LACTOCOCCAL PLASMID REPLICON: VECTOR

CONSTRUCTION AND GENETIC ORGANIZATION

by

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ABSTRACT

The 5.5 kb high-copy number cryptic plasmid pDI25 from *Lactococcus lactis* subsp. *lactis* 5136 was isolated and used as the basis to construct a series of vectors. The vector pFX1 (5.5 kb) was first made by ligating the 4.5 kb *Hpa*II-*Mbo*I fragment of the lactococcal plasmid to the 1 kb chloramphenicol transacetylase gene from the staphylococcal plasmid pC194.

Plasmid pFX1 was further modified by deleting a non-essential 1.9 kb *Cla*I region to construct pFX2 (3.6 kb). Deletion analysis showed an essential region for plasmid replication was located within a 1.2 kb *Cfo*I-*Tha*I-*Cfo*I fragment.

The vector pFX3 was constructed by incorporating the α fragment of the *Escherichia coli lacZ* structural gene, a multiple cloning region and the T7 and T3 promoters from pUBS into pFX2. Recombinant plasmids constructed in *E. coli* using X-gal selection could be subsequently electroporated into lactococci. pFX3 could also be used directly for transcription studies or DNA sequencing of cloned inserts.

A set of lactococcal translational gene-fusion vectors was constructed by incorporating the *E. coli lacZ* gene fusion system (pNM480,481,482) into pFX2. These constructions, pFX4, pFX5 and pFX6, permit the fusion of cloned genes to *lacZ* in all three reading frames. Gene expression can be readily and quantitatively monitored by measuring ß-galactosidase activity.

All the pFX vectors were efficiently transformed into lactococci and *E. coli* by electroporation (10^4-10^6 cfu/µg DNA in each host) and maintained stably in both organisms (>95% cells carrying the Cm marker after 100 generations growth without drug selection).

A cell-wall bound proteinase from *Lactococcus lactis* subsp. *cremoris* H2 was isolated and characterized as a PI type proteinase since it preferentially degraded ß-casein. A 6.5 kb *Hin*dIII fragment of plasmid pDI21 (63 kb) was initially cloned and expressed this enzymatic activity in *E. coli* using vector λNM1149. The restriction map of this pDI21 *prt* gene fragment had minor differences from those of other published lactococcal *prt* fragments.

Using pFX1, the pDI21 *prt* gene fragment was recloned and directly electroporated into lactococci where it was efficiently expressed. The effectiveness of pFX3 was demonstrated by initially cloning a pDI1 4.4 kb *Eco*RI tagatose 1,6-bisphosphate aldolase gene fragment into *E. coli* from where it was electroporated into lactococci. Using the translational fusion vectors pFX4, pFX5 and pFX6, the 6.5 kb *Hin*dIII *prt* gene fragment of pDI21 was identified as having two promoters with opposite orientations. The pDI21 2.0 kb *Eco*RI galactose-6-phosphate isomerase gene fragment was shown to carry a promoter and the direction of gene transcription was determined.

The complete DNA sequence of the lactococcal portion of pFX2 (2508 bp) was determined and the genetic organization analyzed. A lactococcal plasmid plus origin and two replication protein coding regions (*repA* and *repB*) were located. RepA had an αhelix-turn-αhelix motif, a geometry typical of DNA-binding proteins. RepB showed high homology to the plasmid replication initiation proteins from other Gram-positive bacteria and *Mycoplasma*. The transcribed inverted repeat sequence between *repA* and *repB* could form an attenuator to regulate pFX2 replication. Upstream of the plus origin site, and in a region nonessential for replication, a 215 bp sequence identical to the staphylococcal plasmid pE194 and carrying the RS_A site was identified. The genetic organization of this lactococcal plasmid replicon shares significant similarity with the pE194 group of plasmids.

LIST OF PUBLICATIONS

Data from this work has been published in part in the following papers.

1. Yu, P.-L., Xu, F. and Pearce, L.E. (1989). Molecular characterization of the lactose plasmid of *Streptococcus cremoris* H2. Proceedings of Eighth Australian Biotechnology Conference, p349-352. Sydney, Australia.

2. Xu, F., Pearce, L.E. and Yu, P.-L. (1989). Molecular cloning and expression of a proteinase gene from *Lactococcus lactis* subsp. *cremoris* H2 in *Escherichia coli* and *Lactococcus lactis* subsp. *lactis*. Proceedings of Annual Conference of New Zealand Microbiological Society. Hamilton, New Zealand.

3. Xu, F., Pearce, L.E. and Yu, P.-L. (1990). Molecular cloning and expression of a proteinase gene from *Lactococcus lactis* subsp. *cremoris* H2 and construction of a new lactococcal vector pFX1. Arch. Microbiol. 154:99-104.

4. Xu, F., Pearce, L.E. and Yu, P.-L. (1990). Construction of a family of lactococcal vectors for gene cloning and translational fusion. FEMS Microbiol. Lett. (in press).

5. Yu, P.-L., Pearce, L.E. and Xu, F. (1989). Improved recombinant DNA method. New Zealand Patent 229125.

6. Yu, P.-L., Xu, F. and Pearce, L.E. (1990). Gene cloning and vector constructions in fermentative lactococci. p15-21. In: Fermentation Technologies. Industrial Applications. Yu, P.-L. (ed.), Elsevier Applied Science, UK.

7. Xu, F., Pearce, L.E. and Yu, P-L. (1990). Lactococcal replicon: vector constructions and genetic organization. Proceedings of Annual Conference of New Zealand Microbiological Society. Lincoln, New Zealand.

8. Xu, F., Pearce, L.E. and Yu, P.-L. (1990). Sequence analysis of a lactococcal plasmid replicon. Mol. Gen. Genet. (submitted).

9. Xu, F., Yu, P.-L. and Pearce, L. E. (1990). Lactococcal plasmid replicon: vector construction and genetic organization. In: Streptococcal Genetics. American Society for Microbiology, Washington, DC, USA (in press).

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ABBREVIATIONS

kb	kilobases
bp	basepairs
pfu	plaque forming units
cfu	colony forming units
Ap	ampicillin
Cm	chloramphenicol
SDS	sodium dodecyl sulphate
MW	molecular weight
kDa	kilodaltons
PEG	polyethylene glycol
X-gal	5-bromo-4-chloro-3-indoyl galactopyranoside
IPTG	Isopropyl thiogalactopyranoside
ORF	open reading frame
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]

CHAPTER 1: GENERAL INTRODUCTION

PART I: INTRODUCTION

1.1 History

Microorganisms play an essential part in milk fermentations, and as early as about 1850, their roles in the production of fermented dairy products were investigated [Prentice and Neaves, 1986]. Most of the initial work, however, was concerned with understanding the processes already in use, rather than making any attempt to improve them.

Milk containing "starters" that initiate or "start" milk fermentation was kept at room temperature for several hours, during which time the indigenous lactic acid bacteria multiplied and produced lactic acid. The milk was eventually coagulated and used as an inoculum for cheese making. If the subsequent cheese was of good quality, the inoculum was transferred for future use. Mixed strain starter cultures originated via this practice were passed from one generation of cheese makers to the next. Such haphazard methods of starter preparation and maintenance were used until the end of 19th century. Van Niel et al. [1929] first reported using pure cultures of *Lactococcus lactis* subsp. *lactis* or *Lactococcus lactis* subsp. *cremoris* to make ripened-cream butter. It was then established that the best flavoured butter could be produced with mixtures of two different types of bacteria, one of which (*L. lactis* subsp. *lactis* and/or *L. lactis* subsp. *cremoris*) was responsible for lactic acid production and the other (*Leuconostoc*) for flavour production. The first pure cultures for cheese-making were isolated in 1937 in New Zealand from commercial mixed starters and were used to produce good-quality cheese [Whitehead, 1953].

