a preservative in ready-to-eat meats (Rodriguez et al., 2002), but pediocin has yet to be authorized as a fully licensed food additive.

Studies have shown that genes for pediocin production are plasmid-encoded: pSRQ11 (9.4 kb) and pSMB74 (8.9 kb) from *P. acidilactici* and pMD136 (19.5 kb) from *P. pentosaceus* are involved in pediocin production (Gonzalez & Kunka, 1983; Motlagh et al., 1994; Kantor et al., 1997) (Table 1.6). Since pediocin can be used as a selection marker in cloning vectors, plasmids that carry the genes for its production are interesting with regards to the development of food-grade cloning vectors.

Unlike other pediococcal species, *T. halophilus* harbours plasmids that are generally thought to be cryptic (Kayahara et al., 1989). pUCL287 from *T. halophilus*, the first fully sequenced pediococcal plasmid, was shown to be a theta-replicating plasmid and *repA287* encoded a protein involved in plasmid replication (Benachour et al., 1995). No homology could be detected between RepA287 and replication proteins of well-characterized theta-type replicons, such as lactococcal Rep22 (Frere et al., 1993; Seegers et al., 1994) and the enterococcal pAM_1-pIP501 group (Le Châtelier et al., 1993). It was concluded that pUCL287 represented a new theta-type replicon family and RepA287 belonged to a new family of replication proteins. Upon closer analysis, two other pediococcal plasmids, pSMB74 (Benachour et al., 1997) and pMD136 (Giacomini et al., 2000), were found to belong to the pUCL287 family of theta replicons.

1.4.7 Bifidobacterium cryptic plasmids

Bifidobacteria share phenotypic and morphological attributes with many lactobacilli, which led to the belief that they belonged to the genus *Lactobacillus*. Classification by molecular methodologies has provided evidence that this is indeed a

distinct genus that is more closely related to the *Actinomycetacea* family (Stiles & Holzapfel, 1997).

Bifidobacteria are natural inhabitants of the gastrointestinal tract, where they play an important role in the health of the host. They compete with intestinal putrefactive bacteria such as enterobacteria (Ibrahim & Bezkorovainy, 1993) and clostridia (Bezirtzoglou & Romond, 1990). They lower the gut pH by releasing acetate and lactate from carbohydrate metabolism and also produce bacteriocins, factors that combine together to inhibit pathogenic bacteria and prevent intestinal infections. Some epidemiological and clinical studies have also provided evidence that fermented milks containing certain *Bifidobacterium* (*B.*) species have anticarcinogenic and immunostimulating properties (Van't Veer et al., 1989; Reddy & Rivenson, 1993; Lee et al., 1993). Besides occurring naturally in the GI tract, *B. animalis* and *B. lactis* have been found in a variety of commercial dairy products. *B. longum*, *B. bifidum* and *B. infantis* are now being added to foods and therapeutic preparations, i.e. probiotic products, for their health benefits (Stiles & Holzapfel, 1997).

First reported in 1982 by Sgorbati et al., plasmids in the genus *Bifidobacterium* were thought to be present only in a few species. Approximately 20% of bacterial isolates that were screened contained detectable plasmids, and only several species were represented, namely *B. longum*, the predominant species in the human intestine, *B. globosum*, a species common to animals and *B. indicum* and *B. asteroides*, exclusively present in the Western and Asiatic honeybees (Sgorbati et al., 1982). Iwata and Morishita (1989) later demonstrated that *B. breve* also harboured plasmids, as many as four. More recently, a plasmid was discovered in a strain of *B. pseudocatenulatum* (Smeianov et al., 2002). Such discoveries refute the initial belief that plasmids could only be found in a few bifidobacterial species.

To this date, several *Bifidobacterium* plasmids have been fully sequenced (Table 1.7): ten from *B. longum*, and one from each species, including *B. breve*, *B. asteroides* and *B. pseudocatenulatum*. Another *B. breve* plasmid (pNBb1) was partially sequenced (Bourget et al., 1993). Based on the absence of ssDNA intermediates, it was determined that pNBb1 did not replicate via RC (Bourget et al., 1993). However, recent findings have

shown that pNBb1 contains the conserved Pfam domain Rep (GenBank Accession no. PF01446) and could thus replicate via RC (Corneau et al., 2004).

Plasmids pKJ36, pKJ50, pMG1 (Park et al., 1999; 2000; 2003) and pNAC1 (Corneau et al. 2004) have been shown to follow RCR because ssDNA intermediates have been found. Curiously, RepA of pKJ50 and RepB of pKJ36 showed significant amino acid homology with various replication proteins from theta-replicating plasmids (respectively, 43% and 37% with *Lb. acidophilus* pLA103 and 54% and 43% with *T. halophilus* pUCL287). Moreover, the three *B. longum* plasmids (pKJ36, pKJ50 and pMG1) contained iteron-like sequences, much like *B. longum* pNAC1, pNAC2 and pNAC3 (Corneau et al., 2004). Although quite common, iterons are not restricted to theta-replicating plasmids, as these repeating sequences have been reported in plasmids that use the strand-displacement mechanism or the RC mechanism (del Solar et al., 1998).

Bifidobacterial plasmids have been divided into five groups based on Rep amino acid sequence homology (Corneau et al., 2004). Most plasmids were grouped with RCR plasmids, except pMB1 (Matteuzzi et al., 1990), which showed similarity to ColE2, a theta-replicating plasmid from *E. coli*. *B. longum* pNAC3 and *B. asteroides* pAP1 seemed to be more closely related to a replicase coded by pDOJH10L and the three plasmids formed a group of plasmids of unknown replication type (Corneau et al., 2004). *Bifidobacterium* is the only genus where all the isolated and characterized plasmids have been deemed cryptic. To this date, no phenotypic traits have been found to be plasmid-encoded.

1.5 Recombinant DNA technology in lactic acid bacteria

Lactic acid bacteria are mainly used in industrial fermentations resulting in foods destined to human or animal consumption. Recombinant LAB can only be applied to foods if they are modified using food-grade genetic engineering tools. This section discusses the general characteristics of cloning vectors, with special focus given to

vectors derived from lactobacilli plasmids. New developments in food-grade cloning and controlled expression systems are also reviewed.

1.5.1 General properties of cloning vectors

An essential prerequisite for effective genetic manipulation of organisms is the availability of suitable vectors that ensure replication and maintenance of both vector DNA and inserted foreign DNA. Useful cloning vectors must have (i) an origin of replication, *ori*, (ii) selectable markers to readily identify transformed cells, (iii) one or more unique restriction endonuclease sites, (iv) a low molecular weight, smaller vectors being more stable and having a higher copy number, and (v) high segregational and structural stability.

A vector used in industrial fermentations has to remain structurally intact and be maintained in the host cell in the absence of selective pressure during the fermentation process. Structural instability, the deletion of specific plasmid sequences, is very difficult to overcome because it is not fully understood. Segregational instability, the loss of entire plasmid molecules, arises from a failure to distribute plasmid to both daughter cells upon cell division and is affected mainly by mode of replication.

Generally, RCR plasmids are more prone to segregational instability than theta-type plasmids. First, RCR plasmids appear to lack a partitioning function, and plasmids are probably randomly distributed over daughter cells (Novick, 1987). Second, the accumulation of ssDNA intermediates also results in reduced segregational stability, as it disturbs copy number control (Pouwels & Leer, 1993). Segregational instability of RCR plasmid vectors also increases with the size of DNA inserts (Leer et al., 1992).

Segregational instability can be overcome by (i) using theta-replicating plasmids that do not produce ssDNA intermediates and stably maintain DNA inserts (Janniere et al., 1990), (ii) using high copy number plasmid vectors, since there is always a probability that at least one plasmid molecule will be transmitted to a daughter cell at each cell division, (iii) applying constant selective pressure in the growth medium and (iv) integrating foreign DNA into the host chromosome.

Before initiating a cloning trial, compatibility of a vector with other vectors or with endogenous plasmids should be considered. Plasmid incompatibility can be caused by competition of different plasmids with a similar replication mechanism for host cell proteins essential in DNA replication (i.e. Rep proteins). Generally, only one type of plasmid will remain in the host, especially if favoured by selective pressure. However, it has been shown that while only one RCR plasmid can be maintained in a host, four to five theta-replicating plasmids can exist stably in the same host (Kiewiet et al., 1993). Hence, vectors derived from theta-type plasmids tend to suffer less from incompatibility problems.

The past two decades have seen the development of a panoply of cloning vectors for the engineering of various bacterial species, some of which are still used today. Currently used vectors for LAB or other Gram-positive bacteria can be divided in two major classes. Class 1 vectors, represented by streptococcal pIP501 and enterococcal pAMβ1 and their derivatives, are large conjugative plasmids that are resistant to macrolides, lincosamides and spectogramin B (MLS resistance) and other antibiotics (Dao & Ferretti, 1985; Simon & Chopin, 1988). pIP501 and pAMβ1 are theta-replicating plasmids and their derivatives have exhibited segregational and structural stability, even upon insertion of large foreign DNA fragments (Kiewiet et al., 1993). These broad host-range plasmids can replicate in many Gram-positive bacteria, including various LAB such as *Lactococcus* spp., *Lactobacillus* spp. and *Pediococcus* spp. (de Vos & Simons, 1994). However, as streptococci and enterococci are not GRAS organisms, pIP501, pAMβ1 and their derivatives cannot be considered food-grade, and are not suitable for food applications.

Class 2 vectors are derived from small cryptic plasmids from several lactococcal species. Once they are tagged with a selectable marker, usually an antibiotic resistance gene, they can be used as cloning vectors. Prototype vectors, based on lactococcal replicons pSH71 and pWV01, contain one or more genes coding for resistance to antibiotics. These vectors tend to suffer from structural and segregational instability due to their mechanism of replication (RCR) and can rarely maintain large DNA fragments; they are only useful for the cloning of smaller genes. Nonetheless, pSH71 and pWV01 replicons and their derivatives are still useful as they have a broad host-range.

In addition to this class of vectors, cryptic plasmids originating from other LAB species have also been marked with antibiotic resistance genes to serve as broad or narrow host-range cloning vectors. Research is now being focused on the development of stable cloning vectors derived from plasmids with GRAS origins.

1.5.2 Cloning vectors derived from lactobacilli cryptic plasmids

To date, a plethora of cloning vectors has been constructed from *Lactobacillus* cryptic plasmids. Virtually every newly isolated lactobacilli plasmid has been tagged with antibiotic resistance markers, sometimes even with a replication origin of Gram-negative bacteria, and used to clone and express heterologous genes in various *Lactobacillus* species, in Gram-positive hosts such as *Bacillus* spp., *Carnobacterium divergens*, *Carnobacterium piscicola*, *Enterococcus faecalis*, *Lactococcus lactis*, *Staphylococcus aureus*, *Streptococcus sanguis*, *Streptococcus gordonii*, *Streptococcus mutans* as well as in *E. coli* (Pouwels & Leer, 1993; Klaenhammer, 1995; Wang & Lee, 1997). Most derived vectors have broad host-ranges that seem rather limited when compared to the host-ranges of lactococcal replicons (Shareck et al., 2004).

Nevertheless, some lactobacilli replicons have proven to be functional in *E. coli*, namely pPSC20/pPSC22, pLC2, pA1, pGT633, pLA106 and pLF1311 (Table 1.8) (Cocconcelli et al., 1991; Klein et al., 1993; Vujcic & Topisirovic, 1993; Tannock et al., 1994; Sano et al., 1997; Aleshin et al., 2000). They are mostly members of the pE194-family of RC plasmids, a group of replicons known to have a wide host-range (del Solar et al., 1998). Derivatives of these plasmids could be propagated in *Lactobacillus* spp., *Bacillus* spp. and *E. coli*.

Some vectors derived from *Lb. fermentum* (Iwata et al., 1986; Fons et al., 1997), *Lb. reuteri* (Ahrné et al., 1992; Heng et al., 1999; Lin & Chung, 1999; Lin et al., 2001), *Lb. helveticus* (Hashiba et al., 1990; 1992), *Lb. acidophilus* (Damiani et al., 1987), *Lb crispatus* (Pouwels & Leer, 1993) and *Lb. delbrueckii bulgaricus* (Zink et al., 1991; Chagnaud et al., 1992) exhibit a narrow host-range called host-specific replication, as they cannot replicate in other bacteria. Such vectors are interesting with regards to safety

aspects associated with the use of viable recombinant organisms or genetically modified microorganisms in food products or their use as vaccine carriers. Narrow host-range vectors are deemed intrinsically safer, as they are less likely to be horizontally transferred to other bacteria species than vectors based on broad host-range replicons (Pouwels & Leer, 1993).

Concerning stability, RC-based vectors like pULP8 and pULP9 were lost from transformed cells after 20 generations (Bringel et al., 1989); after 13 generations, only 30% of transformants still harboured pLAB1000-derived vectors (Josson et al., 1989); *Lb. delbrueckii bulgaricus* pLE16 transformants lost resistance to chloramphenicol after 28 generations (Chagnaud et al., 1992). It can be argued that industrial strains of LAB used in the production of fermented foods only undergo a few generations, thus implying that even vectors that suffer from segregational instability could still be suitable for cloning.

Nonetheless, vectors based on theta-replicating plasmids showed remarkable stability: 100% of pULA105E transformants still exhibited erythromycin resistance after 100 generations (Kanatani et al., 1992); pKC5b-derived shuttle vector pSP1 was maintained in *Lactobacillus* spp. for 100 generations without selective pressure (Pavlova et al., 2002); pTC82 and its derivative pTC82-RO both showed exceptional segregational stability, as 100% of transformants still harboured recombinant plasmids after 216 generations (Lin & Chung, 1999).

However, not all vectors derived from theta-replicating plasmids are exceptionally stable: pRV566 (*Lb. sakei*) was maintained in transformed cells for only 20 generations. Such a loss rate was deemed suitable considering *Lb. sakei* strains used as starters in sausage making grow for about 12 generations during the fermentation process (Alpert et al., 2003). Some vectors also tend to show excellent segregational stability *in vitro*, but do quite poorly *in vivo*. A recombinant plasmid, pNCHK104 derived from *Lb. reuteri* pGT232 was found to be structurally and segregationally stable under laboratory growth conditions (>97% erythromycin-resistant cells after being cultured 7 days without selective pressure), but was poorly maintained *in vivo* (<33% resistant cells after being associated with mice for 14 days) (Heng et al., 1999).

Selective markers used in cloning vectors permit the selection of positively transformed clones. In lactobacilli, many antibiotic resistance genes cannot be used as

markers, strains exhibiting natural resistance to ampicillin, cloxacillin, gentamycin, kanamycin, neomycin, penicillin, streptomycin and tetracycline, amongst others (Vescovo et al., 1982; Olukoya et al., 1993). In laboratory settings, erythromycin and chloramphenicol resistance genes are used in the development of cloning vectors for lactobacilli. However, for food application purposes, food-grade selection markers are required. In lactobacilli, several markers based on carbohydrate metabolism have been developed, including xylose metabolism in pLP3537-xyl (Posno et al., 1991b) and lactose metabolism in pBG10 (Hashiba et al., 1992). Such markers are interesting as they can be used to exert selective pressure on recombinant LAB in large scale fermentations.

1.5.3 Food-grade cloning vectors

Food-grade cloning vectors have been developed to fulfill a demand for GRAS recombinant products. Although any genetic manipulation of an organism creates a GMO, food-grade genetic modifications, i.e. modifying an organism with its own DNA or with DNA originating from GRAS organisms, might not be as ill perceived as a non food-grade genetic modification. Moreover, self-cloning, i.e. the re-introduction of DNA from a host that is modified or from a closely related strain of the same species, is excluded from the EU Directive on the contained use of genetically modified microorganisms (CEC-219, 1990) (de Vos, 1999b). In this light, organisms that are modified by self-cloning are not considered GMOs and are rather regarded as safe and suitable for food applications.

Food-grade vectors (i) are constructed with DNA of GRAS organisms (i.e. LAB cryptic plasmid) and (ii) do not contain antibiotic resistance genes as markers. Food-grade selective marker systems have been devised: marker genes originate from GRAS organisms (i.e. LAB) and are derived from naturally occurring phenotypes such as carbohydrate metabolism, resistance or immunity to bacteriocins, proteolytic activity and DNA synthesis (de Vos, 1999b).

LAB possess a variety of sugar fermentation phenotypes that can be exploited as selectable markers on cloning vectors. Genes for the metabolism of rare sugars, such as

xylose, inulin and melibiose, have been used as markers in the construction of cloning vectors (Posno et al., 1991b; Hols et al., 1994; Wanker et al., 1995; Boucher et al., 2002). Lactose metabolism, although quite widespread among LAB, can still be used as a selective marker in lactose-deficient strains or mutants. A set of cloning and expression vectors for self-cloning of *L. lactis* using *lacF* as a selection marker, the lactose-inducible *lacA* promotor and the lactococcal pSH71 replicon was developed by Platteeuw et al. (1996), a strategy that had been carried out previously (MacCormick et al., 1995). Lactose complementation markers were also developed for *Lactobacillus* spp. by Takala et al. (2002), who constructed a vector containing *lacG* from *Lb. casei* which could restore the ability of a lactose-deficient *Lb. casei* strain to grow on lactose.

Further complementation marker systems have been developed based on auxotrophies, generally mutations in genes essential for growth. One system was comprised of a vector carrying an ochre suppressor as a selectable marker which could rescue a mutation in *L. lactis*' purine genes for purine biosynthesis (Dickely et al., 1995). Another similar system was based on an amber suppressor, *supD*, encoding an altered $tRNA^{Ser}$ that could complement pyrimidine auxotrophs that had a mutated *pyrF* (Sorensen et al., 2000).

Recently, a spin off of complementation marker systems was reported by Émond et al. (2001). The system involves two plasmids: $RepB^-$ plasmid pCOM1 carries an erythromycin resistance gene, while $RepB^+$ vector pVEC1 harbours the DNA insert. Since pCOM1 can only replicate in *L. lactis* if *trans* complemented by RepB, both plasmids have to be present to transform a host cell and enable its survival in the presence of erythromycin. The marker plasmid pCOM1 can be removed effectively upon curing in antibiotic-free medium.

Selective markers based on genes for nisin resistance (*nsr*) or nisin immunity (*nisI*) have also been reported. The plasmid vectors pVS40, pFM011 and pFK012 containing *nsr* from *L. lactis* were constructed more than a decade ago and were shown to be quite effective in the direct selection of transformants (von Wright et al., 1990; Froseth et al., 1991; Hugues & McKay, 1991). *nisI* was used as a selection marker in pLEB590, which was shown to transform efficiently nisin-sensitive strains of *L. lactis* and *Lb. plantarum* (Takala & Saris, 2002). Similarly, it was suggested that the immunity gene to

lactacin F, a bacteriocin produced by *Lb. johnsonii*, could serve as a suitable marker to develop new food-grade cloning vectors (Allison & Klaenhammer, 1996). This marker is small and versatile and was found to be applicable in various *Lactobacillus* spp.

Selection markers are of prime importance in recombinant DNA technology. Research should definitely focus on the further development of food-grade selective marker systems that are suitable for large scale, industrial applications.

1.5.4 Controlled expression systems

Controlled expression systems are important tools that allow genes of interest to be expressed independently of the growth of the production host (Kuipers et al., 1997). Such systems are important, for they can be used in industrial settings, where recombinant LAB are used in large scale fermentations and the expression of cloned genes can be controlled by the addition of specific inducer molecules.

Several inducible expression systems used in LAB have been described and reviewed (Table 1.9) (Kok, 1996; Kuipers et al., 1997; de Vos et al., 1997; de Vos, 1999a). Controlled gene expression systems can be based on promoters controlled by sugars (*lac* promoter), salt (*gadC* promoter), temperature upshift (*tec* phage promoter), pH decrease (P170) or phage infection (Φ31 promoter). Not all systems fulfill food-grade criteria, as inducing factors should be acceptable in foods: small inorganic molecules (salts), organic molecules (saccharides, fatty acids) or proteinaceous compounds originating from LAB. Moreover, changes in growth conditions including pH, temperature, aeration or even phage infection would be an acceptable way to induce gene expression.