In the modern dairy industry, lactic acid bacteria are used for the manufacture of numerous fermented foods [Sandine, 1987]. They are also of benefit to food flavours

and are antagonistic to harmful microorganisms by producing bacteriocins and other inhibiting products in food and feed. Lactic acid bacteria have been shown to enhance the nutritional quality of some foods, and have been claimed to be important to human health in relation to immunocompetence, prevention of some cancers and reduction of blood cholesterol levels [Ayebo et al., 1981; Grunewald, 1982; Sandine, 1987]. Dairy microbiology has now evolved from the past of poor understanding of microorganisms in milk fermentation to the present, where milk products are produced with defined strains under controlled physiological conditions.

In the future, recombinant DNA technology, which is presently being investigated for its application to dairy products, is likely to provide a new means of deriving suitable starters for milk fermentations with enzymes manipulated for optimum activity and performance.

1.2 Taxonomy of Lactococci

1.2.1 Classification of lactic acid bacteria

Streptococcus, Lactococcus, Enterococcus, Lactobacillus, Pediococcus and *Leuconostoc* comprise the non-endospore Gram-positive lactic acid bacteria. These bacteria typically ferment glucose to lactic acid by either the Embden-Myerhof (EM) glycolytic pathway (homofermentative) or by a combination of the hexose monophosphate pathway (HMP) followed by the phosphoketolase pathway (heterofermentative) (Fig. 1.1).

Traditionally, taxonomy of these organisms was based on their differences in morphology, physiology, and serology. Lactic acid bacteria were classified into genera according to their different modes of fermenting glucose (Table 1.1). The current *Lactococcus* and *Enterococcus* species originally belonged to the *Streptococcus* group.
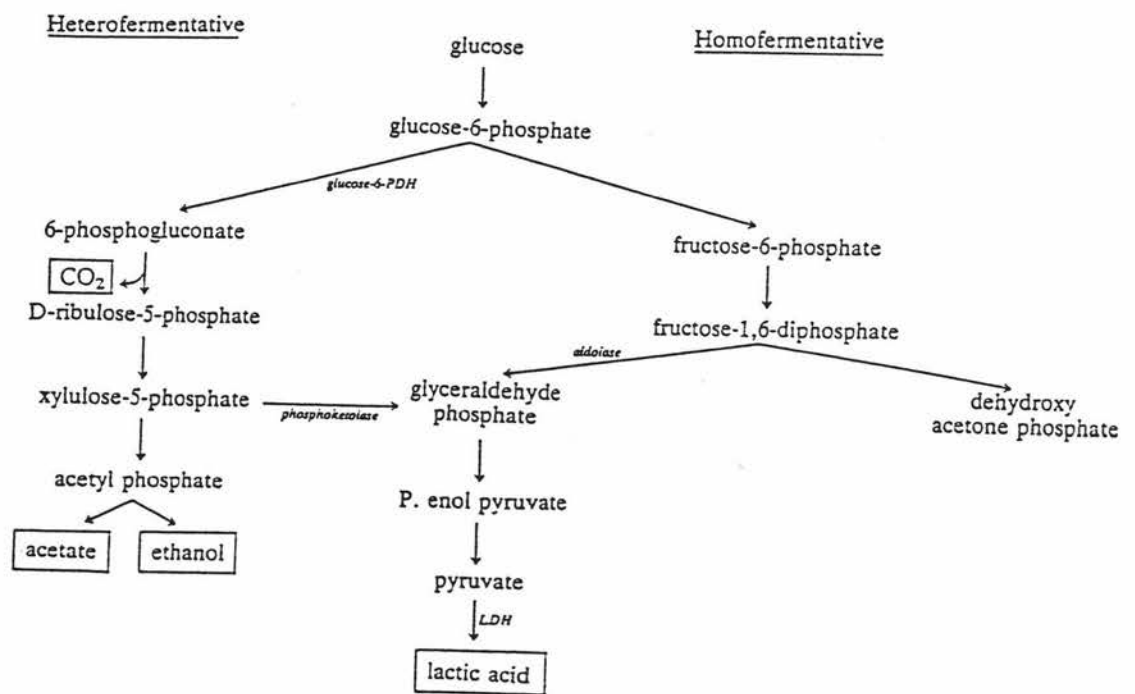
Figure 1.1 Glycolytic pathways in lactic-acid bacteria [from Garvie, 1984].

Table 1.1. Metabolic pathways in lactic acid bacteria [Garvie, 1984].

| | | Metabolic pathway | | |
		Embden–Myerhof	Hexose-monophosphate	Phospho-ketolase
Genus	Sub-genus			
Streptococcus	All species	+	−	−
Leuconostoc	All species	−	+	+
Pediococcus	All species	+(probably)	−	−
Lactobacillus	Thermobacteria	+	−	−
	Streptobacteria	+	(+)	(+)
	Betabacteria	−	+	+

+, present; (+), present but used only under special conditions; −, not present.

Recent comparative analysis of the 16S rRNA [Ludwig et al., 1985] and extensive nucleic acid hybridization studies [Kilpper-Balz and Schleifer, 1981; 1984; Schleifer and Kilpper-Balz, 1984; Schleifer et al., 1985] have shown that *Lactococcus* and *Enterococcus* are genetically distinct and they can be classified into separate groups. The genus *Streptococcus* currently retains only the pneumococci, other pyogenic and the oral streptococci. *Enterococcus* consists of the typical enterococci, while *Lactococcus* encompasses all lactic streptococci and some *Lactobacillus* strains [Schleifer, 1987].

1.2.2 Nomenclature and characteristics of lactococci

For a long time, the classification of lactococci has been subject to much debate and uncertainty. Various systems were used to define the three important dairy organisms (Table 1.2). In the eighth edition of Bergey's Manual of Determinative Bacteriology, *Streptococcus cremoris* and *Streptococcus lactis* were recognized as two species, and *Streptococcus lactis* subsp. *diacetylactis* was a subspecies of *S. lactis*. This nomenclature has been used in the literature even after the 9th edition of Bergey's Manual of Systematic Bacteriology was published in 1986. The 9th edition combined *S. cremoris* with *S. lactis* to form a single extended *S. lactis* species. *S. lactis* now includes *S. lactis* subsp. *lactis*, *S. lactis* subsp. *cremoris* and *S. lactis* subsp. *diacetylactis* [Mundt, 1986]. Recently, the application of genetic techniques in taxonomy of *Streptococci* showed that *S. lactis*, *S. cremoris* and other lactic streptococci as well as some *Lactobacilli* could be classified into a new genus *Lactococcus* [Schleifer, 1987].

Table 1.2 Different systems of nomenclature for important lactococcal types.

System source	lactis type	cremoris type	diacetylactis type
Bergey's 8th edition (1974)	*S. lactis*	*S. cremoris*	*S. lactis* subsp. *diacetylactis*
Bergey's 9th edition(1986)	*S. lactis* subsp. *lactis*	*S. lactis* subsp. *cremoris*	*S. lactis* subsp. *diacetylactis*
Approved new names(1986)*	*L. lactis* subsp. *lactis*	*L. lactis* subsp. *cremoris*	*L. lactis* subsp. *lactis*

* Anonymous, 1986.

The lactococci have the following features. All strains are spherial or ovoid in shape, non-motile, and non-haemolytic. They are able to grow in 4% NaCl with the exception of *L. lactis* subsp. *cremoris* that tolerates only 2% NaCl. These bacteria can grow at 10°C but not at 45°C and this feature distinguishes them from the streptococci and enterococci. The major biochemical and chemical differences of the species and subspecies are summarized in Table 1.3. It will be noted that citrate-fermenting lactococci, formerly known as *S. lactis* subsp. *diacetylactis*, no longer have a subspecies status. Schlelifer et al. [1985] suggest this strain should not be given a subspecies status because citrate fermentation is plasmid-encoded. It should also be noted, however, that only the citrate permease is plasmid-encoded. The citrate lyase is chromosomal. Because the citrate fermenting *diacetylactis* type is commercially important, a new name *L. lactis* subsp. *lactis* var. *diacetylactis* for this strain was proposed more recently [Sandine, 1988].

Currently, the new nomenclature has been generally accepted and will be used in this thesis.

Table 1.3. Biochemical and chemical characteristics differentiating species and subspecies of the genus *Lactococcus* [Schleifer, 1987].

Species and subspecies	Peptidoglycan type	Major menaquinones	Acid production from							
			Galac-tose	Lac-tose	Mal-tose	Meli-biose	Meli-zitose	Raffin-ose	Ri-bose	Hydrolysis of arginine
L. lactis subsp. *lactis*	Lys-D-Asp	MK-9, MK-8	+	+	+	−	−	−	+	+
L. lactis subsp. *cremoris*	Lys-D-Asp	MK-9, MK-8	+	+	−	−	−	−	−	−
L. lactis subsp. *hordniae*	Lys-D-Asp	MK-8, MK-9	−	−	−	−	−	−	−	+
L. garviae	Lys-Ala-Gly-Ala	MK-9, MK-8	+	+	v	v	−	−	+	+
L. plantarum	Lys-Ser-Ala	−	−	−	+	−	+	−	−	−
L. raffinolactis	Lys-Thr-Ala	−	+	+	+	+	v	+	v	v

+ = positive; − = negative; v = variable.

1.3 Biochemistry of Lactococci

1.3.1 Lactose metabolism

Lactococci are used as starter cultures for a variety of industrial fermentations in which lactic acid, acetic acid, aroma compounds and polysaccharides are produced, giving products their specific taste, texture and keeping quality. Their primary function is however the rapid fermentation of lactose into lactic acid, resulting in casein precipitation and milk coagulation. An important secondary effect is that the low pH condition created prevents the growth of spoilage pathogenic bacteria such as clostridia, staphylococci or enterobacteria.

The biochemical pathways involved in lactose transport and degradation are now clearly established. Two different systems for lactose transport have been found in lactococci. (1) The lactose phosphoenolpyruvate (PEP) dependent phosphotransferase system (PTS), in which lactose is phosphorylated during translocation, and (2) the lactose permease system, mediating the entry of unmodified lactose. The lactose transport systems differ with respect to their complexity and bioenergetics. The mechanism by which lactose is transported determines the fermentative pathways (including the tagatose-6-P, Leloir and Embden-Meyerhof-Parnas pathways) [Kandler, 1983] for the further conversion of the sugars into lactate (Fig. 1.2).

Utilization of lactose by the PEP-PTS system is the main pathway in starter lactococci. Recently, various lines of evidence have established that the lactose utilization genes are plasmid-encoded. They include P-ß-gal and the two lactose specific PTS enzymes, Factor III-*lac* and Enzyme II-*lac* as well as the genes encoding the three enzymes of the tagatose-6-P pathway [Crow et al., 1983; Gasson, 1983; Inamine et al., 1986; McKay, 1983; Yu et al., 1989].
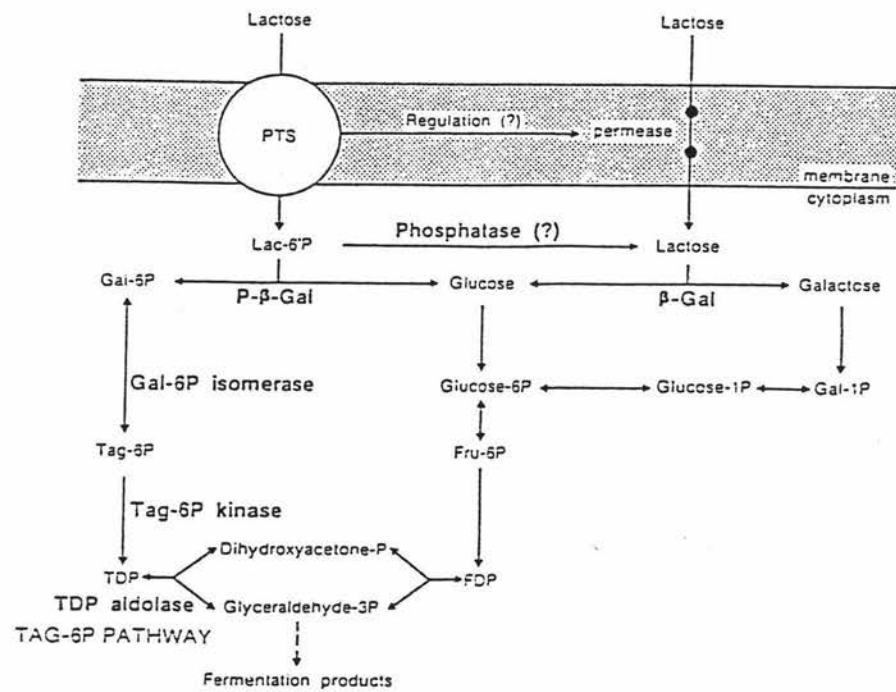
Figure 1.2. Utilization of lactose in lactococci [from Crow et al., 1983]

1.3.2 Lactococcal proteinases

1.3.2.1 Role of proteolysis in cell growth

Starter cultures are required to grow rapidly during milk fermentations to produce sufficient acid for good quality products. The free amino acids and small peptides normally present in fresh milk can however only support the growth of *L. lactis* subsp. *cremoris* to cell densities corresponding to 8-16% of those found in coagulated (fully grown) milk cultures [Mills and Thomas, 1980]. These auxotrophic organisms need at least 10 to 13 amino acids for fast growth [Thomas and Prichard, 1987]. In milk, most of these amino acids are in short supply, thus starters critically depend on their proteolytic systems to break down milk protein to produce sufficient free amino acids and small peptides which can be transported through the bacterial cell membrane (Fig. 1.3).

A survey of the proteolytic activity towards milk-casein revealed that 23 Prt$^+$ *L. lactis* subsp. *cremoris* strains isolated from mixed starter cultures were able to degrade ß-casein [Thomas and Pritchard, 1987]. Marked perference for ß-casein was found with proteinases from *L. lactis* subsp. *cremoris* AC1 [Geis et al., 1985], HP [Exterkate and de Veer, 1985], Wg2 [Hugenholtz et al., 1984] and five *L. lactis* subsp. *lactis* strains [Monnet, 1987]. The proteinases from *L. lactis* subsp. *cremoris* AM1 and SK11, however, were found to be able to degrade ß- and α_{s1}-casein equally well, although their specifities towards ß-casein were different from those of the other proteinases [Visser et al., 1986; Simon et al., 1985].

Detailed studies on the ß-casein degradation by proteinases from *L. lactis* subsp. *lactis* 763 [Monet et al., 1986], *L. lactis* subsp. *cremoris* HP [Visser et al., 1988], AC1 [Monnet et al., 1989], H2 [Ng, 1988] showed that the cleavage sites were limited to the C-terminal region, and that their cleavage patterns had minor differences.
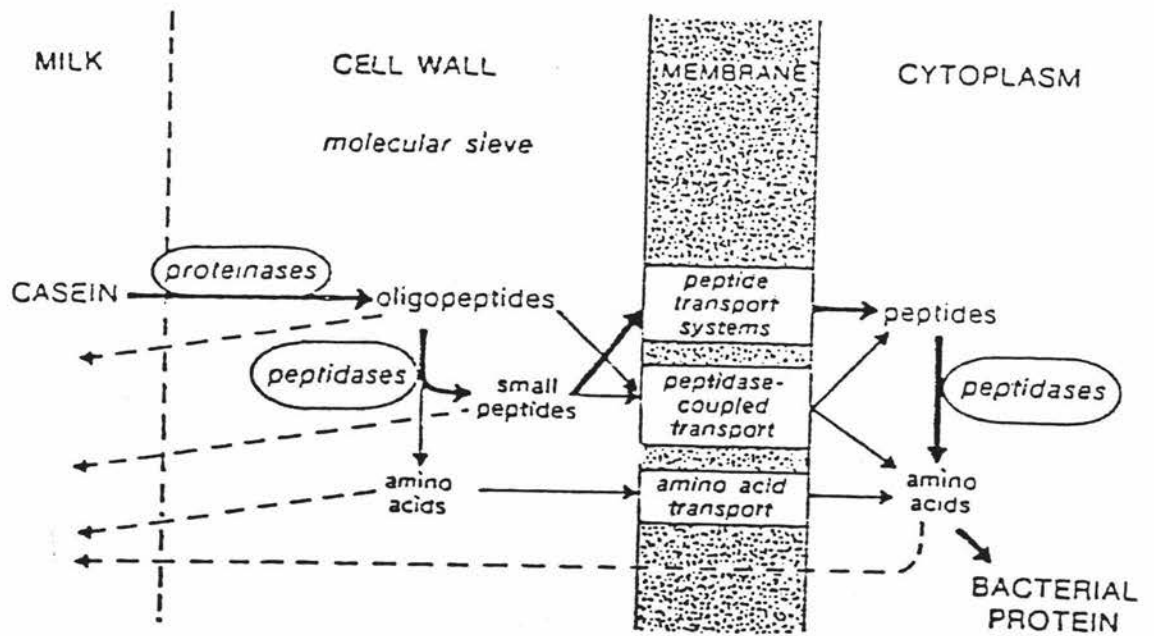
Figure 1.3   Utilization of casein for growth of lactococci in milk [Thomas and Pritchard, 1987]