The most versatile system is the NICE system (nisin-controlled expression). Biosynthesis of the antimicrobial peptide nisin is controlled by a nisin gene cluster. The promoter of *nisA* is autoregulated by a two-component regulatory system, consisting of the sensor kinase NisK and the response regulator NisR, which respond to extracellular nisin. The addition of nisin induces co-transcription of genes of interest with *nisA* via membrane-associated NisK and transcriptional regulator NisR, resulting in the expression

of genes cloned downstream of *nisA* (Geis, 2003). de Ruyter et al. (1996a) have developed a series of vectors and strains specifically suited for regulated gene expression in *L. lactis*, based on transcriptional and translational fusions of the *nisA* promoter. The vectors were derived from lactococcal replicon pSH71 and carried the *E. coli gusA* and *L. lactis pepN* genes either translationally or transcriptionally fused to the *nisA* promoter. Expression of the genes was induced by subinhibitory levels of nisin.

Similarly, Henrich et al. (2002) also reported the development of a set of plasmid vectors suited for nisin-inducible gene expression in any strain of *L. lactis*. Food-grade recombinants of *L. lactis* were constructed which had the nisRK genes and the nisin-inducible promoter P_{nisA} fused to peptidase genes (*pep*). Expression of P_{nisA}::*pep* fusions after induction with nisin was successfully demonstrated.

The nisin system not only functions in lactococci, but also in other LAB, such as *Lactobacillus* spp. and *Leuconostoc* spp. (Kleerebezem et al., 1997; Kuipers et al., 1998). Controlled expression systems that rely on nisin for induction are of great interest for the overproduction of various proteins, as nisin is a food-grade additive and is thus safe to use in food applications. Moreover, the system is suitable for industrial scale applications.

1.6 Conclusion

Many cryptic plasmids have been isolated from LAB and used to construct cloning or integration vectors, especially in the genera *Lactococcus* and *Lactobacillus* and in *S. thermophilus*. There is still much work remaining for the *Leuconostoc*, *Pediococcus* and *Bifidobacterium* genera (Shareck et al., 2004). Research prospects are clear-cut: (i) additional cryptic plasmids should be isolated, especially from the genera that suffer from a deficiency in genetic engineering tools; (ii) plasmids that have been isolated, but have yet to be studied in depth, should be characterized and sequenced, their minimal replicons determined as well as their modes of replication; (iii) vectors that have been derived should be tested for their capacities in cloning and expression of heterologous genes in various hosts; (iv) cloning vectors with high segregational stability and high and low copy number derivatives should also be constructed to offer stable expression of target genes; (v) narrow host-range vectors are important for the development of vaccine delivery

systems *in vivo* and should not be overlooked; (vi) more importantly, food-grade vectors, which use food-grade marker systems, need to be developed to enable the legal acceptation of food products containing recombinant organisms; and finally, (vii) food-grade modification strategies, such as integration and controlled expression systems, should also be considered for the engineering of industrial strains of LAB used in food production; these systems should be derived preferably from LAB cryptic plasmids. The development of efficient, food-grade, industrially suitable genetic engineering tools to modify LAB used for the production of fermented foods, for the overproduction of various food ingredients and metabolites or for the delivery of oral vaccines is of prime importance. These genetic manipulation tools have been awaited, as food-grade recombinant lactic acid bacteria have the potential to revolutionize the market place.

Table 1.1 Characteristics of *Lactococcus lactis* RCR cryptic plasmids

Plasmid	Origin	Size (kb)	Family of replicon	References
pWV01	*L. lactis subsp. cremoris*	2.2	pE194	Kok et al., 1984; Leenhouts et al., 1991
pSH71	*L. lactis*	2.1	pE194	de Vos et al., 1987
pD125	*L. lactis* subsp. *lactis*	5.1	pE194	Xu et al., 1990
pCL2.1	*L. lactis* subsp. *lactis*	2.1	pE194	Chang et al., 1995
pWC1	*L. lactis*	2.8	pC194	Pillidge et al., 1996
pBM02	*L. lactis* subsp. *cremoris*	3.9	pE194	Sanchez et al., 2003

Table 1.2 Characteristics of *Lactococcus lactis* theta-replicating plasmids

Plasmid	Origin	Size (kb)	References
pCI305	*L. lactis* subsp. *lactis*	8.7	Hayes et al., 1990; 1991
pSL2	*L. lactis* subsp. *lactis* biovar diacetylactis Bu2	7.8	Jahns et al., 1991
pVS40	*L. lactis* subsp. *lactis* biovar diacetylactis	7.8	von Wright et al., 1990; von Wright & Raty, 1993
pSK11L	*L. lactis* subsp. *cremoris*	47.3	Horng et al., 1991
pWV02	*L. lactis* subsp. *cremoris*	3.8	Kiewiet et al., 1993
pCI528	*L. lactis* subsp. *cremoris*	46	Lucey et al., 1993
pUCL22	*L. lactis* subsp. *lactis*	40	Frere et al., 1993
pCT1138	*L. lactis* subsp. *lactis* biovar diacetylactis	5.5 MDa	Pedersen et al., 1994
pWV04	*L. lactis* subsp. *cremoris*	19	Seegers et al., 1994
pWV05	*L. lactis* subsp. *cremoris*	27	Seegers et al., 1994
pIL7	*L. lactis*	31	Seegers et al., 1994
pJW563	*L. lactis* subsp. *cremoris*	11.5	Gravesen et al., 1995
pND302	*L. lactis* subsp. *lactis*	8.8	Liu et al., 1996; 1997b
FG2 plasmid	*L. lactis* subsp. *lactis FG2*	1.8	Liu et al., 1997a
pNZ4000	*L. lactis*	40	van Kranenburg & de Vos, 1998
pND324	*L. lactis* subsp. *lactis*	3.6	Duan et al., 1999
pCIS3	*L. lactis* subsp. *cremoris*	6.1	Seegers et al., 2000
pCI2000	*L. lactis* subsp. *lactis*	60	Kearney et al., 2000

MDa: Megadaltons

Table 1.3 Characteristics of *Lactobacillus* spp. cryptic plasmids

Plasmid	Origin	Size (kb)	Mode of Replication	Family of replicon	References
pcaT	*Lb. plantarum*	8.5	NA	NA	Jewel & Thompson-Collins, 1989
pA1	*Lb. plantarum*	2.8	RCR	pE194	Vujcic & Topisirovic, 1993
pLP1	*Lb. plantarum*	2.1	RCR	pC194	Bouia et al., 1989
p8014-2	*Lb. plantarum*	1.9	RCR	pC194	Leer et al., 1992
pC30il	*Lb. plantarum*	2.1	RCR	pC194	Skaugen et al., 1989
pLB4	*Lb. plantarum*	3.5	RCR	pE194	Bates & Gilbert, 1989
pLP2000	*Lb. plantarum*	2.1	RCR	pC194	Daming et al., 2003
pLP9000	*Lb. plantarum*	9.3	RCR	NA	Daming et al., 2003
a 7 kb plasmid	*Lb. plantarum*	7	RCR	pE194	Cocconcelli et al., 1991; 1996
pLKL	*Lb. plantarum*	6.8	NA	NA	Eguchi et al., 2000
pLKS	*Lb. plantarum*	2.0	theta	-	Eguchi et al., 2000
pMD5057	*Lb. plantarum*	11.0	NA	NA	Danielsen, 2002
p256	*Lb. plantarum*	7.2	theta	-	Sorvig et al., 2005
pPB1	*Lb. plantarum*	2.9	RCR	pE194	de las Rivas et al., 2004
pWCFS101	*Lb. plantarum*	1.9	RCR	pC194	van Kranenburg et al., 2005
pWCFS102	*Lb. plantarum*	2.4	RCR	pMV158	van Kranenburg et al., 2005
pWCFS103	*Lb. plantarum*	36	theta	-	van Kranenburg et al., 2005
pLY2	*Lb. fermentum*	15.6	NA	NA	Iwata et al., 1986
pLY4	*Lb. fermentum*	57.8	NA	NA	Iwata et al., 1986
pLEM3	*Lb. fermentum*	5.7	RCR	pC194	Fons et al., 1997

pLF1311	*Lb. fermentum*	2.4	RCR	pE194	Aleshin et al., 1999
pKC5b	*Lb. fermentum*	4.4	theta	-	Pavlova et al., 2002
p353-1	*Lb. pentosus*	1.7	NA	NA	Posno et al., 1991a
p353-2	*Lb. pentosus*	2.3	RCR	pC194	Leer et al., 1992
pLUL631	*Lb. reuteri*	10.2	NA	NA	Axelsson et al., 1988
pLAR33	*Lb. reuteri*	18	NA	NA	Rinckel & Savage, 1990
pGT633	*Lb. reuteri*	9.8	NA	NA	Tannock et al., 1994
pGT232	*Lb. reuteri*	5.1	RCR	pC194	Heng et al., 1999
pTE15	*Lb. reuteri*	15	NA	NA	Lin et al., 1999
pTE80	*Lb. reuteri*	7.0	NA	NA	Lin et al., 1999
pTC82	*Lb. reuteri*	7.0	RCR	pC194	Lin et al., 1996a; 2001
pLAB1000	*Lb. hilgardii*	3.3	RCR	pC194	Josson et al., 1989; 1990
pLAB2000	*Lb. hilgardii*	9.1	NA	NA	Josson et al., 1989
pLC2	*Lb. curvatus*	2.6	RCR	pE194	Vogel et al., 1991; Klein et al., 1993
pWS97	*Lb. delbrueckii*	60	NA	NA	Zink et al., 1991
pLB10	*Lb. delbrueckii bulgaricus*	2.7	NA	NA	Chagnaud et al., 1992
pLBB1	*Lb. delbrueckii bulgaricus*	6.1	theta	-	Azcarate-Peril & Raya, 2002
pJBL2	*Lb. delbrueckii lactis*	8.7	theta	-	Bourniquel et al., 2002
pN42	*Lb. delbrueckii lactis*	8.1	theta	-	Bourniquel et al., 2002
p1	*Lb. acidophilus*	1.6	NA	NA	Damiani et al., 1987
p3	*Lb. acidophilus*	4.2	NA	NA	Damiani et al., 1987
pPM4	*Lb. acidophilus*	NA	NA	NA	Luchansky et al., 1988
pLA103	*Lb. acidophilus*	14.0	theta	-	Kanatani et al., 1992; 1995b
pLA105	*Lb. acidophilus*	3.2	theta	-	Kanatani et al., 1995a

pLA106	*Lb. acidophilus*	2.9	RCR	pE194	Sano et al., 1997
pLJ1	*Lb. helveticus*	3.3	theta	-	Takiguchi et al., 1989
pCP53	*Lb. helveticus*	11.5	NA	NA	Yamamoto & Takano, 1996
pLH1	*Lb. helveticus*	19.4	theta	-	Fortina et al., 1993 ; Thompson et al., 1999
pLH2	*Lb. helveticus*	5.7	RCR	NA	Fortina et al., 1993
pLH3	*Lb. helveticus*	3.4	RCR	NA	Fortina et al., 1993
pLH4	*Lb. helveticus*	2.6	RCR	NA	Pridmore et al., 1994
pSAK1	*Lb. sakei*	19	theta	-	Unpublished; GenBank Accession no. Z50862
pRV500	*Lb. sakei*	13	theta	pUCL287	Alpert et al., 2003
pLZ15	*Lb. casei*	28.3	NA	NA	Chassy & Flickinger, 1987
p121BS	*Lactobacillus* spp.	4.2	NA	NA	Whitehead et al., 2001

NA: not available; -: not applicable to theta replicons

Table 1.4 Characteristics of *Streptococcus thermophilus* cryptic plasmids

Plasmid	Origin	Size (kb)	Mode of replication	Family of replicon	References
pA2	*S. thermophilus*	2	RCR	NA	Mercenier et al., 1990
pA33	*S. thermophilus*	6.9	RCR	NA	Mercenier et al., 1990
pST1	*S. thermophilus*	2.1	RCR	pC194	Janzen et al., 1992
pST1 no.29	*S. thermophilus*	2.8	RCR	pC194	Hashiba et al., 1993
pER8	*S. thermophilus*	2.2	RCR	pC194	Somkuti & Steinberg, 1986
pER371	*S. thermophilus*	2.7	RCR	pC194	Solaiman & Somkuti, 1998
pER341	*S. thermophilus*	2.8	RCR	pC194	Somkuti et al., 1998
pER36	*S. thermophilus*	3.7	NA	NA	Somkuti & Steinberg, 1986
pER13	*S. thermophilus*	4.2	NA	NA	Somkuti & Steinberg, 1986
pER16	*S. thermophilus*	4.5	NA	NA	Somkuti & Steinberg, 1986
pER342	*S. thermophilus*	9.5	NA	NA	Somkuti & Steinberg, 1986
pER35	*S. thermophilus*	11.0	NA	NA	Somkuti & Steinberg, 1986
pER372	*S. thermophilus*	14.8	NA	NA	Somkuti & Steinberg, 1986
pER7	*S. thermophilus*	4.5	NA	NA	Somkuti & Steinberg,1999
pER26	*S. thermophilus*	4.5	NA	NA	Somkuti & Steinberg,1999
pER41	*S. thermophilus*	3.6	NA	NA	Somkuti & Steinberg,1999
pCI65st	*S. thermophilus*	6.5	RCR	pC194	O'Sullivan et al., 1999
pSMQ172	*S. thermophilus*	4.2	RCR	pMV158/pE194	Turgeon & Moineau, 2001
pND103	*S. thermophilus*	3.5	RCR	pC194	Su et al., 2002
pSt04	*S. thermophilus*	3.1	RCR	pC194	Geis et al., 2003
pER1-1	*S. thermophilus*	3.4	RCR	pC194	Geis et al., 2003
pJ34	*S. thermophilus*	3.4	RCR	pC194	Geis et al., 2003
pSt08	*S. thermophilus*	7.5	RCR	pC194	Geis et al., 2003
pER1-2	*S. thermophilus*	4.4	NA	NA	Geis et al., 2003
pSt06	*S. thermophilus*	5.3	NA	NA	Geis et al., 2003
pSt0	*S. thermophilus*	8.1	NA	NA	Geis et al., 2003
pSt22-2	*S. thermophilus*	4.2	NA	NA	Geis et al., 2003
pt38	*S. thermophilus*	2.9	RCR	pC194	Petrova et al., 2003

NA: not available

Table 1.5 Characteristics of *Leuconostoc* and *Oenococcus oeni* cryptic plasmids

Plasmid	Origin	Size (kb)	Mode of replication	Family of replicon	References
pLo13	*Ln. oenos*	3.9	RCR	pC194	Fremaux et al., 1993
pOg32	*Ln. oenos*	2.5	RCR	pT181	Brito et al., 1996
p4028	*Ln. oenos*	4.4	theta	-	Zuniga et al., 1996
pRS1	*O. oeni*	2.5	RCR	pT181	Alegre et al., 1999
pFR18	*Ln. mesenteroides*	1.8	RCR	pT181	Biet et al., 1999
pTXL1	*Ln. mesenteroides*	2.7	theta	-	Biet et al., 2002
pCI411	*Ln. lactis*	2.9	RCR	pE194	Coffey et al., 1994

-: not applicable to theta replicons

Table 1.6 Characteristics of *Pediococcus* spp. cryptic plasmids

Plasmid	Origin	Size (kb)	Mode of replication	References
pSRQ1	*P. pentosaceus*	30 MDa	NA	Gonzalez & Kunka, 1983
pSRQ7	*P. pentosaceus*	12 MDa	NA	Gonzalez & Kunka, 1983
pSRQ8	*P. pentosaceus*	17 MDa	NA	Gonzalez & Kunka, 1983
pSRQ9	*P. acidilactici*	6.7 MDa	NA	Gonzalez & Kunka, 1983
pSRQ10	*P. acidilactici*	23 MDa	NA	Gonzalez & Kunka, 1983
pSRQ11	*P. acidilactici*	9.4	NA	Gonzalez & Kunka, 1983; Marugg et al., 1992
pSMB74	*P. acidilactici*	8.9	theta	Motlagh et al., 1994; Benachour et al., 1997.
pUCL287	*T. halophila*	8.7	theta	Benachour et al., 1995; 1997
pMD136	*P. pentosaceus*	19.5	theta	Kantor et al., 1997

MDa: Megadaltons; NA: not available

Table 1.7 Characteristics of *Bifidobacterium* spp. cryptic plasmids

Plasmid	Origin	Size (kb)	Mode of replication	References
pMB1	*B. longum*	1.9	theta	Matteuzzi et al., 1990
pVS809	*B. globosum*	2.8	NA	Mattarelli et al., 1994.
pTB6	*B. longum*	NA	NA	Matsumura et al., 1997
pKJ50	*B. longum*	5.0	RCR	Park et al., 1997
pKJ36	*B. longum*	3.6	RCR	Park et al., 1997
pMG1	*B. longum*	3.9	RCR	Park et al., 2003
pNBb1	*B. breve*	5.6	RCR	Bourget et al., 1993
pCIBb1	*B. breve*	5.8	RCR	O'Riordan & Fitzgerald, 1999
pAP1	*B. asteroides*	2.1	unknown	Kaufmann et al., 1997; GenBank Accession no. Y11549
p4M	*B. pseudocatenulatum*	4.5	RCR	Smeianov et al., 2002; GenBank Accession no. AF359574
pB44	*B. longum*	3.6	RCR	Smeianov et al., 2002; GenBank Accession no. AY066026
pDOJH10L	*B. longum*	10	RCR	Lee, J. H. et al., 2002; GenBank Accession no. AF538868
pBLO1	*B. longum*	3.6	RCR	Schell et al., 2003; GenBank Accession no. AF540971
pNAC1	*B. longum*	3.5	RCR	Corneau et al., 2004
pNAC2	*B. longum*	3.7	RCR	Corneau et al., 2004
pNAC3	*B. longum*	10.2	unknown	Corneau et al., 2004

NA: not available; unknown: family of replicon is unknown

Table 1.8 Vectors derived from *Lactobacillus* spp. cryptic plasmids and their characteristics

Vector	Replicon	Origin	Size (kb)	Genetic marker	Shuttle vector	Host-range	References
pcaT	pcaT	*Lb. plantarum*	8.5	Cm^r	NA	*C. divergens* *C. piscicola* *Lb. casei* *Lb. plantarum* *C. piscicola*	Ahn et al., 1992
pA1	pA1	*Lb. plantarum*	4.0	Cm^r	no	*E. coli* *Lb. delbrueckii* *Lb. plantarum*	Vujcic & Topisirovic, 1993
pULP8	pLP1	*Lb. plantarum*	6.6	Ap^r Em^r	yes	*Ba. subtilis* *Lb. plantarum*	Bringel et al., 1989
pULP9	pLP1	*Lb. plantarum*	6.8	Ap^r Em^r	yes	*Ba. subtilis* *Lb. plantarum*	Bringel et al., 1989
pLP825	p8014-2	*Lb. plantarum*	7.6	Ap^r Cm^r	yes	*Lb. acidophilus* *Lb. brevis* *Lb. casei* *Lb. fermentum* *Lb. helveticus* *Lb. pentosus* *Lb. plantarum*	Posno et al., 1991a Posno et al., 1991a Leer et al., 1992; Posno et al., 1991a Posno et al., 1991a de los Reyes-Gavilan et al., 1990 Posno et al., 1991a; Leer et al., 1992 Badii et al., 1989; Leer et al., 1992; Posno et al., 1991a; Duckworth et al., 1993

pLP82H	p8014-2	*Lb. plantarum*	5.9	Ap^r Cm^r	yes	*Lb. casei*	Leer et al., 1992
						Lb. pentosus	Duckworth et al., 1993
						Lb. plantarum	
pLPC37	p8014-2	*Lb. plantarum*	3.3	Cm^r	no	*Lb. casei*	Leer et al., 1992
						Lb. pentosus	
						Lb. plantarum	
pPSC1	a 7 kb plasmid	*Lb. plantarum*	4.1	Cm^r	yes	*Ba. subtilis*	Cocconcelli et al., 1991
						Lb. acidophilus	
						Lb. fermentum	
						Lb. helveticus	
						Lb. plantarum	
						Lb. reuteri	
						L. lactis	
pPSC10	a 7 kb plasmid	*Lb. plantarum*	3.0	Em^r	yes	*Ba. subtilis*	Cocconcelli et al., 1991
						Lb. acidophilus	
						Lb. fermentum	
						Lb. helveticus	
						Lb. reuteri	
						L. lactis	
pPSC11	a 7 kb plasmid	*Lb. plantarum*	2.9	Cm^r	yes	*Ba. subtilis*	Cocconcelli et al., 1991
						Lb. acidophilus	
						Lb. fermentum	
						Lb. helveticus	
						Lb. reuteri	
						L. lactis	
pPSC20	a 7 kb plasmid	*Lb. plantarum*	5.5	Cm^r Em^r	yes	*Ba. subtilis*	Cocconcelli et al., 1991; Vescovo et al., 1991
						Lb. acidophilus	