1.3.3.2 Classification of lactococcal proteinases

The problem of how to classify the different cell-wall bound proteinases has led to different classification systems being proposed. Complex profiles of cell wall proteinases have been shown to exist in *L. lactis* subsp. *lactis* [Cliffe and Law, 1985]. Classification work has been carried out only on the relatively simple *L. lactis* subsp. *cremoris* proteinase system. Initial studies were involved in the investigation of the effect of pH and temperature on proteinases. Three types of activities were distinguished; two acid proteinases designated PI and PIII, with optimal temperatures of 40°C and 30°C, respectively, and a neutral proteinase, PII, with an optimal temperature of 30°C. Subsequently, the PII was reported to be an artifact of PI [Visser et al., 1986]. By using the sensitive cross-immunoelectrophoresis (CIE) technique, proteinases were next differentiated into several components; A, B, C and A'. Component A was present in all strains [Hugenholtz et al., 1984] (Table 1.4). In studying the proteolytic system of *L. lactis* subsp. *cremoris* Wg2, two proteolytic components A and B were identified. They were difficult to separate by gel filtration, ion exchange chromatography and isoelectric focusing, indicating that the two proteins had very similar MWs (140 kDa) and isoelectric points (pH 4.5). Separation was ultimately achieved by the CIE technique. The authors postulated that the two proteins A or B could be produced by autoproteolytic digestion from the other. The produced protein had a similar MW, but different antigenic properties when compared with the parent protein [Hugenholtz et al., 1987].

A recent classification system was based on the patterns of degradation of the different casein components by partially purified enzymes [Visser et al., 1986]. All strains belong to one of three types, AM1-type (degrading α_{s1}- as well as ß-casein), HP type (degrading only ß-casein with a specificity different from that of AM1-type), and a third group possessing both types of specificities. These results, however, are difficult to reconcile with other data, which indicated the presence of a common proteolytic component A in all strains examined (Table 1.4).

Table 1.4 Composition of the proteolytic systems of *L. lactis* subsp. *cremoris* strains [Hugenholtz et al., 1984]

L. lactis subsp. *cremoris*	Components of the proteolytic system	Proteolytic activity
Wg2, HP, C13 and ML1	A and B	PI and PIII
E8	A and C	PI
TR, FD27 and US3	A, B and C	PI,PII and PIII
AM1, SK11	A, (B), C and A'	PIII

Future studies involving proteinase gene probes and DNA hybridization might be able to elucidate the relationships of different proteinases in any one strain, or between different strains.

1.3.2.3 Role of proteolysis in cheese and casein production

In cheesemaking, whole milk is initially inoculated with both starter cultures and the highly specific proteolytic enzyme chymosin, and lactic acid is produced by the lactococci in the chymosin-induced coagulum. The whole process typically takes between 4.0-4.5 hours at temperatures ranging from 32-37° C. Casein, on the other hand, uses similar starters growing in skim milk at 26° C to pH 4.6 in order to acid precipitate this class of milk protein.

Starter proteinases, besides playing an important role in degrading milk protein to supply essential amino acids for cell growth, also ultimately contribute significantly to the production yield and development of flavour for both cheese and casein. Different aspects of proteolysis affecting cheese production and quality have been reviewed in the past years, e.g. flavour [Law, 1984a], texture [Lawrence et al., 1987], bitterness [Richardson and Creamer, 1973; Visser, 1981; Stadhouders et al., 1983; Exterkate, 1987] and accelerated ripening [Law, 1984b]. Proteinases function in

cheese ripening by slowly degrading the large peptides produced by chymosin action on casein [O'Keefe et al., 1976; Visser, 1977; Zevaco and Desmazeaud, 1980]. Electron-microscopic examination of 5-month old Cheddar cheese showed extensive degradation of the bacterial cell wall but the spheroplasts remained intact [Umemoto et al., 1978]. This may be due to the semi-solid gel structure in which the cells are embedded in cheese and the osmotic stability provided by the high solute concentrations in the moisture phase [Thomas and Pritchard, 1987]. Consequently, cell wall bound proteinases rather than intracellular proteinases are likely to be predominant in the protein degradation in cheese.

It has also been consistently reported that cheese made with chymosin alone contained very little peptides and amino acids, compared with cheese made using the normal mixture of starter and chymosin [O'Keefe et al., 1976; Visser, 1977]. That is, cell wall bound proteinases from starters played a major role in cheese proteolysis [Mills and Thomas, 1980]. Intracellular proteinases and peptidases would also function during the ripening if the cells were lysed and released into the cheese matrix [Law et al., 1974]. Because they possess wide specificities; the peptidases of lactococci are probably capable of completely hydrolysing casein-derived peptides to amino acids [Mou et al., 1975]. The small peptides and amino acids produced by starter proteolysis have an important influence on Cheddar cheese flavour.

Recently, the possibility of shorting the cheese ripening time by adding plasmid-free starter cells to obtain a high rate of proteolysis has been investigated. Using a Lac⁻Prt⁻ strain of *L. lactis* subsp. *cremoris* C2 with normal single strain cultures of *L. lactis*. subsp. *cremoris* to make cheese, it was discovered that cheese with the modified C2 strain matured faster than normal, and that the rate of proteolysis during storage, the concentration of amino acids and overall flavour were all increased [Kempler et al., 1979; Aston et al., 1983a,b; Grieve and Dulley, 1983].

Although increasing proteolysis can be used to accelerate cheese ripening, excessive

proteolysis, besides causing bitterness, is also likely to decrease cheese or casein yield by producing soluble N in whey. Several reports presented using mixed cultures for lactic casein making [Thomas and Lowrie, 1975] and for cheesemaking [Stadhouders and Hup, 1975; Hugenholtz, 1986] with predominantly (70-80%) Prt⁻ cells have made good cheese [Mills and Thomas, 1980; Richardson et al., 1983; Richardson, 1984]. Here the Prt⁻ cells are able to utilize the protein degradation products formed by the Prt⁺ population.

Because of the significant involvement of the starter proteolytic enzymes in cheese, long-term research goals include using recombinant DNA technology to introduce optimum proteinase activity and specificity into cultures used for cheese and casein manufacture. These studies are likely to lead to improve the flavour, yield and ripening time of these major dairy products.

PART II: GENETICS OF LACTOCOCCI

1.4 Introduction

It has long been known that the important dairy-relevant properties of lactococci are unstable because their genes are encoded in plasmids. Genetic studies on lactococci were first directed at the transfer of plasmids by conjugation into and between lactococcal strains. Other transfer systems such as transduction and transformation have been subsequently established. In the past few years, the application of recombinant DNA techniques in the molecular analysis of lactococci has evolved rapidly, and there has been much progress in the development of gene cloning systems. More recently, electroporation techniques provide an easy means of transferring DNA into lactococcal cells. The prospect of improving dairy starters by molecular genetic techniques looks promising.

1.5 Lactococcal Plasmids

The phenotypic instability of several key metabolic properties of lactococci has long been recognized, e.g. reduced ability to grow in milk, loss of ability to metabolize citrate and loss of phage resistance. In the early 1970s, biochemical studies on lactococci showed that lactose and/or proteinase-deficient variants were readily isolated due to the relatively high spontaneous loss of these properties. In many pure cultures, these "slow variants" could be isolated at frequencies of 0.1-1.0%. These observations led to the speculation that these properties were plasmid-encoded in lactococci [Pearce, 1970; McKay et al., 1972].

Spontaneous or enhanced loss of plasmids by treatment with acridine dyes, elevated temperature and protoplast curing has indicated the plasmid linkage of many important properties in lactococci. In addition to lactose metabolism and proteinase production, linkage also includes glucose-, galactose-, sucrose-, mannose-, xylose- and

citrate metabolism, restriction and modification systems, phage insensitivity, nisin and bacteriocin production, and resistance against inorganic salts and ultraviolet light [Davies and Gasson, 1981; McKay, 1983; Kondo and McKay, 1985].

These speculations were later confirmed by both physical and genetic studies. Several DNA transfer systems are operative in lactococci and have been used to provide both phenotypic and physical evidence for plasmid linkage of essential milk fermentation properties. These will be discussed below.

1.6 Gene Transfer Systems in Lactococci

1.6.1 Transduction

Transduction, the bacteriophage-mediated genetic exchange of bacterial DNA, was the first DNA transfer system to be reported in genetic studies of lactococci. At first, transduction with virulent bacteriophages was used to transfer tryptophan independence in *L. lactis* subsp. *diacetylactis* 18-16 [Sandine et al., 1962] and streptomycin resistance in *L. lactis.* subsp. *lactis* C2 [Allen et al., 1963]. There was no further work on gene transfer of any type in lactococci for the next ten years until a temperate phage transduction system was developed for *L. lactis* subsp. *lactis* C2. Using this system, chromosomal maltose and mannose markers and plasmid encoded lactose genes were transferred [McKay et al., 1973]. Work from the same laboratory also established a 50% frequency of cotransfer of the *lac* and *prt* genes of strain C2 [McKay and Baldwin, 1974].

Transduction results thus provided evidence for plasmid linkage of metabolic traits in lactococci [McKay et al., 1976]. Using bacteriophages induced from a Lac transductant, the frequency of Lac transfer could be increased by a high frequency transfer (HFT) phenomenon [McKay et al., 1973]. Transduction of the 30

kDa plasmid of *L. lactis* subsp. *lactis* C2 to plasmid-free recipients produced Lac$^+$Prt$^-$ and Lac$^+$Prt$^+$ transductants harbouring a plasmid of approximately 20-21 kDa in size. Lac$^-$ derivatives isolated either spontaneously or after acriflavine treatment lost this plasmid. It was speculated that the limiting size of the phage head resulted in a process termed transductional shortening after the isolation of two defective transducing phages [Klaenhammer and McKay, 1976; McKay et al., 1976]. Transduced *lac* genes, besides being plasmid-encoded, were also able to integrate into the chromosome in the homologous transduction system of *L. lactis* subsp. *lactis* strain C2 [McKay and Baldwin, 1978; Snook et al., 1981].

A similar Lac transduction behavour was observed in *L. lactis* subsp. *lactis* 712, the parent of strain C2 [Davies and Gasson, 1981]. These authors also found that the erythromycin resistance plasmid pAMß1, originally introduced by conjugation from *Streptococcus faecalis* DS-5, could be transduced between lactococci [Gasson and Davies, 1980]. Transduction systems have also been reported in other strains of *L. lactis* subsp. *lactis* and in *L. lactis* subsp. *cremoris* [Mckay et al., 1980]. *L. lactis* subsp. *lactis* C2 obtained *lac* genes from transduction with a temperate phage induced from *L. lactis* subsp. *cremoris* C3 [Snook et al., 1981].

Transduction has made a significant contribution to the advancement of genetic studies of lactococcci and a commercial application was described. It was found that the stabilized chromosomal Lac$^+$Prt$^+$ transductant of *L. lactis* subsp. *lactis* C2 which was less proteolytic than the wild type was capable of producing a Cheddar cheese with a reduced bitterness [McKay and Baldwin, 1978; Kempler et al., 1979]. This effect was most likely due to the reduced copy number of the proteinase gene.

1.6.2 Conjugation

Conjugation, the transfer of genes between two cells in close physical contact, was the

second gene transfer system discovered operative in lactococci. Transfer of lactose utilization genes by conjugation was first reported in *L. lactis* subsp. *lactis* 712 [Gasson and Davies, 1979; 1980], and from *L. lactis* subsp. *diacetylactis* 18-16 to a plasmid free derivative of *L. lactis* subsp. *lactis* C2 [Kempler and McKay, 1979]. Subsequently, conjugation of lactose plasmids has been carried out from many lactococci to this same derivative of *L. lactis* subsp. *lactis* C2 and also from *L. lactis* subsp. *cremoris* C3 to derivatives of *L. lactis* subsp. *lactis* ML3 and *L. lactis* subsp. *cremoris* B1 [McKay et al., 1980; Snook, et al. 1981].

Conjugation frequencies in the first described experiments were low (10^{-5}-10^{-9} per donor). Among the transconjugants from matings involving *L. lactis* subsp. *lactis* C2, 712 and ML3 strains, however, high frequency donors were isolated. Transfer frequency of the Lac plasmid could reach as high as 10^{-1} per donor. High frequency donors were observed in *L. lactis* subsp. *lactis* strains 712, C2, ML3, ME2, C20, in *L. lactis* subsp. *cremoris* M43 and in *L. lactis* subsp. *diacetylactis* DRC3 and WM4 [Gasson and Davies, 1980; Anderson and McKay, 1983; Scherwitz et al., 1983; McKay and Baldwin, 1984; Klaenhammer and Sanozky, 1985; Kondo and McKay, 1985; Steenson and Klaenhammer, 1985]. All these variant strains exhibited a cell aggregation phenomenon which caused obvious changes both in the colony morphology and in the appearance of broth cultures. Physical study of lactose plasmids from aggregating progeny revealed the presence of novel enlarged plasmids [Walsh annd McKay, 1981, 1982; Gasson, 1983].

The wide host range plasmids pAMß1 and pIP501 have been conjugated into a wide range of lactococci strains. Plasmid pAMß1 has been proven to be useful for its ability to mobilize nonconjugative or poorly conjugative plasmids. Hays et al. [1987] used this property to mobilize proteinase genes from *L. lactis* subsp. *cremoris* strains UC317, UC205 and UC411 to *L. lactis* subsp. *lactis*. The wide range of host strains in which these plasmids replicate and transfer phenotypes have made pAMß1 and pIP501 potentially useful plasmids both for *in vivo* strain development and as the

basis for new vector constructions [Gasson, 1983; Evans and Macrina, 1983].

Before efficient transformation by electroporation was established (see section 1.6.6), naturally transformable organisms such as *S. sanguis* were used as intermediate donors to conjugatively mobilize plasmid constructs to the dairy lactococci [Smith and Clewell, 1984; Romero et al., 1987]. By using the broad-host-range conjugative plasmid pVA797, the partially homologous cloning vectors pVA838 and pSA3 were introduced into various non-transformable strains. Conjugation therefore has the advantage of technical feasibility and the ability to disseminate genetic information both within and between species. Yu and Pearce [1986], for example, conjugated several plasmids from lactococci to *Clostridium acetobutylicum*.