						Lb. fermentum *Lb. helveticus* *Lb. reuteri* *L. lactis*	
pPSC22	a 7 kb plasmid	*Lb. plantarum*	4.3	Cm^r Em^r	yes	*Ba. subtilis*	Cocconcelli et al., 1991
						Lb. acidophilus *Lb. fermentum* *Lb. helveticus* *Lb. reuteri* *L. lactis*	
NA	cryptic plasmid	*Lb. plantarum*	NA	Ap^r	yes	*E. coli*	Mayo et al., 1989
						Lb. plantarum	
pLPV106	p256	*Lb. plantarum*	NA	Ap^r Em^r	yes	*Lb. plantarum*	Holck et al., 1992
						Lb. sake	
pLPVIII	p256	*Lb. plantarum*	4.2	Em^r *lac*	yes	*Lb. sake*	Axelsson & Holck, 1995
pLPV103	p256	*Lb. plantarum*	5.1	Cm^r	Yes	*Lb. plantarum*	Sorvig et al., 2005
pWCFS104	pWCFS101	*Lb. plantarum*	3.0	Cm^r	no	*Lb. plantarum*	van Kranenburg et al., 2005
						Lb. helveticus *Lb. casei* *Ln. lactis*	
pWCFS105	pWCFS102	*Lb. plantarum*	3.4	Cm^r	no	*Lb. plantarum*	van Kranenburg et al., 2005
						Lb. helveticus *Lb. casei* *Ln. lactis*	

						C. maltaromaticum *Ba. subtilis*	
pWCFS106	pWCFS103	*Lb. plantarum*	4.2	Cm^r	no	*Lb. plantarum*	van Kranenburg et al., 2005
pLY2	pLY2	*Lb. fermentum*	15.6	Tc^r	NA	*Lb. fermentum*	Iwata et al., 1986
pLY4	pLY4	*Lb. fermentum*	57.8	Em^r	NA	*Lb. fermentum*	Iwata et al., 1986
pLEM5	pLEM3	*Lb. fermentum*	3.4	Em^r	no	*Lb. fermentum*	Fons et al., 1997
pLEM7	pLEM3	*Lb. fermentum*	3.5	Em^r	no	*Lb. fermentum*	Fons et al., 1997
pLFVM2	pLF1311	*Lb. fermentum*	5.0	Cm^r	yes	*Lb. brevis* *Lb. buchneri* *L. lactis* *En. faecalis* *En. faecium* *Ba. subtilis* *Ba. thuringiensis* subsp. *galleriae* *Ba. thuringiensis* subsp. *kurstaki* *Ba. thuringiensis* subsp. *finitimus* *Ba. amyloliquefaciens* *Ba. flavum* *E. coli*	Aleshin et al., 1999
NA	pLF1311	*Lb. fermentum*	NA	NA	yes	Gram-positive	Aleshin et al., 2000

						E. coli	
pSP1	pKC5b	*Lb. fermentum*	9.4	Em^r	yes	*Lb. fermentum*	Pavlova et al., 2002
						Lb. jensenii	
						Lb. spp.	
						Lb. gasseri	
						Lb. crispatus	
						Lb. johnsonii	
						Lb. salivarius	
						S. mutans	
						S. gordonii	
						S. sanguis	
pLPE23M	p353-2	*Lb. pentosus*	3.7	Em^r	no	*Lb. plantarum*	Pouwels & Leer, 1993
pLPE24M	p353-2	*Lb. pentosus*	3.7	Em^r	no	*Lb. plantarum*	
pLPE317	p353-1	*Lb. pentosus*	2.9	Em^r	no	*Lb. casei*	Posno et al., 1991a
						Lb. pentosus	
						Lb. plantarum	
pLPE323	p353-2	*Lb. pentosus*	3.6	Em^r	no	*Lb. casei*	Posno et al., 1991a
						Lb. pentosus	
						Lb. plantarum	
pLPE350	p353-2	*Lb. pentosus*	3.6	Cm^r	no	*Lb. casei*	Leer et al., 1992
						Lb. pentosus	
						Lb. plantarum	
pLP3537	p353-2	*Lb. pentosus*	6.3	Ap^r Em^r	yes	*Lb acidophilus*	Posno et al., 1991a
						Lb. casei	
						Lb. pentosus	

pLP3537-xyl	p353-2	*Lb. pentosus*	12.2	Ap^r Em^r *xyl*	yes	*Lb. plantarum* *Lb. casei*	Posno et al., 1991b
pGT633	pGT633	*Lb. reuteri*	9.8	Em^r	NA	*Lb. pentosus* *Lb. plantarum* *Ba. subtilis* *En. faecalis* *Lb. delbrueckii* *Lb. fermentum* *Lb. reuteri* *Lb. gasseri* *Lb. salivarius* *St. aureus* *S. sanguis*	Tannock et al., 1994
NA	pLUL631 + pVS1	*Lb. reuteri*	NA	Em^r	yes	*L. lactis* *E. coli*	Axelsson et al., 1988
pLUL631	pLUL631	*Lb. reuteri*	10.2	Em^r	NA	*Lb. reuteri*	Ahrné et al., 1992
pLUL200	pLUL631	*Lb. reuteri*	6.0	Cm^r	yes	*Lb. reuteri*	
pLUL201	pLUL631	*Lb. reuteri*	7.4	Cm^r Em^r	yes	*Lb. reuteri*	
pLUL202	pLUL631	*Lb. reuteri*	4.3	Cm^r	no	*Lb. reuteri*	
pLUL634	pLUL631	*Lb. reuteri*	5.1	Em^r	no	*Lb. reuteri*	
pNCKH104	pGT232	*Lb. reuteri*	5.7	Em^r	yes	*Lb. reuteri*	Heng et al., 1999
pNCHK103	pGT232	*Lb. reuteri*	6.7	Em^r	yes	*Lb. reuteri*	
pTE15-RO	pTE15	*Lb. reuteri*	6.7	Em^r Ap^r	yes	*Lb. reuteri*	Lin et al., 1999
pTE80-RO	pTE80	*Lb. reuteri*	6.9	Em^r Ap^r	yes	*Lb. fermentum* *Lb. reuteri*	
pTC82-RO	pTC82	*Lb. reuteri*	7	Em^r	no	*Lb. reuteri*	Lin et al., 2001
pERM3.2	pLAB1000	*Lb.*	7.6	Em^r	yes	*Lb. plantarum*	Scheirlinck et al., 1989

		hilgardii		Ap^r			
pLAB1102	pLAB1000	*Lb. hilgardii*	7.5	Cm^r Ap^r	yes	*Ba. subtilis* *En. faecalis* *Lb. casei* *Lb. plantarum*	Josson et al., 1989
pLAB1301	pLAB1000	*Lb. hilgardii*	5.3	Em^r Ap^r	yes	*Ba. subtilis* *En. faecalis* *Lb. casei* *Lb. plantarum*	Josson et al., 1989
pLAB1304	pLAB1000	*Lb. hilgardii*	5.2	Em^r Ap^r	yes	*Ba. subtilis* *En. faecalis* *Lb. plantarum*	Josson et al., 1990
pLAB1321	pLAB1000	*Lb. hilgardii*	6.9	Em^r Ap^r	yes	*Ba. subtilis* *En. faecalis* *Lb. plantarum*	Josson et al., 1990
pJK352	pLC2	*Lb. curvatus*	5.9	Cm^r Ap^r	yes	*Ba. subtilis* *Lb. casei* *L. lactis*	Klein et al., 1993
pJK352d	pLC2	*Lb. curvatus*	3.2	Cm^r	no	*Ba. subtilis* *Lb. casei* *L. lactis*	Klein et al., 1993
pJK353	pLC2	*Lb. curvatus*	5.8	Cm^r Ap^r	yes	*Ba. subtilis* *Lb. casei* *L. lactis*	Klein et al., 1993
pJK354	pLC2	*Lb. curvatus*	5.8	Cm^r Ap^r	yes	*Ba. subtilis*	Klein et al., 1993

						Lb. casei *L. lactis*	
pJK355	pLC2	*Lb. curvatus*	3.2	Cm^r	no	*Ba. subtilis*	Klein et al., 1993
						Lb. casei *L. lactis*	
pJK356	pLC2	*Lb. curvatus*	3.2	Cm^r	no	*Ba. subtilis*	Klein et al., 1993
						Lb. casei *L. lactis*	
pJK300	pWS97	*Lb. delbrueckii*	6.8	Cm^r Ap^r	yes	*Lb. delbrueckii*	Zink et al., 1991
pLE16	pLB10	*Lb. delb. bulgaricus*	7.6	Cm^r Tc^r	yes	*Lactobacillus* spp. 89	Chagnaud et al., 1992
pSS1	pLBB1	*Lb. delb.* ssp. *bulgaricus*	7	Cm^r Em^r Tc^r	no	*L. lactis* *Lb. johnsonii*	Azcarate-Peril & Raya, 2002
pN42 + pJDC9	pN42	*Lb. delb. lactis*	NA	Em^r	yes	*L. lactis*	Bourniquel et al., 2002
pLHR	pLJ1	*Lb. helveticus*	8.5	Ap^r Em^r	yes	*Lb. helveticus*	Hashiba et al., 1990
pBG10	pLJ1	*Lb. helveticus*	6.0	_-*gal*	yes	*Lb. helveticus*	Hashiba et al., 1992
pCP53d	pCP53	*Lb. helveticus*	4.7	Tc^r	no	*Lb. helveticus* *Lb. casei*	Yamamoto & Takano, 1996
pPV751	p1	*Lb. acidophilus*	NA	Tc^r	yes	*Lb. acidophilus*	Damiani et al., 1987
pPV754	p3	*Lb. acidophilus*	NA	Tc^r	yes	*Lb. acidophilus*	

pTRK13	pPM4	*Lb. acidophilus*	12.5	Cm^r	NA	*Lb. acidophilus*	Luchansky et al., 1988
pTRK159	pPM4	*Lb. acidophilus*	10.3	Cm^r Em^r Tc^r	yes	*Lb. acidophilus*	Muriana & Klaenhammer, 1991
pULA105E	pLA105	*Lb. acidophilus*	7.8	Ap^r Em^r	yes	*Lb. acidophilus* *Lb. casei*	Kanatani et al., 1992
pLA106PVem	pLA106	*Lb. acidophilus*	3.6	Em^r	no	*Lb. acidophilus.* *Lb. casei* *E. coli*	Sano et al., 1997
pRV566	pRV500	*Lb. sakei*	7.3	Em^r Ap^r	yes	*Lb. sakei* *Lb. plantarum* *Lb. curvatus* *Lb. casei*	Alpert et al., 2003
pLZ15	pLZ15	*Lb. casei*	27	*lac*	NA	*Lb. casei* *L. lactis*	Chassy & Flickinger, 1987 Heme et al., 1994
pAZ20	pNCDO151	*Lb. casei*	8.3	Ap^r Cm^r	yes	*Lb. delbrueckii*	Zink et al., 1991

Cm^r: chloramphenicol resistance; Ap^r: ampicillin resistance; Em^r: erythromycin resistance; Tc^r: tetracycline resistance; *L.*: *Lactococcus*; *Lb.*: *Lactobacillus*; *S.*: *Streptococcus*; *Ln.*: *Leuconostoc*; *St*: *Staphylococcus*; *Ba*: *Bacillus*; *C.*: *Carnobacterium*; *E.*: *Escherichia*; *En.* : *Enterococcus*

Table 1.9 Characteristics of inducible expression systems for lactic acid bacteria

LAB	Induction treatment	Inducible element	Induction factor	References
L. lactis	Lactose	*lacA* or *lacR* promotor	<10	Van Rooijen et al., 1992; Eaton et al., 1993; Payne et al., 1996
L. lactis	Lactose	LacR	<10	Gosalbes et al., 2000
L. lactis	Lactose	*lacA/T7* promotor	<20	Wells et al., 1995; Steidler et al., 1995
S. thermophilus	Lactose	*lacS*-GalR	~10	Mollet et al., 1993
Lb. pentosus	Xylose	*xylA* promotor	60-80	Lokman et al., 1994; 1997
L. lactis	High temperature	*dnaJ* promoter	<4	Van Asseldonk et al., 1993
L. lactis	High temperature	*tec*-Rro12	>500	Nauta et al., 1996; 1997
L. lactis	Low pH, low temperature	PA170 promotor	50-100	Israelsen et al., 1995
L. lactis	Low pH	*gadC*-GadR	>1000	Sanders et al., 1997; 1998a; 1998b
L. lactis	Aeration	*sodA* promoter	2	Sanders et al., 1995
L. lactis	Absence of peptides	*prtP* or *prtM* promotor	<8	Marugg et al., 1995; 1996
L. lactis	Mitomycin C	repressor/operator Φr1t	70	Nauta et al., 1996
L. lactis	Φ31 infection	Φ31 promotor and *ori*	>1000	O'Sullivan et al., 1996
L. lactis	Nisin	*nisA* or *nisF* promotor	>1000	Kuipers et al., 1995; de Ruyter et al., 1996a; 1996b; Henrich et al., 2002
Lb. helveticus	Nisin			
Lb. casei	Nisin	*nisA* or *nisF* promotor	>1000	Kleerebezem et al., 1997
Ln. lactis	Nisin			

Chapter 2. Characterization of pLJ42, a novel *Lactobacillus plantarum* theta-replicating plasmid

In this chapter, results of the research are summarized and discussed. A cryptic plasmid was isolated from a strain of *Lactobacillus plantarum*, and characterized. Its replicative features were analyzed and a cloning vector based on its replicon was constructed and transformed into various hosts. This manuscript was written by Shareck, J. under the supervision of Choi, Y., Miguez, C. B. and Lee, B. It was submitted to Applied and Environmental Microbiology for publication.

2.1 Abstract

In this research, strains of *Lactobacillus* spp. were screened for plasmids. *Lb. plantarum* L42 was found to harbour two plasmids of estimated sizes 5.5 kb (pLJ42) and >10 kb (pLF42). The sequence and genetic organization of pLJ42 (5,529 bp) were determined. It was revealed that the plasmid was composed of two modules, a replication region and a mobilization operon. Structural elements identified in the origin of replication (*ori*) were typical of theta-replicating plasmids. The replication protein RepA was found to be homologous with Rep proteins from theta plasmids, which confirmed the assumption pLJ42 replicated by the theta mechanism. An *E. coli-Lactobacillus* shuttle vector ($pLJ42_v$) was constructed using the pLJ42 replicon. *E. coli* was effectively transformed to express erythromycin resistance (Em^r) and the green fluorescent protein (*gfp*) gene. Transformation of several strains of *Lactobacillus* with $pLJ42_v$ indicated that the pLJ42 replicon had a limited host-range.

2.2 Introduction

Lactic acid bacteria (LAB), a group of generally recognized as safe (GRAS) organisms that metabolize sugars into primarily lactic acid, have traditionally been used for the production of various fermented foods and beverages (Stiles & Holzapfel, 1997). Several strains of LAB are well known for their health-benefiting properties, and have shown to be effective in the treatment of bacterial gastroenteritis, *Helicobacter pylori* infections and inflammatory bowel diseases (Reid et al., 2003). More recently, LAB have been exploited as hosts for the overexpression of heterologous proteins, a promising avenue for the pharmaceutical and food technology fields (de Vos, 1999a). There is increasing interest in the genetic manipulation of LAB to improve existing characteristics or introduce novel, industrially pertinent phenotypes. However, because LAB have food-related applications, their genetic modification requires the use of genetic engineering tools that are deemed food-grade. Cloning vectors derived from LAB plasmids that do not contain antibiotic resistance genes as markers are suitable to engineer recombinant LAB, as they are composed solely of DNA from GRAS organisms (de Vos, 1999b).

Plasmids are naturally present in LAB, and many have been isolated and characterized (reviewed by Shareck et al., 2004). Two modes of replication are commonly found among LAB plasmids, rolling-circle (RC) and theta. RC plasmids encode a site-specific nuclease that nicks double-stranded DNA at its target site (*dso*), initiating the synthesis of the leading strand of DNA (del Solar et al., 1998). Single-stranded DNA (ssDNA) intermediates are generated during RC replication, and their accumulation decreases the segregational stability of cloning vectors based on RC replicons (Gruss & Ehrlich, 1989). In theta replication, strand synthesis occurs covalently and simultaneously on both strands, and ssDNA intermediates are not generated (de Vos & Simons, 1994). Cloning vectors derived from theta replicons are more stable and can maintain larger DNA inserts (Kiewiet et al., 1993). However, such vectors have a narrow host-range compared to their RC-based counterparts (Hayes et al., 1991). Nevertheless, narrow or limited host-range vectors are suitable for certain applications where horizontal transfer is of concern, i.e. in the use of recombinant organisms in food fermentations or as vaccine carriers (Pouwels & Leer, 1993).

Lactobacillus plantarum is commonly found in spontaneous food fermentations, and is also one of the main species of lactobacilli in the human small intestine (Stiles & Holzapfel, 1997). Strains of *Lb. plantarum* are used industrially as starters for vegetable and meat fermentations (Hammes et al., 1990), and in probiotic preparations: a probiotic fruit drink containing *Lb. plantarum* 299v-fermented oatmeal gruel is available in Sweden (Molin, 2001). Many plasmids have been isolated and characterized in different *Lactobacillus* spp., and used to derive cloning vectors, most of which are RC-based (Wang & Lee, 1997; Shareck et al., 2004). To our knowledge, only two theta plasmids from *Lb. plantarum* have been used to construct cloning vectors, plasmids p256 and pWCFS103 (Sorvig et al., 2005; van Kranenburg et al., 2005).

In this research, *Lb. plantarum* L42 was found to harbour two plasmids of estimated sizes 5.5 kb (pLJ42) and >10 kb (pLF42). The sequence and genetic organization of pLJ42 (5,529 bp) were determined and revealed the plasmid is composed of two modules, a replicon and a mobilization operon. Sequence analysis showed pLJ42 may replicate by theta mechanism. A cloning vector based on the plasmid replicon which uses the green fluorescent protein (*gfp*) gene as a reporter gene and erythromycin resistance (Em^r) as a selection marker was constructed. Its heterologous gene expression level was studied in various hosts.

2.3 Material and methods

2.3.1 Bacterial strains, plasmids and growth conditions

Lactobacillus strains were provided by the Centre de recherche et développement sur les aliments (Saint-Hyacinthe, Qué.) or originated from a lab collection (Dr. B. Lee, Macdonald Campus, McGill University) and are listed in Table 2.1. Lactobacilli were grown aerobically and statically in MRS broth (Oxoid, Nepean, ON; de Man et al., 1960) at 37°C. When cells were grown for the purpose of isolating plasmid DNA, 20 mM L-threonine (Aldrich Chemical Company, Milwaukee, WI) were added to MRS broth to hinder the formation of strong cell walls and facilitate cell lysis. For the making of

lactobacilli electro-competent cells, MRS broth was supplemented with 1% glycine (Bio-Rad, Mississauga, ON).