The plasmid linkage of several important lactococcal characteristics was established by conjugation experiments, including lactose metabolism and proteinase production [McKay and Baldwin, 1984], resistance to nisin and bacteriophages [McKay and Baldwin, 1984], production of the bacteriocin, diplococcin [Davey, 1984; Neve et al., 1984] and a restriction and modification system [Chopin et al., 1984]. As for commercial application, conjugation has been used to select a nisin-producing strain which dramatically enhanced the production yield [Tsai and Sandine, 1987] and for the construction of bacteriophage-resistant dairy starter strains [Sanders et al., 1986].

1.6.3 Transposition

Transposons are genetic elements which are able to transfer from one DNA molecule to another. Transposons isolated from other systems have been conjugatively transferred into lactococci. Tn919 from *Streptococcus sanguis* FC1 and Tn916 from *S. faecalis* DS16 are the best studied transposons that have been introduced into lactococci [Tomich et al., 1980; Frank and Clewell, 1981; Hartley et at., 1984; Clewell et al., 1984; Fitzgerald and Clewell, 1985; Clewell et al., 1985]. These transposons

which range from 15 to 17 kb in size, encode tetracycline resistance, and share the property of conjugative transfer in the absence of plasmid DNA at frequencies ranging from 10^{-5} to 10^{-9} per donor. Both elements have been transferred to a number of lactococcal species by the filter mating technique and have also been cloned in *Escherichia coli*.

Tn916 has been shown to conjugatively transfer among strains of *S . faecalis*, *Streptococcus mutans*, *Streptococcus agalactiae*, and *L. lactis* subsp. *lactis* [Clewell, 1986]. Tn919 has been observed to transfer conjugatively among *S. faecalis*, *L. lactis* subsp. *lactis*, *L. lactis* subsp. *diacetylactis*, *L. lactis* subsp. *cremoris*, *Lactobacillus plantarum*, and *Leuconostoc cremoris* [Hill et al., 1985].

Tn916 has been shown in several systems to insert at different sites on the bacterial chromosome, and in at least some strains, there may be insertional hot-spots. Tn916 and its related transposons have been useful in locating the genes of hemolysin production on the *S. faecalis* plasmid pAD1 [Clewell et al., 1982] and genes involved in the malolactate, maltose, mannose and arginine metabolic pathways in *L. lactis* subsp. *lactis* IL1441 [Renault and Heslot, 1987]. Tn919 was reported to insert in a hot spot in *L. lactis* subsp. *lactis* MG1363 [Hill et al., 1985].

Originally, the frequencies of conjugative transfer of Tn919 observed with lactococcal recipients were generally too low (10^{-8} per recipient) for practical mutant screening purposes. The introduction of pMG600 (a deletion plasmid derived from pLP712) into a *L. lactis* subsp. *lactis* CH919 donor (an MG1363 strain containing Tn919 on its chromosome), generating *L. lactis* subsp. *lactis* CH001, resulted in a significant improvement in the transfer frequency of Tn919 to *L. lactis* subsp. *lactis* CK50 (1.25×10^{-4} per recipient) [Hill et al., 1987].

Similar high frequency transfer of Tn919 into *L. lactis* subsp. *diacetylactis* 18-16S has been observed, with inserts in transconjugants occurring at different sites on the

chromosome and also in the plasmid DNA. *L. lactis* subsp. *lactis* CH001 was however unable to transfer the transposon into *L. lactis* subsp. *cremoris*, *Lactobacillus* or *Leuconostoc* strains in agar surface matings. This indicated that the high frequency delivery system was somewhat strain specific, and that the conjugal functions specified by pMG600 were incompatible with some recipient cell types [Hill et al.,1987].

The application of conjugative transposons to gene transfer is an exciting development in the genetic analysis of lactococci. This type of work provides a useful genetic tool for the exploration of chromosomal genes of lactococci.

1.6.4 Protoplast fusion

Protoplast fusion as a genetic system was first reported in 1980 for lactococci [Gasson, 1980]. In this system, osmotically stabilized protoplasts were induced to fuse together and regenerated into recombinant cells. Cell walls were removed by either lysozyme [Gasson, 1980; Okamoto, 1985] or mutanolysin [Cocconcelli et al., 1986; Reed, 1987], and protoplasts were fused after treatment with either polyethylene glycol [Gasson, 1980; Simon et al., 1985; Vossen et al., 1988], or in the presence of an electric field [Reed, 1987]. Strains of *L. lactis* subsp. *lactis* or *L. lactis* subsp. *cremoris* could be fused [Okamoto et al., 1985; Simon et al., 1985] as well as lactococci to *Lactobacillus* [Cocconcelli et al., 1986; Reed, 1987] or *B. subtilis* [Baigori et al., 1988; Vossen et al., 1988]. Compared with plasmid transfer, transfer of chromosomal markers was relatively low [Okamoto et al., 1985].

Protoplast fusion appears to have broad application in inducing genetic recombination. The fusion process has been shown to generate large numbers of random recombinants [Okamoto et al., 1985]. Furthermore, because the fusion is neither strain- nor species-specific, this method has the potential of making a wider range of genetic material available for future lactococcal strain improvement.

1.6.5 Protoplast transformation

Though there have been few reports on whole cell transformation [Sanders and Nicholson, 1987], the thick peptidoglycan which characterizes the cell wall of lactococci has been considered to be a barrier to DNA uptake and thus a hinder to the development of successful transformation systems. To circumvent this barrier, the possibility of protoplast transformation was investigated. Gasson [1980] first reported a method to produce and regenerate lactococcal protoplasts. Two years later, the first paper describing the transformation of a Lac plasmid into *L. lactis* subsp. *lactis* LM3302 protoplasts was published [Kondo and Mckay, 1982]. The protoplasts were induced to take up DNA by using polyethylene glycol (PEG), a procedure commonly used in the transformation of *Streptomyces* and *Bacillus* [Bibb et al., 1978; Chang and Cohen, 1979]. Geis [1982] proceeded in a similar way to transfect bacteriophage DNA into *L. lactis* subsp. *diacetylactis* F7/2 at a frequency of 5×10^4 cfu/μg DNA [Geis, 1982]. Since then, several protocols have been adapted and optimised to increase protoplast transformation (10^4 to 10^6 cfu/μg DNA) of a limited number of strains of *L. lactis* subsp. *lactis* [Kondo and Mckay, 1984; Gasson and Anderson, 1985; von Wright et al., 1985; Simon et al., 1986; Woskow and Kondo, 1987], *S. faecalis* [Smith, 1985; Wirth et al., 1986] and *Streptococcus thermophilus* [Mercenier et al., 1988]. A very low frequency transformation of *L. lactis* subsp. *cremoris* has also been reported [Simon et al., 1985; Woskow, 1987].

The success of protoplast transformation directly depends on the ability to achieve a controlled cell wall digestion such that the osmotically fragile cells can be later regenerated to their normal state. Many parameters such as conditions for protoplast formation, PEG treatment, regeneration and etc. require careful optimization for each new recipient. Usually, published procedures have been found to be difficult to reproduce in another laboratory. Protoplast transformation has been succesfully used to transform plasmid constructs from intermediate hosts such as *E. coli* or *B. subtilis* to lactococci [de Vos, 1987]. There have been very few reports of directly cloning

genes into lactococci by this method.

1.6.6 Electroporation

Electroporation, which involves the application of a brief and high voltage pulse to a suspension of cells and DNA, is now recognized as one of the most promising tools for the introduction of DNA into lactococci. The pulse results in transient membrane permeability allowing uptake of the DNA. Harlander [1986] first reported the transfer of plasmids pSA3 and pGB301 into *L. lactis* subsp. *lactis* LM0230 by electroporation. By now, a wide range of lactic acid bacteria can be transformed using this technique. These are *L. lactis* subsp. *lactis* [Powell et al., 1988; Luchansky et al., 1988], *L. lactis* subsp. *cremoris* [van der Lelies et al., 1988] and *S. thermophilus* [Somkuti and Steinberg, 1988], *Lactobacillus asidophilus*, *Lactobacillus casei*, *Enterococcus faecalis* and *Leuconostoc dextranicum* [Chassy and Flickinger, 1987; Luchansky et al., 1988].