Escherichia coli TOP10 (Invitrogen, Burlington, ON) was used as a host for the propagation of recombinant plasmids and sequencing templates. Cells were grown in 2X YT broth (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl) (w/v) at 37°C and aerated by vigorous shaking.

All media were solidified by adding 1.5% bacto agar (Difco) and used as agar plates. When appropriate, ampicillin (Sigma, Oakville, ON) and kanamycin (Invitrogen) were used at final concentrations of 100 and 50 μg/ml, respectively for *E. coli*. Erythromycin (Sigma) was used at a final concentration of 250 and 10 μg/ml for *E. coli* and *Lactobacillus* spp., respectively.

All plasmids used in this study are listed in Table 2.1.

2.3.2 DNA manipulation

Plasmid DNA was isolated from *Lactobacillus* spp. by using a modified version of the method of O'Sullivan & Klaenhammer (1993). Bacterial cells (40 ml, 0.1% inoculum) were grown (16-17 h) in the presence of 20 mM L-threonine to weaken the cell wall. Cells were washed with TES buffer (30 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, pH 8.0), resuspended in solution I (25% sucrose, 50 mM Tris-HCl, 1 mM EDTA, 30 mg/ml lysozyme), and transferred to Eppendorf tubes (eppi) in volumes of 250 μl. The mixture was incubated at 37°C for 1 h. To lyse the cells, one volume of lysis buffer (200 mM NaOH, 1% SDS (w/v)) (QIAGEN, Mississauga, ON) was added and the eppi was gently inverted. The lysate was allowed to clear for 7 min at 37°C and neutralized by adding 1.5 volume of neutralization buffer (3 M potassium acetate, pH 5.5) (QIAGEN). The eppi was inverted gently and centrifuged for 12 min at 15,000 *g* and 4°C.

DNA was precipitated from the supernatant by adding one volume ice-cold isopropanol (-20°C) and collected by centrifugation (15,000 *g*, 20 min, 4°C). The pellet was dried in a speed-vacuum and resuspended in 320 μl sterile distilled water. 200 μl of 7.5 M ammonium acetate were added and the mixture was extracted with 350 μl

phenol/chloroform/isoamyl alcohol (25:24:1) (15,000 *g*, 5 min, 25°C or RT). DNA in the aqueous upper phase was precipitated with 1 ml ethanol (-20°C) (15,000 *g*, 20 min, 4°C). The pellet was dried in a speed-vacuum and resuspended in 30 µl sterile distilled water and treated with 0.1 mg/ml RNase for 30 min at 37°C.

Plasmid DNA was also recovered from neutralized cellular lysates using QIAprep Spin Miniprep columns (QIAGEN) according to the instructions provided by the manufacturer. For purification of plasmid DNA from *E. coli* cells, the QIAprep Spin Miniprep Kit was used following the manufacturer's instructions.

Restriction endonuclease digestions were performed according to the supplier's directions (NEB, Mississauga, ON). Total plasmid DNA and digested DNA were analyzed in 0.7% (w/v) agarose gels in 1X TAE buffer (40 mM Tris-acetate/1 mM EDTA). Gels were stained in a solution of ethidium bromide (0.5 µg/ml) for one minute and destained in a solution of 1 mM $MgCl_2$ (15 min to 6 h). DNA was detected upon exposure to UV light. Selected plasmids and digested DNA fragments were excised and purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN). Ligations were performed using T4 DNA ligase (NEB) according to the manufacturer's directions.

Chemically competent cells were prepared by growing *E. coli* cells to $OD_{600} \approx$ 0.5-0.8. The cells were put on ice for 10 min and centrifuged (3,200 *g*, 10 min, 4°C). The pellet was resuspended in ice-cold $CaCl_2$ (0.1 M) and left on ice for 30 min. Cells were centrifuged (3,200 *g*, 10 min, 4°C) and the pellet was dissolved in $CaCl_2$ (100 mM). Competent cells were kept on ice before being transformed.

10 µl of ligation mixture were added to 100 µl of chemically competent *E. coli* cells and iced for 30 min. Cells were transformed by a 1 min heat-shock at 42°C and iced for 1 min. 800 µl of 2X YT were added, and transformed cells were incubated for 1 h at 37°C with shaking, after which they were plated on 2X YT agar supplemented with 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Sigma). Positive selection of recombinant molecules was effected using blue/white screening of colonies.

Electro-competent cells of *Lactobacillus* spp. were prepared following the method of Aukrust & Blom (1992) with slight modifications. Briefly, cells were grown in MRS broth with 1% glycine and harvested at $OD_{600} \approx$ 0.4-0.6. The pellet was washed with an equal volume of ice-cold washing solution (1 mM $MgCl_2$), then with the electroporation

solution, 30% PEG 1500 (Aldrich Chemical Company, Milwaukee, WI). Cells were resuspended to one hundredth of initial volume in 30% PEG 1500 and kept on ice. 12 µl of DNA solution (0.5 µg plasmid DNA) were added to 100 µl competent cells, mixed gently and iced for 3 min. The mixture was transferred to a prechilled electroporation cuvette (0.2 cm) (Bio-Rad, Mississauga, ON) and electroporated at 2.0 kV, 25 µF and 400 Ω (Bio-Rad Gene Pulser). After electroporation, cells were diluted 10 times in MRS and incubated for 2-3 h at 37°C with shaking. Cells (100 µl) were plated on MRS agar containing the appropriate antibiotic.

Total DNA of L42 was obtained following the te Riele et al. (1986) protocol with modifications. Cells were grown to $OD_{600} \approx 0.8$-1.0 (4-5 ml MRS). Prior to harvesting, cells were divided into two samples and one was incubated for 1 h with rifampicin (100 µg/ml) (Sigma). Rifampicin is an inhibitor of RNA polymerase. Its addition inhibits the conversion of single-stranded DNA (ssDNA) to double-stranded DNA (dsDNA) and ensures the presence of ssDNA. Cells were harvested (3,200 *g*, 12 min, 4°C) and washed with EDTA (0.1 M, pH 8.0)/NaCl (0.15 M). Pelleted cells were resuspended in EDTA (0.01 M, pH 6.9)/NaCl (0.15 M). Lysozyme was added (30 mg/ml) and the mixture was incubated for 1 h at 37°C. Cell lysis was completed by the addition of Sarkosyl (Sigma) to a final concentration of 1%. The cell lysate was heated at 65°C for 20 min and extracted with phenol/chloroform (1:1). Finally, the lysate was incubated for 20 min at 37°C in the presence of 50 µg/ml RNase. If samples were not used immediately, they were stored at -20°C.

2.3.3 DNA sequencing and sequence analysis

For sequencing purposes, the largest fragment of *Kpn*I-digested pLJ42 (≈ 5 kb) was subcloned into pUC19 (Yanisch-Perron et al., 1985). The LI-COR-DNA Sequencer 4200 (LI-COR, Lincoln, Nebraska, USA) in conjunction with the dideoxynucleotide (ddNTP) termination procedure were used to carry out sequencing. Universal forward and reverse primers were used to initiate the first sequencing reactions as they hybridized with the plasmid DNA in the pUC19 region. Based on the first sequences, walking primers

were synthesized to sequence pLJ42, 600 base pairs (bp) at a time, until the entire nucleotide sequence had been determined. Overlapping and contiguous sequences were assembled together.

Primers pL42*Kpn*F (5'-CAATTAAAACAGCAACTAGC-3') and pL42*Kpn*R (5'-TAGAGAGAAGAATGGGAGCA-3') were designed to amplify the missing *Kpn*I fragment (≈ 400 bp). The polymerase chain reaction (PCR) was performed using HotStarTaq Master Mix® (QIAGEN) and a GeneAmp® PCR System 2700 (Applied Biosystems, Perkin Elmer), and the conditions were 15 min at 95°C, followed by 30 cycles of amplification (95°C for 1 min, 54°C for 1 min and 72°C for 1 min) and by stabilization at 72°C for 7 min. The amplified fragment was cloned into pCR®2.1-TOPO® (Invitrogen). Universal primers were used for sequencing.

To identify open-reading frames (Orfs), repeats and dyad symmetries, the software Clone Manager Suite 7 (Scientific and Educational Software, Durham, NC) was used. Ribosome-binding sites (RBS) for putative Orfs were identified manually. Promoter sequences were assessed using the software available at the web site softberry.com. Homology searches were done using Basic Local Alignment Search Tool (Altschul et al., 1997) at the NCBI web site (www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignments were performed using the CLUSTAL-W program (Thompson et al., 1994) at the EBI site (www.ebi.ac.uk) after retrieving sequences from GenBank. Secondary structures were predicted using the neural network algorithm PHD (Rost et al., 1994) at the Pôle BioInformatique Lyonnais website (npsa-pbil.ibcp.fr). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.0 (Kumar et al., 2004).

Species identity was confirmed by amplifying a highly conserved region of 16S ribosomal RNA (16S rRNA) using the primers P3LbF (5'-GGGAATCTTCCACAATGG ACG-3') and P4iR (5'-ATGCTTTCGAGCCTCAGCGTC-3'). The PCR conditions were 15 min at 95°C, followed by 30 cycles of amplification (95°C for 1 min, 55°C for 1 min and 72°C for 1 min) and by stabilization at 72°C for 7 min. The fragment was cloned into pCR®2.1 (Invitrogen) and sequenced using universal primers. The nucleotide sequence was submitted to a homology search using BLAST.

2.3.4 *Plasmid constructions*

The plasmids used and constructed in this study are listed in Table 2.1. $pLJ42_s$, an *E. coli-Lactobacillus* shuttle vector, was constructed by amplifying the replicon region of pLJ42 (1561 bp) using the primers L42F 5'-TAGCGAGTGAACGAAGTGAACGCTG-3' and L42R 5'-CTAACATCTTTCAAGTCTTCGATT-3', the PCR conditions set to 15 min at 95°C, followed by 30 cycles of amplification (95°C for 1 min, 54°C for 2 min and 72°C for 1 min) and by final stabilization at 72°C for 7 min. The 1.5 kb fragment was ligated into the multiple-cloning site (MCS) of pCR®2.1-TOPO®. An erythromycin resistance cassette (Em^r), originating from pJIM2278, was amplified with the primers EmR(F) 5'-CTCTAGATTAAGAAGGAGTGATTACATGAACAA-3' and EmR(R) 5'-GTCTAGATTATTTCCTCCCGTTAAATAATAGATAAC-3', which introduced two *Xba*I sites. The cassette was ligated into the unique *Xba*I site of $pLJ42_s$, yielding an Em^r shuttle vector ($pLJ42_s$-Em^r). The *pepN* promoter from *Lb. rhamnosus* pUPN54 was amplified with the primers ProF 5'-CGCGGCCGCACATTGGCTGCTGGCGTT CTGTTG-3' and ProR 5'-CGCGGCCGCGCACCAATTCCTCCTTGTGTGTGAGCG-3', adding two *Not*I restriction sites into the original promoter sequence. The fragment was inserted into the unique *Not*I site of $pLJ42_s$-Em^r, resulting in $pLJ42_s$-P-Em^r. The *gfp* gene was amplified with primers *Not*I-*gfp* (5'-CGCGGCCGCATGGCTAGCAA AGGAGAAGAAC-3') and *Xba*I-*gfp* (5'-CTCTAGATCAGTTGTACAGTTCAT CCATG-3'), introducing *Not*I and *Xba*I at both extremities of the gene. *gfp* was inserted downstream of the promoter, resulting in $pLJ42_v$ ($pLJ42_s$-P-*gfp*-Em^r, 7.5 kb).

All amplified fragments were first cloned into pCR®2.1 and recombinant plasmids were propagated in *E. coli*. Recombinant plasmids were isolated from positive clones and fragments were cleaved out of pCR®2.1 with the appropriate restriction enzymes, after which they were ready for ligation into $pLJ42_s$.

2.3.5 *Transformation and expression of gfp*

Lactobacillus plantarum strains L21, L42, ATCC 14431, ATCC 14917 electro-competent cells were transformed following the method described by Aukrust & Blom (1992) and plated on MRS plates containing erythromycin (10 μg/ml). Positive clones were confirmed based on growth in presence of erythromycin and expression of *gfp* as seen upon exposure to fluorescent light using a fluorescence microscope (Laborlux-S, Leitz).

2.3.6 *Southern hybridization*

Southern hybridization of ssDNA intermediates was carried out according to the method of te Riele et al. (1986). Total DNA samples were adjusted to 30 mM sodium acetate, pH 4.6/0.5 M $ZnCl_2$/250 mM NaCl and digested with or without nuclease S1 (8000 U/ml) (Sigma) for 30 min at 37°C. Samples of nuclease S1-treated and untreated pLJ42 DNA were separated on a 0.7% agarose gel. To assess the quality of the DNA, the gel was stained briefly in ethidium bromide (0.5 μg/ml) and photographed under UV illumination. The DNA in the gel was depurinated (250 mM HCl, 10-20 min), denatured (0.5 M NaOH/1.5 M NaCl, 30 min) and neutralized (0.5 M Tris-HCl, pH 7.5/1.5 M NaCl, 30 min). The gel was equilibrated in 20X SSC buffer (3 M NaCl, 300 mM sodium citrate, pH 7.0, 10 min) and the DNA was transferred by capillary transfer to a positively charged nylon membrane (Boehringer-Mannheim, Mannheim, Germany). The DNA was fixed to the blot by soaking the membrane in 2X SSC buffer (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) and exposing it to UV light for 3 min. The membrane was also baked for 2 h at 80°C. Labelling and detection of nucleic acids were performed with the DIG High Prime DNA Labelling and Detection Starter Kit 1 (Roche, Laval, Qué.), using the DIG-labelled pLJ42 replicon as a probe. Overnight hybridization was performed in a Wheaton rolling bottle at 42°C. Before detection, the blot was washed twice in low-stringency buffer (2X SSC, 0.1% SDS) followed by two washes in high-stringency buffer (0.5X SSC, 0.1% SDS). The blot was subjected to immunological detection using anti-digoxigenin antibody

conjugated to alkaline phosphatase followed by CDP-*Star*, ready-to-use (Roche, Laval, Qué.). CDP-*Star* ready-to-use is an ultra-sensitive chloro-substituted 1,2-dioxetane chemiluminescent substrate for alkaline phosphatase which exhibits extremely rapid light signal generation. The visible light signal was recorded on X-ray film (Kodak X-OMAT AR Film).

2.4 Results

2.4.1 DNA sequence and general organization of pLJ42

A total of 32 strains of *Lactobacillus* spp. were screened for plasmids (Table 2.2). On agarose gel, *Lb. plantarum* strain L42 presented two plasmids of estimated sizes 5.5 kb (pLJ42) and >10 kb (pLF42) (Fig. 2.1). pLJ42 was chosen for further work.

In preparation for sequence determination, pLJ42 was cleaved using *Kpn*I restriction enzyme, resulting in two fragments. The larger fragment was subcloned into pUC19. The recombinant plasmid was sequenced using universal forward and reverse primers. From each partial sequence, primers were designed in order to walk around the entire plasmid, until an overlap was reached. The contiguous and overlapping sequences were assembled together. From this sequence, primers were designed to amplify the remaining 400-bp *Kpn*I fragment, which was cloned into pCR®2.1-TOPO® and sequenced. The sequence was added to the partial pLJ42 sequence.

The complete nucleotide sequence of pLJ42 was determined. The plasmid consisted of 5,529 bp (Fig. 2.2). The G + C content of pLJ42 (42.4 mol%) was consistent with that reported for *Lb. plantarum* plasmid pLKS (42.7 mol%) (Eguchi et al., 2000) and for the chromosome of *Lb. plantarum* strain WCFS1 (44.5 mol%) (Kleerebezem et al., 2003). Upon computer analysis (Clone Manager Suite 7), a total of seven putative open-reading frames (Orf) preceded by ribosome-binding sites (RBS) and encoding polypeptides of more than 60 amino acids were identified (Fig. 2.2). Coding sequences accounted for 77.5% of the total pLJ42 nucleotide sequence. Five Orfs were oriented in the same direction, while Orf4 and Orf8 were oriented in the opposite direction. Deduced

products of Orfs were submitted to homology searches (www.ncbi.nlm.nih.gov/BLAST/), and five showed high degrees of similarity to proteins with putative or known functions (Table 2.3).

Based on homology results, pLJ42 appeared to be composed of two different regions. One region spanned nucleotides (nt) 1261-2579 and contained Orf1 which encoded a gene for replication (*repA*). Structural elements characteristic of the origin of replication (*ori*) of theta plasmids were identified upstream of *repA*. It was assumed this region constituted the pLJ42 replicon (*ori*, *repA*), shown in Fig. 2.3. Another region (nt 2750-5521) contained a cluster of genes (*mobA*, *mobB*, *mobC*) that may be involved in plasmid mobilization. Specific sequences typically found in the origin of transfer (*oriT*) of mobilization regions were identified upstream of *mobC* (Furaya & Komano, 1997). This confirmed the hypothesis that the region constituted a mobilization module. Both regions will be discussed in detail in the next sections.

2.4.2 Replication features of pLJ42 deduced from sequence analysis

The region encompassing Orf1 and its flanking sequences (nt 1261-2579) was found to have a G + C content (40.3 mol%) similar to that of the entire plasmid (42.4 mol%). The ATG start codon of Orf1 (nt 1486-2412) was preceded by a RBS (GGAG) and by a putative promoter region. The potential –10 promoter box (5'-TATAAT-3'; coordinates 1437-1443) was identical to the prokaryotic consensus 5'TATAAT, while the –35 promoter sequence (5'-TTGACTA-3'; coordinates 1456-1461) was very similar to the –35 consensus sequence 5'TTGACA (Rosenberg & Court, 1979). A rho-independent stem-loop terminator was identified 106 bp downstream of Orf1 (nt 2515-2579) with a ΔG of –7 kcals/mol. It was followed by a stretch of A residues, a typical feature of stem-loop terminators which are generally found downstream of Orfs. These structures signal to DNA polymerase the termination of RNA synthesis, ending gene transcription and protein synthesis (Rosenberg & Court, 1979).

The gene product of Orf1 had a size of 308 amino acids and showed conservation of the pfam01051 domain of Rep_3 initiator replication proteins, a family that includes RepA, a protein that can bind to DNA repeats that flank *repA* (Marchler-Bauer et al., 2005). The putative polypeptide encoded by Orf1 also showed significant homology to replication proteins of *Lb. plantarum* plasmids pLKS and pMD5057 (84% and 68%, respectively), *T. halophilus* plasmid pUCL287 (69%) and *Lb. sakei* plasmid pRV500 (67%) (Eguchi et al., 2000; Danielsen, 2002; Benachour et al., 1997; Alpert et al., 2003). These plasmids belong to the pUCL287 family of theta-replicating plasmids (Benachour et al., 1997). Based on these homologies, Orf1 was assumed to encode a replication protein, RepA.

pLJ42 RepA was aligned with homologous replication proteins and with Rep proteins of class A theta plasmids (Fig. 2.4). While a central region was well conserved among all Rep proteins, the N-terminal region was only conserved among plasmids of the pUCL287 subfamily. More importantly, pLJ42 RepA could be included in that subfamily. Secondary structures (α-helices and β-strands) were predicted using the neural network algorithm PHD on the output of CLUSTAL-W at the Pôle BioInformatique Lyonnais website (npsa-pbil.ibcp.fr) (Fig. 2.5). Two motifs characteristic of Rep proteins were found, the leucine-zipper (LZ) and the α-helix-turn-α-helix (HTH) motifs, at the N-terminal and C-terminal, respectively, and may be involved in protein-protein interaction and DNA-protein binding, respectively (del Solar et al., 1998).

The non-coding region upstream of *repA* (nt 1261-1477) contained an AT-rich sequence (72.4 mol%) consisting of 11-bp direct repeats (DR) (5'-CCTCTTTTAAA-3') repeated four times, coordinates 1261-1307. Further downstream, 22-bp sequences were tandemly iterated four and a half times, coordinates 1336-1444. These sequences are known as iterons, and constitute the Rep binding site. The pLJ42 iterons were adjacent to the *repA* promoter, the last one overlapping the –35 motif. A stretch of 37 bp containing GC clusters separated the DRs from the iterons, an arrangement reported in other plasmids (Benachour et al., 1997).

Long DRs and iterons, repeated four and four and a half times, respectively, are structural elements typically found in *ori* of theta plasmids (Alpert et al., 2003). Based on the presence of such structures in pLJ42, it was assumed that the untranslated region

upstream of *repA* constituted the plasmid *ori*. In a plasmid, *ori* is the region where plasmid replication initiates, after double-stranded DNA is nicked by RepA (del Solar et al., 1998).

The 11-bp DRs were almost identical to the ones reported for pUCL287 and pRV500, differing only by one nucleotide (Fig. 2.6). As for the iterons, they presented a stretch of conserved nucleotides, obtained by the juxtaposition of repeated units (TTGTCTGTTTAT), which was also found in other plasmids, including pUCL287, pMD5057, pLKS and pRV500 (Benachour et al., 1997; Danielsen, 2002; Eguchi et al., 2000; Alpert et al., 2003;).

Upon alignment, pLJ42 *ori* (nt 1261-1477) showed 73% identity to pUCL287 *ori* (nt 2062-2297), 66% identity to pLKS *ori* (nt 543-781) and 64% identity to pRV500 *ori* (nt 472-681). High homology among these *ori* sequences supports the conservation of *ori* among members of the pUCL287 family of theta replicons.

Based on the presence of a gene encoding a replication protein preceded by its target site *ori*, the region spanning nt 1261-2579 was assumed to constitute the pLJ42 replicon, a region essential for plasmid replication.

2.4.3 Mobilization module of pLJ42

A region in pLJ42 encompassing nt 2750 to 5521 had a G + C composition (43.7 mol%) consistent with that of the total plasmid (42.4 mol%). Three putative Orfs were identified, encoding polypeptides of 112 amino acids (aa), 445 aa and 229 aa, respectively. While Orf5 (nt 3428 to 4765) overlapped Orf3 (nt 3111 to 3449) by 21 nt, the start codon of Orf7 (nt 4762 to 5451) overlapped the stop codon of Orf5 by two nucleotides (TG).

A RBS was located 8 bp upstream of Orf3 (position 3098). It was preceded by a putative –10 promoter region 56 bp upstream (5'-GTTTATACT-3'), which was separated by 11 bp from a putative –35 promoter region (5'-GTGACC-3') (P_1). Two other RBS sequences were located 13 bp and 9 bp upstream of Orf5 and Orf7, respectively. While a potential promoter region (P_2) exhibiting homology to the consensus –10 and –35

sequences was present 11 bp upstream of the Orf5 RBS, the only promoter region (P_3) that could be identified for Orf7 was located 206 bp upstream of its RBS.

Hairpin structures were identified downstream of all three Orfs, with ΔG (kcals/mol) of -6.9, -3.6 and -13, respectively. The last hairpin structure was assumed to be the rho-independent transcription terminator. Located 24 bp downstream of the Orf7 stop codon (nt 5476 to nt 5521), two 14-bp perfect inverted repeats (IR) had the potential of forming a stem-loop structure with a high ΔG (-13 kcals/mol). The presence of one transcription terminator downstream of the gene cluster suggests that all three genes may be transcribed simultaneously.

To associate a function to the three Orfs, their gene products were compared to protein sequences in the database. Orf5 was found to encode the largest polypeptide of pLJ42, a 445 amino acid protein with a molecular weight (MW) of 51 kDa that presented significant homology with MobA proteins from *L. lactis* plasmids pCI528 (47%) and pNZ4000 (47%) (Lucey et al., 1993; van Kranenburg & de Vos, 1998), and moderate homology with a mobilization protein from *Pediococcus* (*P.*) *pentosaceus* plasmid pMD136 (30%) (Giacomini et al., 2000) and with relaxase proteins from *Staphylococcus* (*St.*) *aureus* plasmids pC223 (27%) and pC221 (27%) (Accession no. CAA31314 and CAA26106). Moreover, the N-terminal of the protein (residues 9-280) showed conservation of the relaxase/mobilization nuclease domain pfam03432 (Marchler-Bauer et al., 2005).

Multiple sequence alignment of the Orf5 protein with homologous MobA and *rlx* proteins showed the conservation of three motifs characteristic of MobA proteins: the Tyr and His residues involved in cleaving-joining reaction (Motifs I and III), and the Ser residue involved in DNA binding (Motif II) (Fig. 2.7a). These motifs form part of the catalytic centre of MobA (Francia et al., 2004). Based on homology with other MobA proteins and conservation of the MobA motifs, Orf5 was deemed to encode MobA, a relaxase involved in bacterial conjugation that nicks *oriT* at its *nic* site.

Upstream of and overlapping Orf5, Orf3 encoded a protein of 112 amino acids with a MW of 13 kDa. It showed significant homology to the gene products of pCI528 Orf1(47%) and pMD136 Orf1 (34%) (Lucey et al., 1993; Giacomini et al., 2000) in addition to being homologous to various MobC-like proteins from staphylococcal

plasmids pSERP, pSE-12228-02 and pC223, GenBank Accession no. AAW52769, NP 863254, NP 943088, respectively. Moreover, the C-terminal of the protein (residues 64-111) showed conservation of the pfam05713 MobC domain, thus prompting the re-naming of Orf3 to *mobC*. MobC-like proteins are relaxases involved in conjugation and are thought to bind to *oriT* and assist MobA in its site- and strand-specific cleavage of DNA (Marchler-Bauer et al., 2005).

Located directly downstream of Orf5 (*mobA*), Orf7 encoded a putative polypeptide of 229 amino acids and MW of 26 kDa that showed 33% identity to pNZ4000 MobB (van Kranenburg et al., 2000). Despite the fact that no conserved domain could be detected, Orf7 was still deemed to encode MobB.

The presence of *mob* genes suggests pLJ42 may be mobilizable. The mobilization region of a plasmid typically contains a *cis*-acting *oriT* region and a *trans*-acting gene encoding a relaxase that can recognize the nick site in *oriT*, initiating DNA transfer in conjugation (Furaya & Komano, 1997). Upon analysis, the non-coding region upstream of Orf3 (nt 2750-3111) was found to harbour three large IRs that could potentially form stem-loop structures with high ΔG (kcals/mol) of -8.3, -6.6 and –8.8, in order of position. One large (11-bp) DR was identified at position 2855 and repeated at position 2949. These structural elements are characteristic of the *oriT* in mobilization modules, a short region (100-300 bp) which contains several IRs with a high ΔG and one significant DR (Lucey et al., 1993).

No significant results were obtained from a homology search using the nucleotide sequence upstream of Orf3 as query (www.ncbi.nlm.nih.gov/BLAST/). However, an imperfect IR (nt 2936-2976) was found to be homologous to *oriT1* and *oriT2* of pNZ4000 (van Kranenburg & de Vos, 1998) and *oriT* of pCI528 (Lucey et al., 1993). Alignment of these sequences with the *oriT* of IncI1 plasmid R64 showed conservation of a region encompassing the right arm of the imperfect IR, which corresponds to the binding site of the R64 mobilization protein NikA (Furaya & Komano, 1997) (Fig. 2.7b). As reported for other plasmids, the imperfect IR was centered around the nucleotide sequence 5'-GAA-3' (Lucey et al., 1993; Wang & Macrina, 1995; van Kranenburg & de Vos, 1998). A few nucleotides downstream of the IR, the nucleotide hexamer 5'-CTTGCA-3' proposed by Francia et al. (2004) as the *oriT nic* site was also conserved. Based solely on the presence

of specific structures homologous to *oriT* structures, the non-coding region upstream of Orf3 was assumed to be the *oriT* of pLJ42.

Based on (i) the conservation of pfam03432 relaxase/mobilization and pfam05713 MobC domains, (ii) the homologies of Orf3, Orf5 and Orf7 with MobC, MobA and MobB proteins, respectively and (iii) the presence of an *oriT* upstream of the gene cluster, the region spanning nt 2750-5521 was deemed to constitute the mobilization module of pLJ42. The presence of mobilization genes (*mob*) encoding specific relaxases and an origin of transfer (*oriT*), the site where *mob* genes bind and nick plasmid DNA, suggests pLJ42 is mobilizable. While these plasmids can prepare their DNA for transfer, they are not self-transmissible and require additional conjugative functions, unlike conjugative plasmids that can form effective cell-to-cell contact and are self-transmissible (Furaya & Komano, 1997).

2.4.4 Description of other putative open reading-frames

Orf2 was located 2 bp downstream of Orf1 (*repA*). A RBS (AAGGG) was identified 11 bp from the ATG start codon, but no promoter sequence could be located using the promoter finding software available at softberry.com. The putative polypeptide of 109 amino acids showed no significant homology to any protein sequence in the database and was deemed to have an unknown function. Based on the presence of a stem-loop with a high ΔG (–7 kcals/mol) located 100 bp downstream of Orf2, this DNA fragment may only ensure the proper termination of the transcription of *repA*.

A distance of 32 bp separated the ATG start codon of Orf4 (nt 3511-3320) from its putative RBS (AAGG). A promoter region was identified 45 bp upstream of the RBS (nt 3642-3593). A rho-independent stem-loop terminator (coordinates 3320-3296) with a ΔG of –2.3 kcals/mol was present immediately downstream of Orf4. When compared to sequences in the database, no function could be assigned to the 63 amino acid product of Orf4.

Orf8 (nt 482-6) was preceded by a RBS (AG) and a putative promoter region, -35 motif (5'-GTGTAA-3') and –10 site (5'-TGTAAT-3'), coordinates 546-541 and 524-518,

respectively. A short distance downstream of its stop codon (5529-5476), an IR with the potential of forming a stem-loop with a ΔG of –24 kcals/mol was assumed to be the rho-independent transcription terminator. The gene product of Orf8, a 158 amino acid protein, showed conservation of the RepL domain, spanning residues 19 to 152. RepL is the prototype replication protein for plasmids that belong to the firmicute family of low GC Gram-positive organisms (Marchler-Bauer et al., 2005). pLJ42 RepL showed significant homology to *Selemonas ruminantium* plasmid pSRD191 RepL (55%) and to replication proteins of other plasmids (Table 2.3).

2.4.5 Detection of ssDNA intermediates

No ssDNA intermediates were detected by Southern hybridization (Fig. 2.8). Among the various samples analyzed, there were no differences in the hybridization patterns of the pLJ42 replicon probe. Strong signals were obtained with both plasmids present in L42, indicating pLJ42 and pLF42 have homologous replicons.

2.4.6 Vector construction and host-range of pLJ42

Based on the pLJ42 replicon, a cloning vector ($pLJ42_v$) was constructed, containing the erythromycin resistance (Em^r) gene from pJIM2278 as a selective marker and *gfp* as a reporter gene. The cloning strategy is illustrated in Figure 2.9. To investigate the host-range of the pLJ42 replicon, the constructed plasmid $pLJ42_v$ was transformed to *E. coli* and four strains of *Lactobacillus plantarum*. *E. coli* cells maintained the vector in presence of erythromycin and showed a high level of *gfp* expression (Fig. 2.10 A, B). Based on transformation results, pLJ42 demonstrated a narrow host-range that seemed restricted to *Lb. plantarum* strains L21 and L42. While both strains were able to grow under erythromycin selective pressure (Fig. 2.10 C, E), L42 was the only strain in which *gfp* was expressed. Moreover, the expression level was much lower than that obtained in *E. coli* (Fig. 2.10 D).

2.5 Discussion

The aim of this work was to screen *Lactobacillus* spp. for cryptic plasmids and to isolate and characterize a plasmid. Originally, *Lb. plantarum* L42 was isolated from a commercial dairy product. Comparison of the 16S rRNA sequence confirmed L42 was a *Lb. plantarum* strain (Fig. 2.11). Plasmid isolation revealed the presence of two plasmids, one of which was sequenced and characterized. pLJ42 consisted of 5,529 bp and had a higher G + C content (42.4 mol%) than that commonly found in *Lb. plantarum* plasmids (35-38 mol%) (Skaugen, 1989; Leer et al., 1992; Vujcic & Topisirovic, 1993; Danielsen, 2002; Daming et al., 2003; de las Rivas et al., 2004; Sorvig et al., 2005). However, its GC composition was almost identical to that of *Lb. plantarum* pLKS (42.7 mol%) (Eguchi et al., 2000) and similar to that reported for the chromosome of *Lb. plantarum* strain WCSF1 (44.5 mol%) (Kleerebezem et al., 2003), suggesting pLJ42 is a *Lb. plantarum* plasmid, and has not been acquired by horizontal transfer.

Seven Orfs were identified within the pLJ42 sequence, each containing a start codon preceded by a potential RBS. Based on homology search results, functions were associated to the putative polypeptides encoded by five of the Orfs. pLJ42 was shown to be composed of two important regions, a replicon and a mobilization module. The replication region contained a gene for replication *repA* preceded by a non-coding sequence, the origin of replication *ori*. The mobilization module consisted of a cluster of *mob* genes preceded by the origin of transfer *oriT*. Another Orf was found to encode *repL*, a gene for replication in plasmids originating from low GC Gram-positive organisms.

The pLJ42 replicon contained all the structural and genetic elements essential for plasmid replication, namely *repA* encoding a replication initiator protein and *ori*, the region where DNA strands are separated, initiating the plasmid replication process. In *ori*, 11-bp direct repeats were repeated four times and were followed by 22-bp sequences (iterons), repeated four and a half times. The AT-rich region containing the 11-bp DRs is the area where opening of DNA strands and assembly of host factors occur, and the iterons function as binding sites for the replication protein (del Solar et al., 1998).

Novel replicons can be classified based on comparison of the amino acid sequence of their replication proteins. Rep proteins are encoded by most plasmids and share common functions (del Solar et al., 1998). In depth sequence analysis revealed that pLJ42 replicated by theta mechanism and belonged to the pUCL287 family, itself related to class A theta-type plasmids of the pUCL22 and pSC101 families. Rep proteins of the pUCL287 family, which showed significant homology with the pLJ42 Rep, were included in an alignment of class A Rep proteins. While a central region was conserved among all Rep proteins, a N-terminal region was highly conserved only inside the pUCL287 subfamily, as Alpert et al. (2003) had reported.

Two motifs common to Rep proteins were also located, the leucine-zipper (LZ) and α-helix-turn-α-helix (HTH) motifs. At the N-terminal, hydrophobic heptad residues characterize the LZ motif. Leucine residues drive the dimerization of Rep proteins by interacting hydrophobically to form tight dimer structures. The LZ motif is thought to be a protein-protein interface that regulates the equilibrium between monomers and dimers of RepA (del Solar et al., 1998).

Rep monomers bind to the iterons of the origin, initiating plasmid replication, while dimers of the protein bind to the promoter region, repressing transcription of *repA*. The HTH motif of Rep is involved in these two processes. Generally located at the C-terminal end of Rep proteins, HTH is composed of two α-helices: one is involved in recognition, while the other is a stabilization helix. HTH plays a role in binding to specific regulatory DNA regions. The HTH motif of Rep monomers can bind to the 3' region of the iterons, involving the motif in plasmid replication. RepA dimers also bind to the promoter region via the HTH motif, thereby exerting control over the synthesis of RepA (del Solar et al., 1998).

Autoregulation implies the promoter region contains a sequence that is homologous to the iterons recognized by Rep. As previously reported, the last iteron of pLJ42 overlapped the –35 promoter region, and can be recognized by the Rep dimer in the autoregulation process (Hayes et al., 1991; Benachour et al., 1997).

Multiple alignment of the nucleotide sequences of *ori* regions of pUCL287-type plasmids showed significant conservation of 11-bp DR and iterons. The pLJ42 11-bp DRs were almost identical to the ones reported for pUCL287 and pRV500. While the

sequences of the different iterons present in *ori* of pLJ42 were not identical, they did adjust to a consensus motif (TTGTCTGTTTAT) that was obtained by the juxtaposition of repeated units. This motif was also found in pUCL287-type plasmids, including pUCL287, pLKS, pMD5057, pRV500, (Benachour et al., 1997; Eguchi et al., 2000; Danielsen, 2002; Alpert et al., 2003). Significant homology among structural elements present in replication origins (DR and iterons) of pUCL287-type plasmids supports the conservation of the *ori* sequence among members of this subfamily of theta plasmids. This is not surprising, considering homologous Rep proteins should recognize homologous sequences in *ori.*

In addition to homologies of pLJ42 *ori* and Rep with pUCL287 theta-type elements, two other findings suggested pLJ42 replicated via the theta mechanism. First, a double-stranded origin of replication (*dso*), a structure typically found upstream of *repA* in rolling-circle (RC) plasmids, could not be identified. Second, single-stranded DNA replication intermediates, the hallmark of RC replication, were not detected by Southern hybridization. These findings confirmed pLJ42 followed theta replication and indicated it may be a new member of the pUCL287 subfamily of class A theta plasmids.

To confirm the relationship between pLJ42 and other pUCL287-type plasmids, a phylogenetic tree was constructed using the amino acid sequences of homologous Rep proteins, shown in Figure 2.12 A. As previously observed, the pLKS Rep protein was the most similar to the pLJ42 Rep, indicating both plasmids have a common ancestor.

While Southern hybridization results did not reveal the presence of ssDNA intermediates, strong signals were obtained with both plasmids present in *Lb. plantarum* L42, indicating pLJ42 and pLF42 possess similar theta replicons. It has been reported that many theta plasmids can be maintained stably in the same cell (Seegers et al., 1994), which supports the apparent compatibility of homologous replicons in strain L42.

Among class A theta plasmids, different subfamilies have been identified according to the degree of similarity of their Rep proteins. However, all plasmids are independent of DNA polymerase I, encode their own replication protein(s) and possess origins of replication, with an AT-rich region generally containing repeats and Rep-binding iterons (del Solar et al., 1998). In these plasmids, replication initiates when the Rep protein monomer binds to the iterons in the origin, forming a nucleoprotein complex.

Rep (a nuclease) cleaves the double-stranded DNA in the AT-rich region. The GC clusters that separate the 11-bp DR and the iterons may act as clamps to limit DNA melting during strand opening to the region implicated in the formation of the replisome (Hayes et al., 1991). Once the parental strands are opened, a primer RNA (pRNA) is synthesized and covalently extended. DNA synthesis is coupled and occurs continuously on the leading strand and discontinuously on the lagging strand. DNA Pol III is required for elongation of plasmid DNA replication (del Solar et al., 1998).

To verify the host-range of the pLJ42 replicon, $pLJ42_v$ was transformed into *E. coli* and various *Lb. plantarum* strains. *E. coli* did maintain the recombinant plasmid under erythromycin pressure and expressed *gfp*, indicating the *Lb. rhamnosus* promoter was functional. However, the replication of $pLJ42_v$ in *E. coli* was driven by the pUC *ori*, not by the *Lb. plantarum ori*.

Of the four *Lb. plantarum* strains tested, $pLJ42_v$ transformed strains L42 and L21, indicating the pLJ42 replicon was strain-specific. It can be speculated that this is due to pLJ42 replicating by theta mechanism, theta replicons being known to have narrow host-ranges (Hayes et al., 1991; Sorvig et al., 2005). *Lb. plantarum* L42 was transformed with $pLJ42_v$ and exhibited a low *gfp* expression level, which may be related to the low copy number of the recombinant plasmid. While the recombinant L21 was able to grow under erythromycin selective pressure, Gfp could not be detected. Recombinant L21 exhibited erythromycin resistance, indicating the Em^r cassette was transcribed. In this event, *gfp* should have been expressed, considering the reporter gene is located upstream of Em^r and under the control of the same promoter. An explanation for this outcome has yet to be found.

Theta plasmids may have limited host-ranges, but several of their characteristics can be exploited in the construction of species-specific cloning vectors. These plasmids replicate by means of double-stranded rather than single-stranded DNA intermediates, which results in better structural and segregational stability, allowing for the insertion of larger foreign DNA fragments (Kiewiet et al., 1993). In addition, theta plasmids have a lower risk of being transferred horizontally to other bacterial species, compared to broad host-range plasmids. This safety aspect is of great importance with regards to the use of live recombinant bacteria in food products or as vaccine carriers (Pouwels & Leer, 1993).