Electroporation now provides the simplest and quickest method of transforming DNA into lactococci. Cells can be frozen for future use; ligation mixtures and "miniprep" DNA can be successfully transformed at high frequency. However, not every strain can be electroporated and optimal conditions varied with different strains. The culture medium, growth phase and ionic strength of electroporation buffer are influential to the success. Perhaps the most important parameters are field strength, duration of pulse and shape of the pulse, which should be optimised in order to obtain the highest efficiency.

PART III: LACTOCOCCAL VECTORS AND GENE CLONING

1.7 Construction of Lactococcal Vectors

As a consequence of the establishment of gene transfer systems, gene cloning techniques have been successfully applied in the molecular analysis of lactococcal genes in the last few years. This part summarizes the major progress in lactococcal vector construction and their use in gene cloning.

1.7.1 Vector construction based on non-lactococcal plasmid replicons

The first lactococcal vectors were constructed by combining the plasmid replicons from *E. coli* (pACYC184) and *S. sanguis* (pGB305 and pVA749). The commonly-used vectors in this class are pSA3 [Dao and Ferretti, 1985], pVA series [Macrina et al., 1982, 1983] and the pMU series [Achen et al., 1986]. Although these vectors were used for cloning some lactococcal genes, it was found that they were transformed into lactococci with low efficiency. Gene cloning with these vectors was usually conducted first in *E. coli* or *S. sanguis* to construct recombinant plasmids, which were then introduced into lactococci. Several studies reported that the recombinant plasmids obtained using these vectors were usually very unstable in lactococci [Dao and Ferretti, 1985; Kim and Batt, 1988; Macrina et al., 1983; Simon and Chopin 1988; Thompson and Collins, 1989; Pillidge, C.J. and Ward, L.J.H., personal communications].

1.7.2 Construction based on broad host-range plasmid replicons

The second class of lactococcal vectors were constructed using the broad host-range plasmid replicons pIP501 (30 kb) or pAMß1 (26 kb), which cannot replicate in *E. coli*.

Deletion derivatives of these two plasmids initially resulted in pGB301 [Behnke et al., 1981] and pHV1301 [Chopin et al., 1986], respectively. Both of these derivatives retained the ability to replicate and express the antibiotic resistance markers (Em^r) in lactococci [de Vos, 1987]. pGB301, the first gene cloning vector described for lactococci [Kondo and McKay, 1984], was however frequently found unstable when inserts were cloned into it [Behnke et al., 1981]. Recently a new series of pIL vectors was constructed by introducing multiple cloning sites into various small pAMß1 deletion derivatives. These vectors could be used to clone large inserts up to 30 kb. The recombinant constructs were however also unstable in lactococci [Simon and Chopin, 1988].

1.7.3 Construction based solely on lactococcal plasmid replicons

The third class of vectors was derived from small cryptic lactococcal plasmids. In this class are the pGK series [Kok et al., 1985; van der Vossen et al., 1985] based on the *L. lactis* subsp. *cremoris* plasmid pWV01 (2.3 kb), pCK series [Anderson and Gasson 1985] and pNZ series [de Vos, 1986a] based on the *L. lactis* subsp. *lactis* plasmid pSH71 (2.1 kb). Subsequent studies showed that pWV01 and pSH71 had comparable physical maps and shared common sequences [de Vos, 1987]. This class of vectors was found to be functional in a wide range of hosts including *E. coli*. Recently, it was reported that lactococcal pCI305 plasmid replicon, which showed no homology to pWV01 or pSH71 by DNA hybridization, was used for vector construction. Vectors derived from this replicon, however, could not replicate in *E. coli* [Hayes et al., 1990].

1.7.4 Vector construction for genetic studies in lactococci

Besides the general cloning vectors which are described above, several vectors have

been constructed for other types of genetic studies in lactococci. These are promoter-probe vectors for identifing DNA sequences which regulate gene expression, e.g. pGKV110 [van der Vossen et al., 1985], pNZ220 [de Vos, 1987], pCK509 [Gasson, 1988] and pMU series [Achen et al., 1986]; the expression vector pMG36, constructed using the lactococcal gene expression signal for expression of heterologous genes in lactococci [van de Guchte et al., 1989]. The chromosome integration vector pHV60 was also constructed for stablizing genes in the lactococcal chromosome [Leenhouts et al., 1989].

1.8 Lactococcal Gene Cloning and Studies

With the various cloning vectors, many lactococcal genes have been cloned. Initial cloning was of plasmid-linked genes such as proteinase and lactose utilization. More recently, chromosomal gene cloning has also been reported (Table 1.5). Some of these cloned genes have now been sequenced including proteinase [Kok et al., 1988; Kiwaki et al. 1989; Vos et al., 1989], phospho-ß-galactosidase [de Vos and Gasson, 1989], nisin [Kaletta and Entian, 1989], phage lysin [Shearman et al., 1989], phage repressor [Lakshmidevi et al., 1990], and insertion element proteins [Haandrikman et al., 1990]. These gene sequence data and the translation initiation signal sequences identified by promoter-probe vectors have provided us an understanding of gene transcription in lactococci [de Vos, 1987; van der Vossen et al., 1987; Lakshmidevi et al., 1990]. Several lactococcal gene expression initiation signals from the sequenced DNA are listed in Table 1.6, and can be described with the following features: (1) typical -35 and -10 sequences similar to the *E. coli* consensus promoter sequences TTGACA and TATAAT, respectively [Harley and Reynolds, 1987]; (2) 15-18 base spacing between the hexanucleotides; (3) the start point for transcription at a ATG sequence; (4) and the Shine-Delgarno sequences showing high complementarity with the 3'end of lactococcal 16S ribosomal RNA (3'AUUUUCCUCC) [de Vos, 1987]. In transcription termination regions, a typical rho-independent termination structure

Table 1.5 Summary of lactococcal genes cloned

Genes	Origins	Hosts for cloning	Vectors	References
Lac[a]	L.lactis C2 (pLM2001)	L.lactis S. sanguis	pGB301 pDB101	Kondo & McKay 1984 Harlander et al. 1986
Lac[b]	L.lactis H2 (pDI21)	E.coli	λ1149	Yu et al. 1989
pbg	L.lactis 712 (pLP712)	E.coli L.lactis E.coli	pAT153 pNZ12 pNZ12	Maeda & Gasson 1986 de Vos & Gasson 1989 de Vos & Gasson 1989
pbg	L.cremoris H2 (pJI70)	E.coli	pUC18/19	Inamine et al. 1986
T6A	L.lactis 4122 (pDI1)	E.coli	pACYC184	Limsowtin et al. 1986
prtP	L.cremoris Wg2 (pWV05)	L.lactis B.subtilis	pGKV2 pGKV2	Kok et al. 1985 Kok et al. 1985
prtP	L.lactis 712 (pLP712)	L.lactis B.subtilis	pCK1 pCK1	Gasson et al. 1987 Gasson et al. 1987
prtP	L.cremoris SK11 (pSK111)	L.lactis E.coli	pNZ122 λEMBL3	de Vos et al. 1989 de Vos et al. 1989
prtP	L.lactis 763 (pLP763)	L.lactis B.subtilis	pGKV11 pGKV11	Kiwaki et al. 1989 Kiwaki et al. 1989
prtM	L.cremoris Wg2 (pWV05)	L.lactis E.coli	pGKV2 pT712	Haandrikman et al. 1989
prtM	L.cremoris SK111 (pSK111)	L.lactis E.coli	pNZ511 pT7-5	Vos et al. 1989
Prt	L.lactis SSL135 (chr.)	L.lactis	pVS2	von Wright et al. 1987
repAB	L.lactis UC317 (pCI305)	E.coli	pBR322	Hayes et al. 1990
UV[r]	L.lactis IL594 (pIL7)	L.lactis	pHV1301	Chopin et al. 1986
Tc[r]	L.lactis IL1458 (Tn916)	L.lactis	pIL	Behnke et al. 1988
bpi (φBK5-T)	L.cremoris	L.lactis E.coli	pMU1328 pACYC184	Lakshmidevi et al. 1990
lyn	L.lactis (φvML3)	E.coli	λgt10	Shearman et al. 1989
Hsp	L.lactis LMA12-4 (pTR2030)	L.lactis E.coli	pSA3 pSA3	Hill et al. 1989 Hill et al. 1989
Bac	L.lactis WM4 (pCS26)	L.lactis	pGB301	Harmon & McKay 1987
mleR	L.lactis (chr.)	L.lactis	pIL275	Renault et al. 1989
Nis[r]	L.lactis DRC3 (pNP40)	L.lactis	pIL252	Simon & Chopin 1988
Nis	L.lactis 6F3 (plasmid)	E.coli	pUC19	Kaletta & Entian 1989
Nis	L.lactis ATCC 11454 (chr.)	E.coli	λJ1	Buchman et al. 1988
Nis	L.lactis FI5876 (chr.)	E.coli L.lactis	λEMBL3 pTG262	Dodd et al. 1990 Dodd et al. 1990
IS-N1	L.cremoris Wg2	E.coli	M13mp18,19 pUC19	Haandrikman et al. 1990
IS-W1 IS-W2	L.cremoris SK11	E.coli	M13mp18,19	Haandrikman et al. 1990
thyA	L.lactis 712	E.coli	pLAFR1, pBR322 pACYC184, pUC19	Ross et al. 1990b