The presence of a mob operon in the plasmid suggested pLJ42 may be a mobilizable plasmid. Such plasmids typically carry a *mob* gene encoding a relaxase and the origin of transfer (*oriT*) (Francia et al., 2004). While mobilizable plasmids encode the genes to process DNA and prepare it for transfer, they require a co-resident conjugative plasmid to supply other essential functions, such as cell-to-cell contact and mating bridge formation (Lucey et al., 1993). Mobilizable plasmids should not be confused with conjugative plasmids, which are self-transmissible (Lanka & Wilkins, 1995).

Bacterial conjugation involves the formation of the relaxosome, a DNA-protein complex formed by *mob* proteins. Mobilization proteins belong to the family of relaxases, and have the capacity to bind to a specific site in the *oriT* region. Conjugation initiates when a relaxase is loaded onto the *oriT* by accessory binding proteins and catalyzes the cleavage of a specific phosphodiester bond at the *nic* site. The relaxase becomes covalently linked to the 5' end of the cleaved DNA strand through a tyrosyl residue. Single-stranded DNA is transferred to the recipient cell and ligated through the cleaving-joining activity of the relaxase, a process which resembles the leading-strand replication by RC plasmids (Lanka & Wilkins, 1995).

The pLJ42 mobilization operon was composed of three genes, *mobA*, *mobB* and *mobC*, preceded by a non-coding sequence. This region presented features that are common to transfer origins, including a high AT content (60.3 mol%), facilitating strand separation, and extensive secondary structures conferred by direct and indirect repeats, which may function as recognition sites for DNA-binding proteins (Lanka & Wilkins, 1995).

pLJ42 MobA showed significant homology to mobilization proteins encoded by plasmids pCI528, pNZ4000 and pMD136 (Lucey et al., 1993; van Kranenburg & de Vos, 1998; Giacomini et al., 2000), all members of the ColE1-superfamily of mobilizable plasmids (Francia et al., 2004). Multiple alignment of ColE1 relaxases with pLJ42 MobA showed the conservation of three motifs which form part of the catalytic centre of the relaxase. Motif I displayed the catalytic Tyr residue which becomes attached to the 5' end of the cleaved DNA after nicking. The Ser residue of Motif II, which plays a role in DNA recognition and binding, was also conserved. Three His residues were present in Motif III, and are thought to be involved in the nucleophilic activity of the Tyr residue in Motif

I by coordination of the required Mg^{2+} ions and direct activation of the active Tyr (Pansegrau et al., 1994).

The 3H motif in Motif III is typical of the ColE1-3H relaxases, proteins that belong to a subfamily of the ColE1-superfamily. Since these 3H relaxases are homologous to TraI of plasmid RP4 (the prototype of conjugative MOB_P relaxases), they were included within the MOB_P family. pLJ42 MobA showed the ColE1-3H signature $Hx(D/E)(x_{4-6})HxH(x_3)n$ in Motif III, and was deemed to belong to the MOB_P relaxases, a subdivision of the ColE1-superfamily of mobilizable plasmids (Francia et al. 2004).

The ColE1-3H-type mobilization regions are thought to differ in terms of number and orientation of constituent genes, in agreement with a wider dispersion in relaxase sequences. The only gene that is always present is the relaxase (Francia et al., 2004). However, the organization of the pLJ42 mobilization operon was found to be very similar to that reported for related plasmids, including, pS194, pC221, pCI528 and pMD136 (Projan et al., 1988; Projan & Archer, 1989; Lucey et al., 1993; Giacomini et al., 2000). In these plasmids, MobA encoding a relaxase was always present, and was preceded by another Orf (*mobC*), which overlapped *mobA* by 21 nt, as reported for pLJ42. For plasmids pLJ42, pMD136 and pNZ4000, a third Orf (*mobB*), followed and overlapped *mobA* (Giacomini et al., 2000; van Kranenburg et al., 2000). All genes were also oriented in the same direction.

The putative *oriT* of pLJ42 showed homology with the MOB_P-type *oriT*, proposed by Francia et al., 2004. The region could be aligned with *oriT1* and *oriT2* of pNZ4000 and *oriT* of pCI528, and presented an imperfect IR homologous to the NikA binding site of plasmid R64. In addition, the nucleotide hexamer 5'-CTTGCA-3' proposed as the *nic* site was also conserved, several nucleotides downstream of the putative binding site.

To investigate the molecular evolution of the pLJ42 mobilization operon, a phylogenetic tree was constructed using the amino acid sequences of homologous MobA proteins. Based on the tree obtained (Fig. 2.12 B), all proteins appeared to have a common ancestor. Moreover, plasmids containing the ColE1-3H motif originated from a variety of genera, including *Lactobacillus*, *Streptococcus*, and *Staphylococcus*, which all belong to the same taxon. Nonetheless, Francia et al. (2004) constructed the phylogenetic

tree of the ColE1-superfamily of relaxases, and the 3H motif was dispersed among a number of bacterial classes, indicating the acquisition of the motif is not recent.

Conjugation in lactobacilli has not been studied as much as it has in *L. lactis*, where it was originally exploited as a food-grade means for the construction of phage-resistant dairy starters for commercial cheese production (Sanders et al., 1986). Several systems based on *L. lactis* conjugative plasmids have been developed to genetically enhance lactococcal starters. A conjugative lactococcal plasmid (pMRC01) was used to construct mobilizable food-grade vectors (Hickey et al., 2001). *L. lactis* pCBG104, a self-transmissible, phage resistance plasmid encoding the bacteriocin lacticin 481 was used to improve the phage resistance of a number of Cheddar starter strains (Mills et al., 2002). Considering that electroporation is not suitable to modify all dairy starters, as this method is not always recognized as being food-grade and some strains are difficult to transform, food-grade conjugation appears to be an interesting alternative for strain improvement.

In addition to the two important regions that constitute pLJ42, Orf8 was shown to encode RepL, a replication protein commonly found in plasmids that originate from low GC Gram-positive organisms. The G + C content of Orf8 (36.9%) was lower than the total GC content of pLJ42, suggesting *Lb. plantarum* may not be the original host of *repL* and may have acquired this replication gene. However, RepL is a replication protein found in the Firmicutes, a bacterial class that includes lactobacilli. Nothing besides the lower GC content of the Orf indicates that *repL* has been acquired by pLJ42. Nonetheless, it can be assumed *repL* does not play an essential role in plasmid replication, as the replicon (*ori* and *repA*) of pLJ42 drove the replication of $pLJ42_v$ when it was introduced in *Lb. plantarum* L42.

In summary, *Lactobacillus plantarum* L42 was shown to possess two plasmids, pLJ42 and pLF42, which have similar replicons. The molecular characterization of pLJ42 revealed it followed theta replication, which may explain the limited host-range demonstrated by $pLJ42_v$. Not many theta-replicating plasmids have been found in *Lb. plantarum*, and $pLJ42_v$ is one of the few lactobacilli cloning vectors based on a theta replicon. Although theta plasmids have a limited host-range, they nonetheless have interesting applications that ought to be developed. Narrow host-range and species-specific cloning vectors could be used for the genetic improvement of industrially used

Lb. plantarum strains, where horizontal transfer is a concern. Ultimately, *Lb. plantarum* strains could be engineered as vaccine carriers, i.e. for the mucosal delivery of antigens exhibiting vaccine properties. Such activities are made possible because cloning vectors derived from LAB plasmids are recognized as food-grade and are deemed safe for human and/or animal consumption.

Table 2.1 Bacterial strains and plasmids used in this study

Strain or plasmid			Characteristics	Source
Strains				
Escherichia coli		TOP10	Type strain, Plasmid-free	Invitrogen
Lactobacillus spp.				
Lb. acidophilus	L1	ATCC 11975		ATCC
Lb. acidophilus	L2	ATCC 33323		ATCC
Lb. acidophilus	L3	ATCC 4357		ATCC
Lb. acidophilus	L4	ATCC 43121		ATCC
Lb. acidophilus	L5	ATCC 53976		ATCC
Lb. acidophilus	L6	ATCC 53546-D		ATCC
Lb. acidophilus	L7	ATCC 53546-A		ATCC
Lb. acidophilus	L11	CH-5		Lab. collection
Lb. acidophilus	L12	145		Lab. collection
Lb. acidophilus	L13	NCFM		Lab. collection
Lb. acidophilus	L14			Lab. collection
Lb. acidophilus	L15			Lab. collection
Lb. acidophilus	L16			Lab. collection
Lb. acidophilus	L17	SNUL		Lab. collection
Lb. acidophilus	L18	R052		Rosell
Lb. acidophilus	L19	LAMA		Lab. collection
Lb. acidophilus	L20	KYOPHILUS		Lab. collection
Lb. plantarum	L8	ATCC 14917		ATCC
Lb. plantarum	L21	R215		Lab. collection
Lb. plantarum	L42		Type strain carrying pLJ42 (5.5 kb)	Lab. collection
Lb. plantarum		ATCC 14431		ATCC
Lb. casei	L9	ATCC 4646		ATCC
Lb. casei	L23	LC10		Lab. collection
Lb. casei	L24	CH-1		Lab. collection
Lb. casei	L25			Lab. collection
Lb. casei	L26			Lab. collection
Lb. casei	L27	YIT9018		Lab. collection
Lb. casei	L29	LLG		Lab. collection
Lb. casei	L41	R202		Rosell
Lb. casei		ATCC 393		ATCC
Lb. casei		ATCC 334		ATCC
Lb. rhamnosus	L32	R049		Rosell
Lb. reuteri	L51			Lab. collection
Lb. bulgaricus	L61			Lab. collection

Plasmids	Characteristics	Source
pUC19	Cloning vector (2.7 kb)	
pCR®2.1-TOPO®	Cloning vector (3.9 kb)	Invitrogen
pCR®2.1	Cloning vector (3.9 kb)	Invitrogen
pL42seq	pUC19 carrying partial pLJ42	This work
pL42m	pLJ42 *Kpn*I missing fragment (400 bp) cloned into pCR®2.1-TOPO®	This work
pJIM2278	Source of Em^r A (4.8 kb)	Lab. collection
pUPN54	Source of promoter	Lab. collection
pLJ42	5.5 kb	This work
$pLJ42_s$	pCR®2.1-TOPO® carrying the 1.5-kb pLJ42 replicon	This work
$pLJ42_s$-Em^r	$pLJ42_s$ carrying the 700-bp *Xba*I-*Xba*I Em^r	This work
$pLJ42_s$-P-Em^r	$pLJ42_s$-Em^r carrying the 500-bp *Not*I-*Not*I *Lb. plantarum* promoter (P)	This work
$pLJ42_v$	$pLJ42_s$-P-Em^r carrying the 700-bp *Not*I-*Xba*I *gfp* gene	This work

ATCC: American Type Culture Collection

Table 2.2 Plasmid screening results

I. D. no.	Species	Strains	Plasmid	Size (kb)
L1	*Lb. acidophilus*	ATCC 11975	-	
L2	*Lb. acidophilus*	ATCC 33323	-	
L3	*Lb. acidophilus*	ATCC 4357	-	
L4	*Lb. acidophilus*	ATCC 43121	+	3.0
L5	*Lb. acidophilus*	ATCC 53976	-	
L6	*Lb. acidophilus*	ATCC 53546-D	-	
L7	*Lb. acidophilus*	ATCC 53546-A	-	
L11	*Lb. acidophilus*	CH-5	-	
L12	*Lb. acidophilus*	145	-	
L13	*Lb. acidophilus*	NCFM	-	
L14	*Lb. acidophilus*	YOGURT	-	
L15	*Lb. acidophilus*	YOGURT	-	
L16	*Lb. acidophilus*	YOGURT	-	
L17	*Lb. acidophilus*	SNUL	-	
L18	*Lb. acidophilus*	R052	-	
L19	*Lb. acidophilus*	LAMA	-	
L20	*Lb. acidophilus*	KYOPHILUS	-	
L8	*Lb. plantarum*	ATCC 14917	-	
L42	*Lb. plantarum*	YOGURT	+	5.5, >10
L21	*Lb. plantarum*	R215	+	1.8, 6, >10
L9	*Lb. casei*	ATCC 4646	-	
L23	*Lb. casei*	LC10	+	>10
L24	*Lb. casei*	CH-1	-	
L25	*Lb. casei*	YOGURT	-	
L26	*Lb. casei*	YOGURT	-	
L27	*Lb. casei*	YIT9018	+	3.5, 7, >10
L29	*Lb. casei*	LLG	-	
L41	*Lb. casei*	R202	-	
L32	*Lb. rhamnosus*	R049	-	
L51	*Lb. reuteri*	YOGURT	-	
L61	*Lb. bulgaricus*	YOGURT	-	

Table 2.3 General features of the Orfs in plasmid pLJ42 and the putative functions of their gene products

Orf	Position	% G+C	Size (aa)	MW (kDa)	Putative RBS	Organism and plasmid with protein database similarity	%Identity/ Positives	Accession no.
Orf1 (*repA*)	1486-2412	43.4	308	36	GGAG	Plasmid pLKS, *Lactobacillus plantarum*, RepA (305)	84/91	BAA87064
						Plasmid pUCL287, *Tetragenococcus halophilus*, RepA287 (311)	69/83	CAA53278
						Plasmid pMD5057, *Lactobacillus plantarum*, RepA (311)	68/82	AAN40880
						Plasmid pRV500, *Lactobacillus sakei*, RepA (311)	67/81	AAN61991
						Plasmid pSMB74, *Pediococcus acidilactici*, RepB (319)	67/81	AAP55632
						Plasmid pLA105, *Lb. acidophilus*, RepA (127)	73/84	BAA08501
Orf2	2415-2744	34.6	109	13	AAGGG	unknown		
Orf3 (*mobC*)	3111-3449	39.5	112	13	GAA	Plasmid pCI528, *L. lactis*, Orf1 gene product (164)	46/63	AAB28189
						Plasmid pMD136, *P. pentosaceus*, Orf1 gene product (111)	34/59	AAD25893
						Plasmid pUCL22, *L. lactis*, ATP-dependent protease (310)	47/67	CAA48032
						Plasmid pSERP, *S. epidermidis*, mobilization protein	36/56	AAW52769
						Plasmid pSE-12228-02, *S. epidermidis*, mobilization protein	35/56	NP 863254
						Plasmid pC223, *S. aureus*, MobC	34/52	NP 943088

Orf	Position	% G+C	Size (aa)	MW (kDa)	Putative RBS	Organism and plasmid with protein database similarity	%Identity/ Positives	Accession no.
Orf4	3511-3320	43.5	63	7	AAGG	unknown		
Orf5 (*mobA*)	3428-4765	45.4	445	51	AGGAGG	Plasmid pCI528, *L. lactis*, mobilization protein (410)	47/65	A48493
						Plasmid pNZ4000, *L. lactis ssp. cremoris*, mobilization protein (MobA, 410)	47/64	AAC64330
						Plasmid pMD136, *P. pentosaceus*, mobilization protein (386)	30/45	AAD39619
						Plasmid pC221, *S. aureus*, relaxase	27/45	CAA26106
						Plasmid pC223, *S. aureus*, relaxase	27/43	CAA31314
Orf7 (*mobB*)	4762-5451	45	229	26	AGGAGG	Plasmid pNZ4000, *L. lactis*, mobilization protein (MobB, 207)	33/57	AAC64331
Orf8 (RepL)	482-6	36.9	158	18	AG	Plasmid pSRD191, *Selenomonas ruminantium*, RepL (172)	55/70	AAT80666
						Shuttle vector pNAK1, replication protein (156)	48/70	AAV86077
						Plasmid pSN2, *S. aureus*, hypothetical protein product (158)	37/67	NP 976275

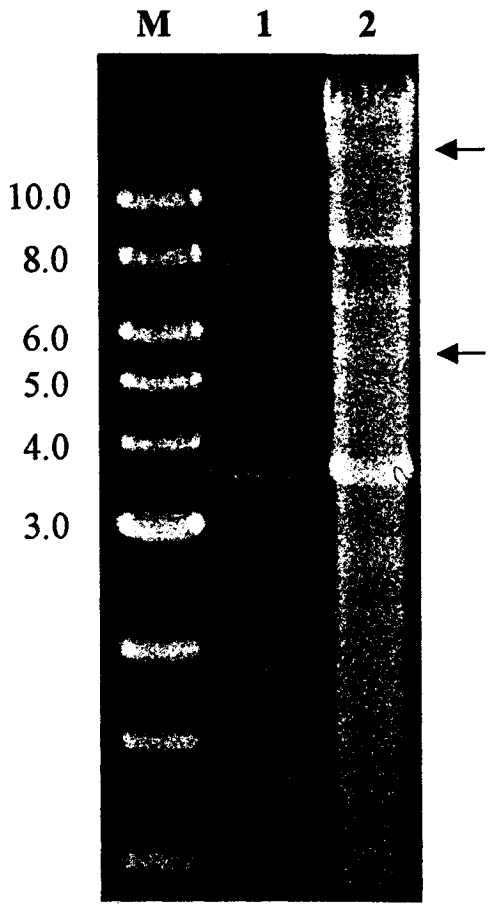

Figure 2.1 Plasmid DNA profile of *Lb. plantarum* L42.

Purified DNA of *Lb. plantarum* L42 was electrophoresed on a 0.7% agarose gel and stained in EtBr. The arrows indicate the two linearized plasmids, pLJ42 (5.5 kb) and pLF42 (>10 kb). M, molecular weight marker (λ-DNA *Hind*III-digested); lane 1, purified pLJ42; lane 2, total plasmid DNA from L42.

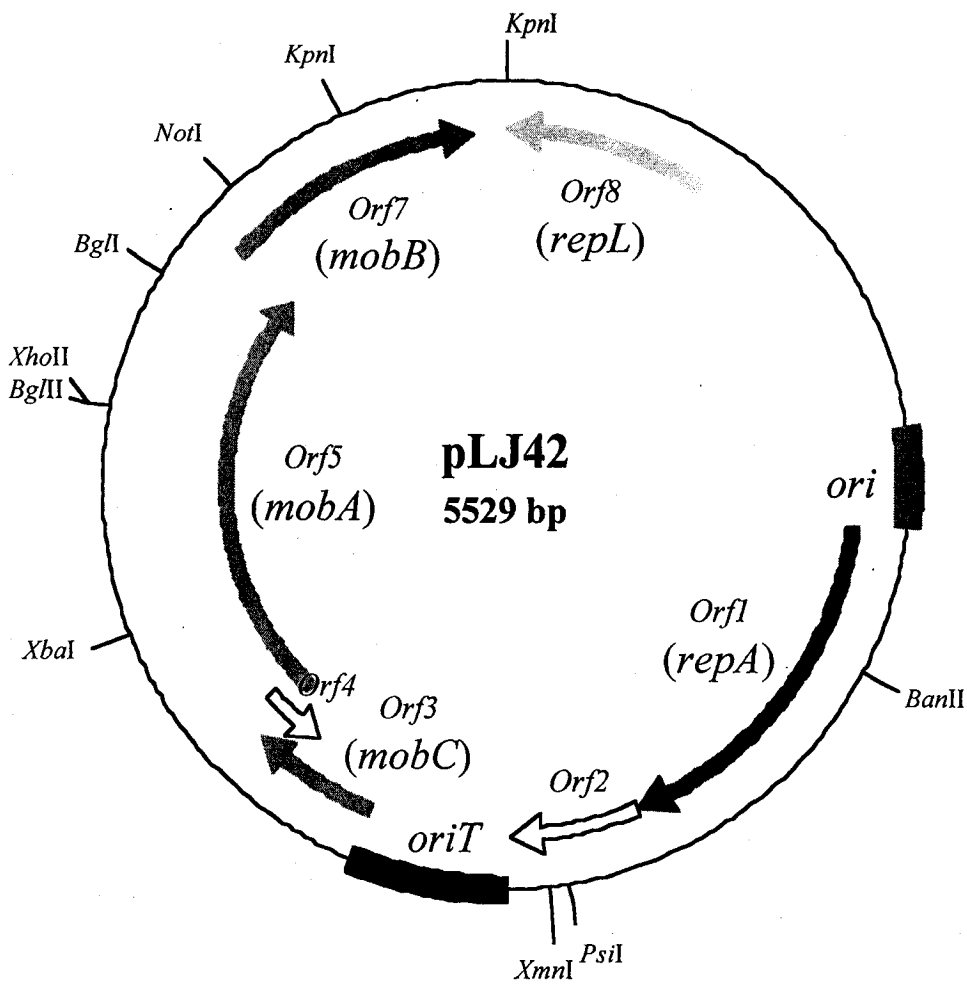

Figure 2.2 General organization of plasmid pLJ42 from *Lb. plantarum* L42.

Solid arrows indicate identified Orfs with their putative functions. Non-coding regions are shown by solid boxes. For a detailed description of the plasmid, refer to the text.

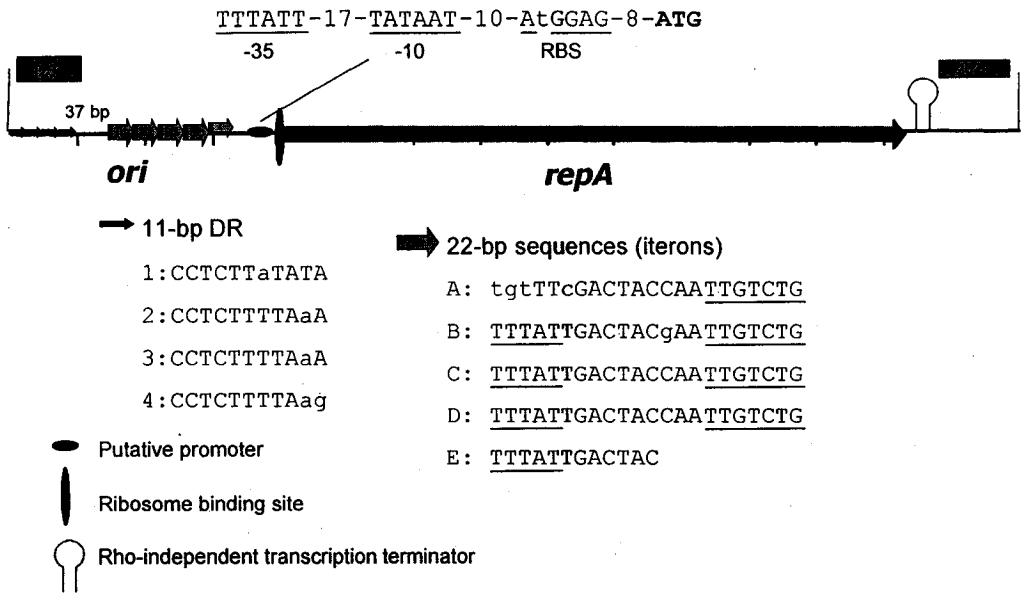

Figure 2.3 Structural organization of the pLJ42 replicon.

Coordinates of the schematized fragment are indicated at each end. The –35 and –10 regions of the *repA* promoter are indicated, in addition to the ribosome-binding site (RBS). The 11-bp direct repeats (DR) and iterons are represented by solid arrows. The sequence of the repeat units are also indicated. Lowercase letters indicate alternative sequences found in the units. Underlined letters indicate the nucleotides conserved in the iterons.

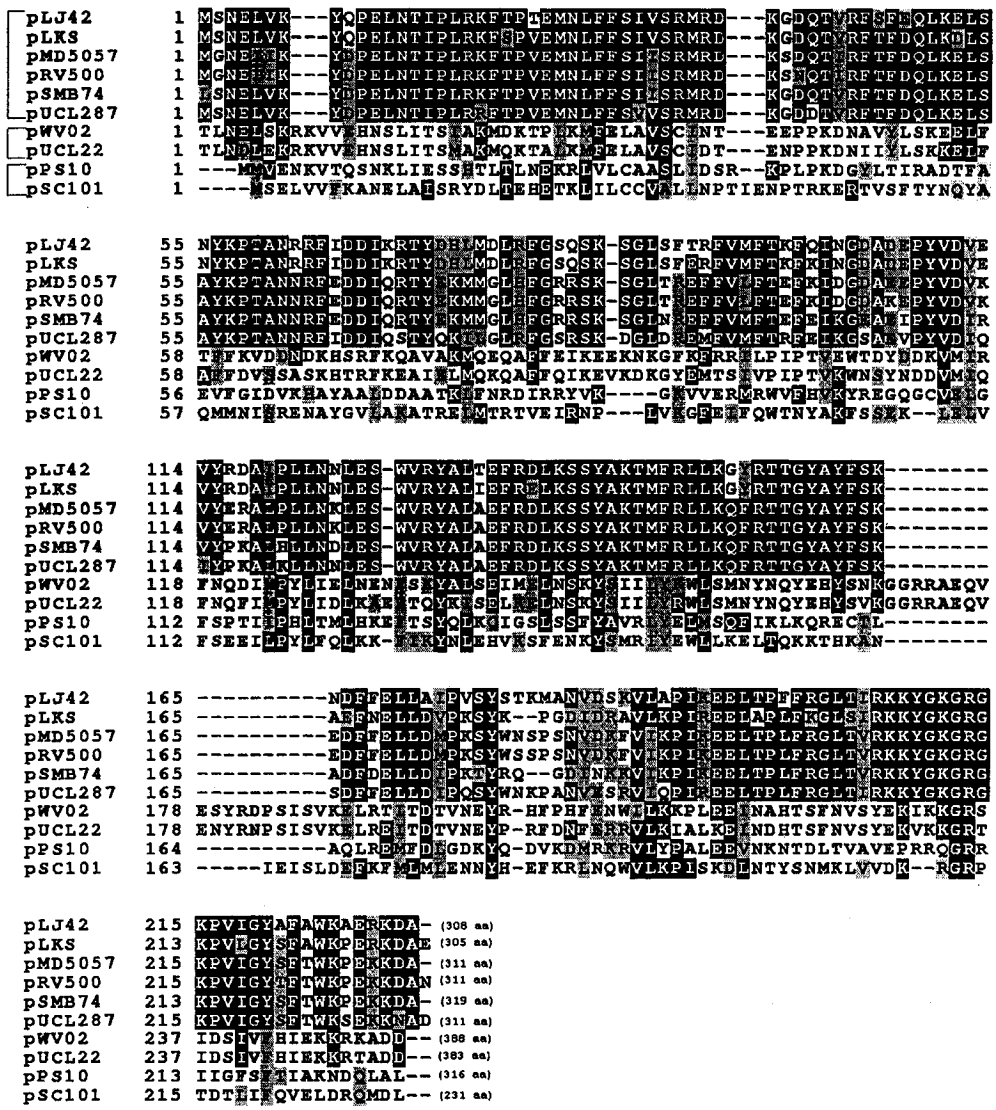

Figure 2.4 Multiple alignment of homologous RepA proteins from three subfamilies of class A theta plasmids.

Amino acid sequences were aligned with the program CLUSTAL-W (version 1.8) using the protein weight matrix BLOSUM30 and default parameters for pairwise alignment. Residues that are identical and similar to the consensus are shaded black and grey, respectively. A central region is conserved among all Rep proteins, but a N-terminal region is only conserved among the members of the pUCL287 family of theta-replicating plasmids. The total number of residues for each protein is given in parentheses.

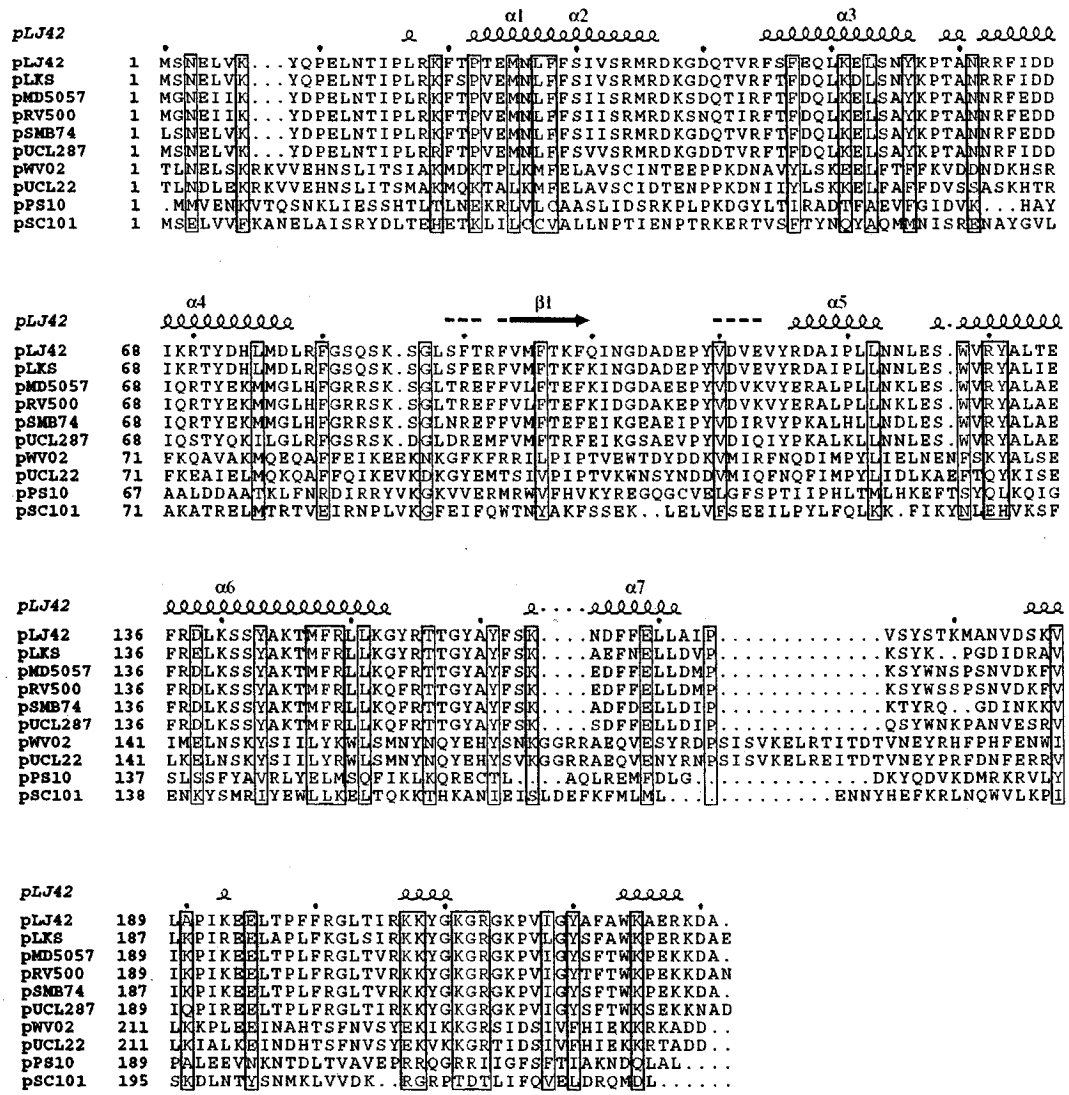

Figure 2.5 Secondary structures of homologous Rep proteins.

An alignment of Rep proteins of ten plasmids from the class A family was performed with the program CLUSTAL-W (Thompson et al., 1994), using the protein weight matrix BLOSUM30, and gap opening and extension penalties of 10 and 0.2. The delay of divergent sequences was set to 30%. A secondary structure prediction was performed using the neural network algorithm PHD (Rost et al., 1994) on the output from CLUSTAL-W at the Pôle BioInformatique Lyonnais website (npsa-pbil.ibcp.fr). To visualize results, the output was processed at espript.ibcp.fr/ESPript/ESPript/ (Gouet et al., 1999). α-Helical regions and β-turns were predicted for pLJ42 RepA and are indicated above the alignment. The LZ motif is located at the N-terminal, and characterized by the presence of hydrophobic leucine residues. The HTH motif is located at the C-terminal, and composed of α-helices 6 and 7.

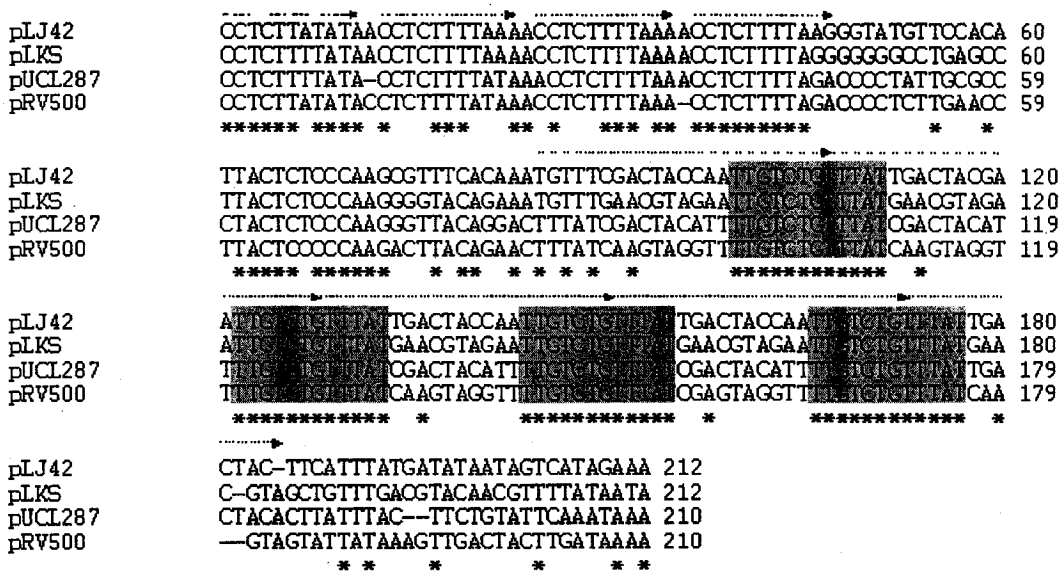

Figure 2.6 Multiple alignment of homologous *ori* sequences of plasmids pLJ42, pLKS (AB035265), pUCL287 (X75607) and pRV500 (NC004942).

Conserved nucleotides are indicated by asterisks. 11-bp direct repeats and iterons are indicated by dotted arrows. The shaded areas show the consensus motif (TTGTCTGTTTAT) obtained by the juxtaposition of repeated units in the iterons.

a.

```
pLJ42 MobA      (11-32) SAASALNYALGKDKELHNDTK      (65-99)  ANEQFKIVQQAF-NQTARRNQVLRITQSFALNELDP
pCI528 Orf2     (11-32) SAASALNYALGQDRPMHEKTE      (65-99)  AKEQFDVIRQLH-NQTKESNQVLRITQSFALDELNP
pNZ4000 Mob     (11-32) SAASALNYALGQDRPMHEKTE      (65-99)  AKEQFDVVRQLH-NQTKESNQVLRITQSFALDELNP
pMD136 MobA     (11-21) SASRLVNYA--------EK-       (36-69)  AKSQLKQVREVYGNQGKTQAYASRI--SFSPKELDP
pC223 Orf1      (11-28) STSRAINYA---EKRAEEKSA      (36-69)  AKSSFKATREMYGKTDGNEGHV--VIQSFKPNEVTP
pC221 Rlx       (11-21) SASRAINYA--------EK-       (36-69)  AKSAFKQTRALYGKE--DGIQAHTVIQSFKPGEVTP
                        ----------                          ----------
                        Motif I                             Motif II

pLJ42 MobA      (120-158) PDYQTAVYTHLDGQNHILHNHIIINKVNLQTGKKLDERK
pCI528 Orf2     (120-158) PNHQSAVYTHLDGKNHVLHNHIIVNKVNLETGKKLREQK
pNZ4000 Mob     (120-158) PNHQSAVYTHLDGKNHVLHNHIIVNKVNLETGKKLREQK
pMD136 MobA     (91-127)  PNQQVALYEHAD--TNALHVHAVIGAIDLETGRKMHGNW
pC223 Orf1      (86-122)  PNHQVAVYTHND-TDHV-HNHIVINSIDLETGKKFNNNK
pC221 Rlx       (85-121)  PNHQVAVYTHTD-KDH-YHNHIVINSVDLETGKKYQSNK
                          ----------
                          Motif III
```

b.

```
pLJ42 oriT      AAGCGGGAATGGGCATACTTTTGCGAAGCAAAATGTATGTCATTCCG-AA AG[redacted]GTATTTGTTTTTTA
pNZ4000 oriT1   AAGCCACATTGTAATACAAGAACGAAGTGATT-TGTATTACAATGTG-AT AG[redacted]GTATTTATGGTTTT
pNZ4000 oriT2   AAGCAACATTGTAATACAAGAACGAAGTGATT-TGTATTACAATGTG-AT AG[redacted]GTATTTATGGTTTT
pCI528 oriT     AAGCCACATTGTAATACAAGAACGAAGTGTTT-TGTATTACAATGTG-AT AG[redacted]GTATTTATGGTTTT
R64 min oriT    GATGGCAATTGTAATAGCGTCGCG.TGTGACG--GTATTACAAT-TGCAC ATGC[redacted]TCCCG-TTTTCGGG
                *     * **                ****  ** *  * *  * * **      ** *
```

Figure 2.7 Alignment of pLJ42 Mob A and *oriT* with homologous *mob* proteins and *oriT*, respectively.

a) Amino acid sequence comparison of the three conserved regions involved in relaxase activity. The alignment includes MobA from *Lb. plantarum* pLJ42, Orf2 from *L. lactis* pCI528, Mob (MobA) from *L. lactis* pNZ4000, MobA from *P. pentosaceus* pMD136, Orf1 and Rlx from *S. aureus* plasmids pC223 and pC221, respectively. Residues shown are given in parentheses. The Tyr (Motif I) and three His (Motif III) residues involved in cleaving-joining reactions, and the Ser residue (Motif II) involved in DNA binding, are indicated in boldface. The ColE1-3H signature Hx(D/E)(x_{4-6})HxH(x_3)*n* in Motif III is indicated by a dotted line. b) Alignment of the imperfect IR of *oriT* of several mobilizable plasmids. The pLJ42 *oriT* region was aligned with *oriT1* and *oriT2* of plasmid pNZ4000, *oriT* of pCI528, and with the *oriT* of the IncI1 plasmid R64. For pLJ42, the imperfect IR is shown with facing arrows. The shaded area represents the conserved hexanucleotide proposed as the putative *nic* site for plasmids pLJ42, pNZ4000 and pCI528. For R64, the IR is underlined, the NikA binding site is shown in boldface and the *nic* site is indicated with an arrowhead.

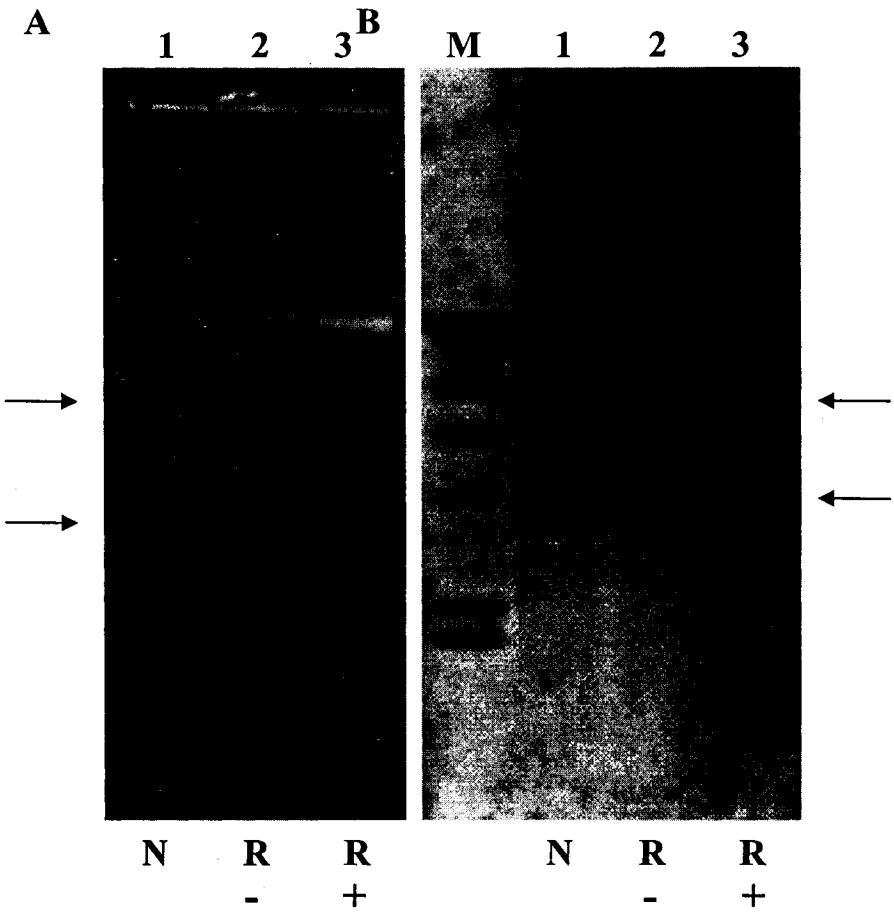

Figure 2.8 Southern blot analysis of pLJ42 replication intermediates using the DIG-labelled pLJ42 replicon as a probe.

(A) Purifed DNA from L42 was electrophoresed on a 0.7% agarose gel before (N) or after incubation of the cells for 1 h with rifampicin, and with (+) or without (-) prior S1 nuclease treatment. DNA was transferred to a positively charged nylon membrane under denaturing conditions. (B) Southern hybridization, using the pLJ42 replicon as a probe. M, molecular weight marker (λ-DNA *Hind*III-digested) showing fragments with the following base pair lengths: 23130, 9416, 6557, 4261, 2322 and 2027. The arrows point to the plasmids that hybridized. pLJ42 and pLF42 both hybridized with the probe, demonstrating they possess similar replicons.

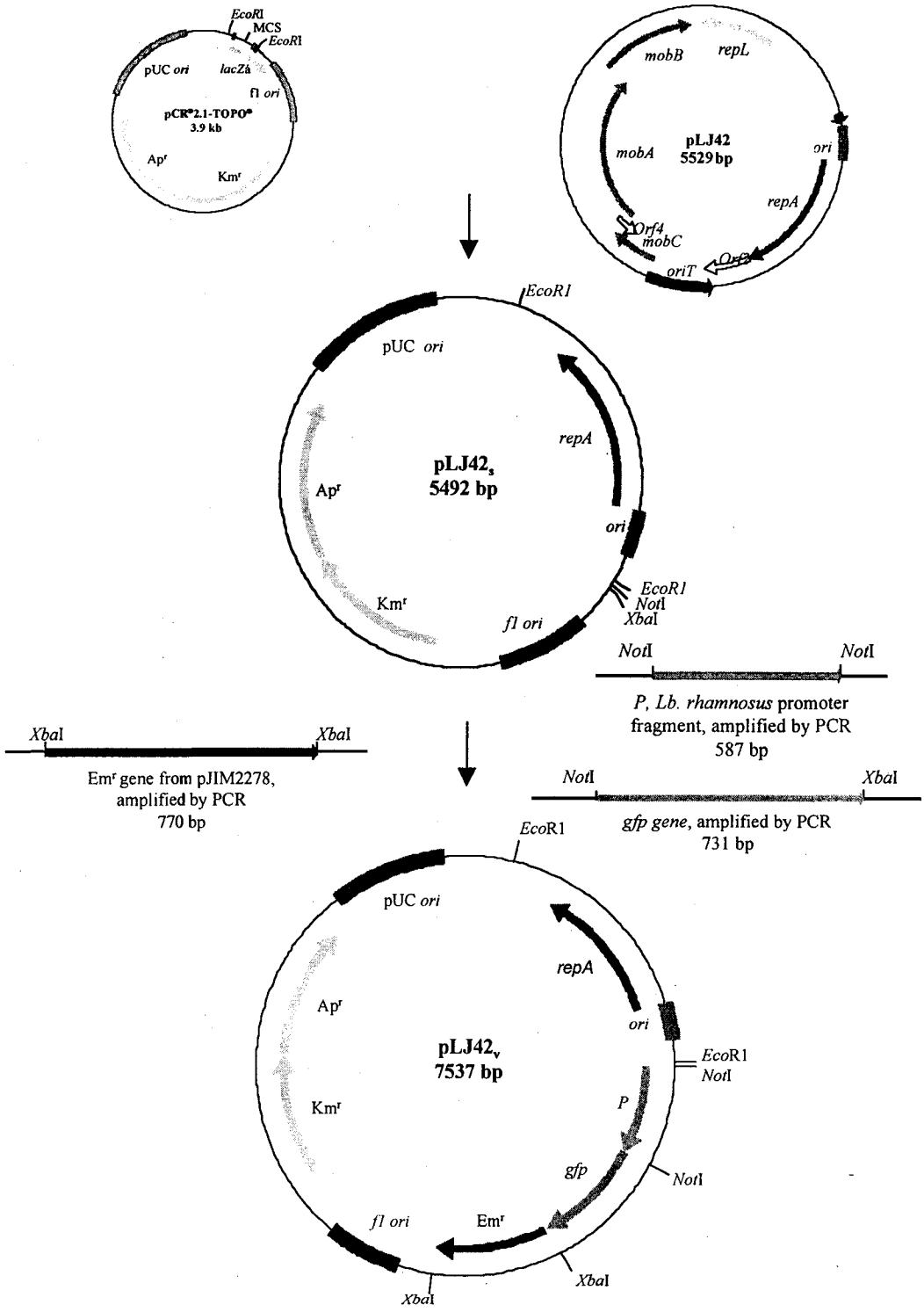

Figure 2.9 Construction of pLJ42v, an *E. coli-Lactobacillus* shuttle vector that carries Em^r as a selective marker and *gfp* as a reporter gene.

Relevant cloning sites are indicated. For details on cloning strategy, refer to text.

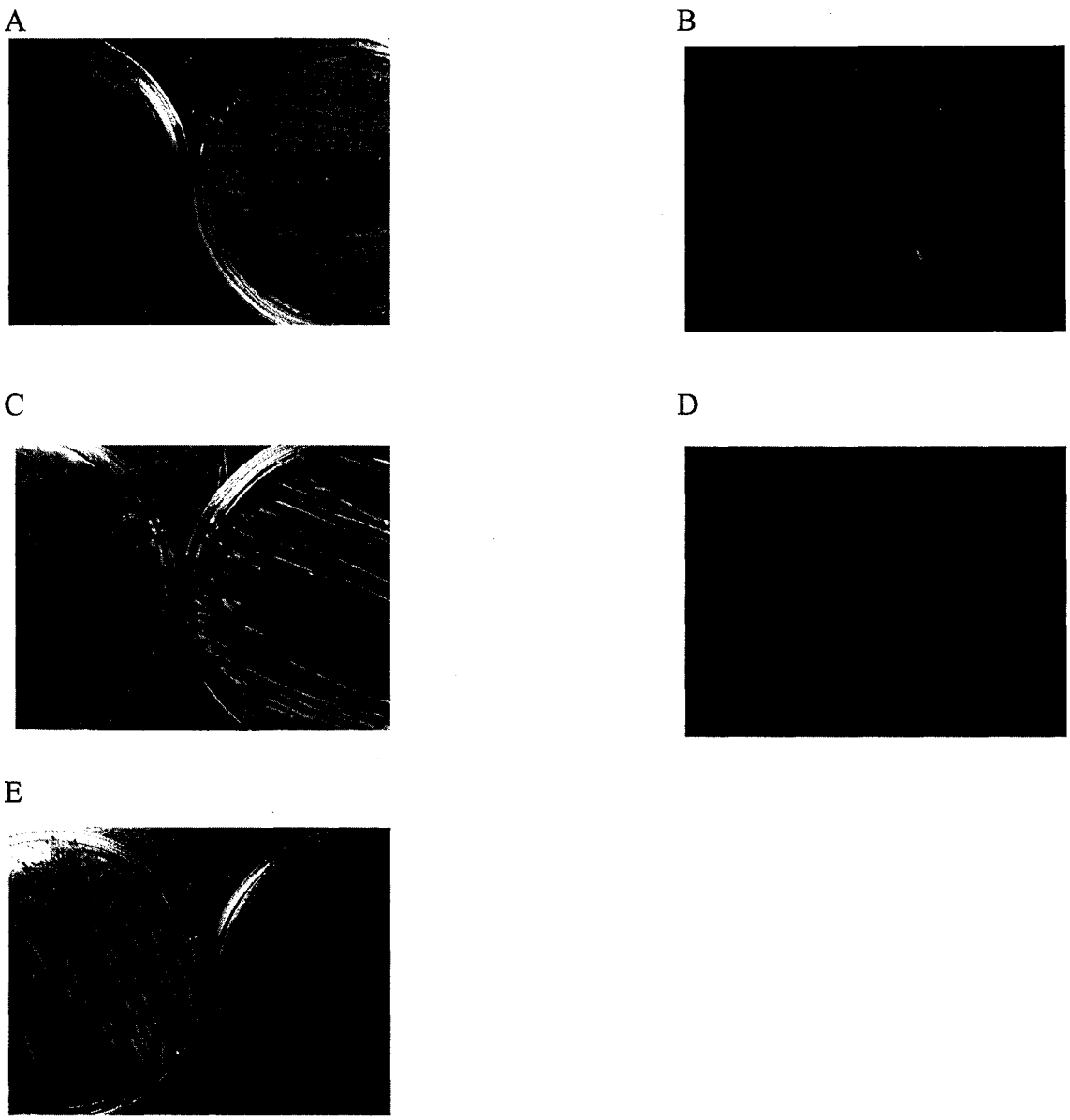

Figure 2.10 Heterologous gene expression in positive clones.

A and B show *E. coli* cells transformed with pLJ42$_v$ expressing Em^r and *gfp*, respectively. C and D show *Lb. plantarum* L42 transformants expressing Em^r and *gfp*, while E shows *Lb. plantarum* L21 growing under erythromycin selection. The level of *gfp* expression in *E. coli* is higher than in *Lb. plantarum*, reflected by the intensity of the fluorescence.

```
L42        GAATTCGGC--TTGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTG 87
MiLAB14    GGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTG 420
4.1        GGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTG 275
LP2        GGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTG 403
LP1        GGAGGCGGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTG 403
           * *  * **  * **************************************************

L42        AGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTA 147
MiLAB14    AGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTA 480
4.1        AGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTA 335
LP2        AGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTA 463
LP1        AGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTA 463
           ************************************************************

L42        ACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC 207
MiLAB14    ACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC 540
4.1        ACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC 395
LP2        ACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC 523
LP1        ACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC 523
           ************************************************************

L42        GCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGC 267
MiLAB14    GCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGC 600
4.1        GCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGC 455
LP2        GCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGC 583
LP1        GCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGC 583
           ************************************************************

L42        GGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGG 327
MiLAB14    GGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGG 660
4.1        GGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGG 515
LP2        GGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGG 643
LP1        GGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGG 643
           ************************************************************

L42        GAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATA 387
MiLAB14    GAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATA 720
4.1        GAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATA 575
LP2        GAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATA 703
LP1        GAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATA 703
           ************************************************************
                      .

L42        TATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGA 447
MiLAB14    TATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGA 780
4.1        TATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGA 635
LP2        TATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGA 763
LP1        TATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGA 763
           ************************************************************
```

Figure 2.11 CLUSTAL-W alignment of partial 16s ribosomal RNA sequences of *Lactobacillus plantarum* strains L42, MiLAB14 (AY383631), 4.1 (AY845198), LP2 (AY735409) and LP1 (AY735404).

Residues shown are indicated at the right of the sequences. Identical residues are indicated by asterisks.

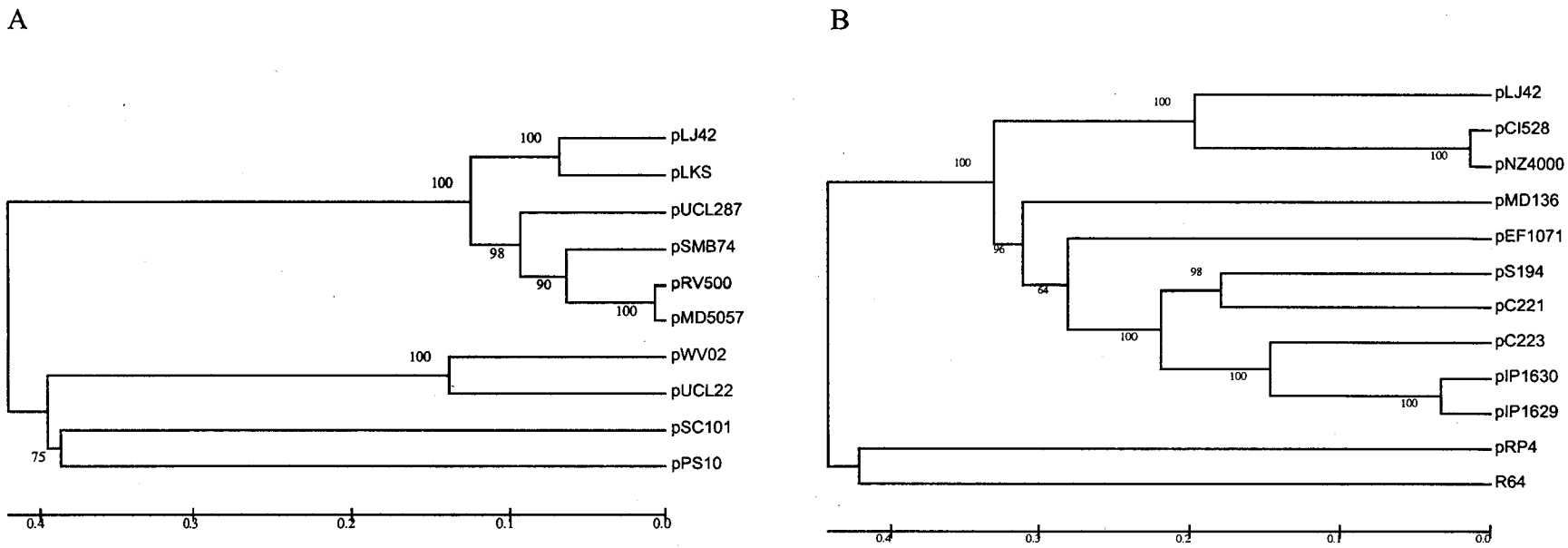

Figure 2.12 Phylogenetic trees of the deduced pLJ42 proteins RepA (A) and MobA (B).

The unrooted trees were generated by the MEGA 3.0 program using p-distance values and by the neighbor-joining method (Kumar et al., 2004). The bootstrap value was set to 500. The alignments were generated using CLUSTAL-W (Thompson et al., 1994) set to the default parameters. For the MobA proteins, only the 300 first amino acid residues were aligned.

Conclusion

To summarize the discoveries of this research, results can be listed as such: (1) strains of *Lactobacillus* spp. were screened for cryptic plasmids; (2) *Lb. plantarum* L42 was found to harbour two plasmids with similar replication functions; (3) pLJ42 (5,529 bp) was isolated and characterized, and revealed it harboured a theta replicon and a mobilization operon; (4) the shuttle vector $pLJ42_v$ was constructed using the pLJ42 replicon combined to the pUC *ori*; (5) the cloning vector was shown to be functional in *E. coli*, which expressed the reporter gene (*gfp*) and erythromycin resistance; (5) $pLJ42_v$ proved to be a species-specific cloning vector, as it could only transform *Lactobacillus plantarum* L21 and L42. This is the first phase in the development of a new family of species-specific cloning vectors for lactobacilli.

Generally, theta-based cloning vectors have narrow host-ranges, but they nonetheless have interesting characteristics, including compatibility with endogenous RCR or theta replicons in a host, high structural and segregational stability, and capacity to carry larger DNA fragments. Moreover, theta cloning vectors are intrinsically safer, as they are less likely to be horizontally transferred to other bacterial species, a safety concern when using recombinant LAB in fermentations. Theta cloning vectors could be quite interesting to engineer recombinant LAB for large scale fermentations, where vector stability is of great importance and selective pressure cannot always be applied.

Narrow host-range and species-specific cloning vectors ought to be developed for further applications. The pLJ42-family of cloning vectors could be used for the genetic improvement of industrially used *Lb. plantarum* strains, where horizontal transfer and vector stability are concerns. Ultimately, *Lb. plantarum* strains could be engineered as vaccine carriers, i.e. for the mucosal delivery of antigens exhibiting vaccine properties. Such activities are made possible insofar as cloning vectors derived from LAB plasmids are recognized as food-grade and safe for human and/or animal consumption.

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Appendix

Biosafety Declaration

- Biosafety Declaration –

Throughout the work I performed at BRI in view of obtaining my Master's degree from McGill University under the supervision of my (co) director[1], I recognize that I manipulated the following microorganisms in the following labs according to Health Canada's Laboratory Biosafety Guidelines (1996) and BRI's Health and Safety procedures.

Between September 2003 and May 2005.

Scientific Name Pure strain or cell lines, Environmental samples (provide details) Clinical samples (provide details)		**Risk Group # (1 or 2)** According to the Health Canada Guideline	**Manipulated in Lab #**	**Lab's Confinement Level (1 or 2)**
Escherichia coli Top10	Invitrogen	1	L-449	1
Lb. acidophilus	ATCC 11975	1	L-449	1
Lb. acidophilus	ATCC 33323	1	L-449	1
Lb. acidophilus	ATCC 4357	1	L-449	1
Lb. acidophilus	ATCC 43121	1	L-449	1
Lb. acidophilus	ATCC 53976	1	L-449	1
Lb. acidophilus	ATCC 53546-D	1	L-449	1
Lb. acidophilus	ATCC 53546-A	1	L-449	1
Lb. plantarum	ATCC 14917	1	L-449	1
Lb. casei	ATCC 4646	1	L-449	1
Lb. acidophilus L11	Lab. collection	1	L-449	1
Lb. acidophilus L12	Lab. collection	1	L-449	1
Lb. acidophilus L13	Lab. collection	1	L-449	1
Lb. acidophilus L14	Lab. collection	1	L-449	1
Lb. acidophilus L15	Lab. collection	1	L-449	1
Lb. acidophilus L16	Lab. collection	1	L-449	1
Lb. acidophilus R052	Rosell	1	L-449	1
Lb. acidophilus L18	Lab. collection	1	L-449	1
Lb. acidophilus L19	Lab. collection	1	L-449	1
Lb. acidophilus L20	Lab. collection	1	L-449	1
Lb. plantarum L21	Lab. collection	1	L-449	1
Lb. casei L23	Lab. collection	1	L-449	1
Lb. casei L24	Lab. collection	1	L-449	1
Lb. casei L25	Lab. collection	1	L-449	1
Lb. casei L26	Lab. collection	1	L-449	1

[1] this person is a researcher working at BRI, yet is still affiliated to McGill University

Lb. casei L27	Lab. collection	1	L-449	1
Lb. casei L29	Lab. collection	1	L-449	1
Lb. rhamnosus R049	Rosell	1	L-449	1
Lb. casei R202	Rosell	1	L-449	1
Lb. plantarum L42	Lab. collection	1	L-449	1
Lb. reuteri L51	Lab. collection	1	L-449	1
Lb. bulgaricus L61	Lab. collection	1	L-449	1
Lb. plantarum	ATCC 14431	1	L-449	1
Lb. casei	ATCC 393	1	L-449	1
Lb. casei	ATCC 334	1	L-449	1

Lb.*: *Lactobacillus

Student: _____Julie Shareck_____; Thesis (co) Director: ___Carlos Miguez___;

signed _____ June 15 2005 signed _____, 15-06 2005

Approved by:

Biosafety Chairperson: _____Denis Rho_____; signed _____

Health and Safety Advisor: C Dodd ; signed _____