Abbreviations: Lac[a], Lac-PTS (enzyme II, factor III) and phospho-β-galactosidase; Lac[b], tagatose-6-phosphate pathway enzyme genes, Lac-PTS and phospho-β-galactosidase; pbg, phospho-β-galactosidase; T6A, tagatose-1,6-bisphosphate aldolase; prtP, proteinase; prtM, proteinase maturation protein; Prt, Prt[+] phenotype; repAB, plasmid replication function; UV[r], resistance to UV irradiation; Tc[r], resistance to tetracycline; bpi, BK5-T promoter inhibitor; lyn, phage lysin; Hsp, bacteriophage resistance mechanism; Bac, bacteriocin production; mleR, activation function for malolactic system; Nis[r], resistance to nisin; Nis, nisin production; chr., chromosome; IS, insertion element protein; thyA, thymidylate synthase.

[Rosenberg andCourt, 1979] was found in the lactococcal *prt* gene [Kok et al., 1987] and in pSH71 plasmid DNA [de Vos, 1987] as a GC-rich inverted repeat, followed by a stretch of U-residues.

Table 1.6 List of lactococcal gene expression signals

Sequence origin	-35	-10	-35 & -10 space	RBS	Start codon	RBS & start codon space	References
L. cremoris Wg2 chr.							van der Vossen et al. 1987
21	TTGACA	TATTAT	18 bp				
23	ATGACA	TAAAAT	17 bp	AAAGGGAGG	ATG	9 bp	
32	TAGAAA	TATACT	17 bp	GGAGG	ATG	9 bp	
44	TTGTTT	AATAAT	18 bp	GAAAGGAGT	ATG	7 bp	
59	TTGACA	TAGAAT	17 bp				
L. cremoris φBK5-T							Lakshmidevi et al. 1990
pa1	CTTCCA	TACTAT	18 bp				
pf2	TGGACT	TGTAAT	17 bp				
pa3	TTTATA	TATAGT	18 bp				
pg2	CAGACA	TATATT	16 bp				
pf1	TTGGAG	TACTCC	17 bp	AGGA	ATG	10 bp	
φSK11							
1	TAGACA	TAAAAT	15 bp				de Vos et al. 1987
2	TTGAAC	TAAAAT	16 bp				
L. lactis 712 pbg	TTGATT	TATTTG	17 bp	AAAGGA	ATG	9 bp	de Vos & Gason 1989
L. lactis φVML3	TTGAGG	TTATAA	16 bp	GGGAA	ATG	3 bp	Shearman et al. 1990
L. cremoris pSK111 IS-N1	TTGCAA	TATTTT	16 bp	AGAGGA	ATG	7 bp	Haandrikman et al. 1990
pWV05 IS-W1	TTGCAA	TATTTT	16 bp	AGAGGA	ATG	7 bp	

Abbreviations: chr, chromosome; pbg, phospho-ß-galactosidase; φBK5-T, φSK11 and φvML3, lactococcal phages; IS, insertion element protein.

1.9 Genetics of Lactococcal Proteinases

Because the lactococcal proteinases play an essential role in milk fermentations as discussed previously, they have been the subject of extensive genetic research during the past few years.

The plasmid linkage of proteolytic activity in a number of lactococcal strains was initially based on curing studies. Subsequently, genetic transfer systems including transduction, conjugation and protoplast transformation, have been used to provide further evidence for this linkage [Kok and Venema, 1988]. Von Wright et al. [1987] reported that a proteolytic activity was associated with a chromosomal fragment from *L. lactis* subsp. *lactis* SSL135. Later, however, it was found that this chromosomal fragment actually encoded a peptide transport system [Tynkkynen et al., 1989]. So far, all the cell bound proteinase genes identified have been plasmid-encoded.

With the development of lactococcal gene cloning systems, Kok et al. [1985] first cloned a lactococcal proteinase in *B. subtilis* using the pWV05 plasmid of *L. lactis* subsp. *cremoris* Wg2. They then transferred the shuttle plasmid carrying the cloned proteinase gene (*prt*) into a lactococcal Prt⁻ recipient, which became Prt⁺ after transformation [Kok et al., 1985]. Subsequently, another two proteinase genes were isolated from plasmids pLP712 of *L. lactis* subsp. *lactis* 712 and pSK111 of *L. lactis* subsp. *cremoris* SK11, and a striking high degree of conservation of restriction sites was found in and around the three proteinase gene regions [de Vos, 1987]. By now, utilizing hybridization studies, many lactococcal Prt⁺ plasmids with various sizes have been found to share homology with the pWV05 *prt* gene probe [Kok and Venema, 1988].

More recently, DNA sequencing analyses of the *prt* genes from plasmids pWV05 [Kok et al., 1988b], pSK111 [Vos et al., 1989] and pLP763 [Kiwaki et al., 1989] showed that the genetic organization of these three *prt* genes was almost identical. The translated

amino acid sequences of *prt* genes have significant homology with a number of subtilisins produced by various *Bacillus* [Kok et al., 1988] and various proteinases from *Thermoactinimyces vulgaris, Tritrirachium album* and *Yarrowia lypolytica* [Vos et al. 1989a]. Deletion of the C-terminal 130 amino acids did not prevent the truncated proteinase from degrading ß-casein [Kok et al., 1988].

After further studies on the flanking regions of proteinase coding sequences in pWV05 and pSK111, a proteinase maturation [Haandrikman et al., 1989; Vos et al., 1989b] and insertion element coding region [Haandrikman et al., 1990] was found in both cases. The genetic organization in and around the *prt* gene regions of pWV05 and pSK111 is shown in Fig. 1.4. Directly upstream of the proteinase coding region *prtP* (coding for ca. 200 kDa), an oppositely orientated open reading frame *prtM*, encoding a 33 kDa lipoprotein, involved in, the maturation of *prtP* product. They provided evidence that the *prtP* product was unable to change a Prt⁻ strain phenotype unless the *prtM* product was also present [Haandrikman et al., 1989; Vos et al., 1989b]. Downstream of *prtM*, an almost identical insertion element sequence (IS element) with 18 bp terminal inverted repeats, was present in both plasmids pWV05 and pSK111. These elements were designated as IS*S1*W and IS*1*N, respectively [Haandrikman et al., 1990]. IS*1*W and IS*1*N differ individually in their orientation with respect to the *prtM* gene. In pWVO5, IS*1*W was flanked on one side by only part of a second IS element. It was proposed that *prt* gene plasmid pWV05 evolved as a deletion derivative of a precursor plasmid that carried IS elements [Haandrikman et al., 1990].
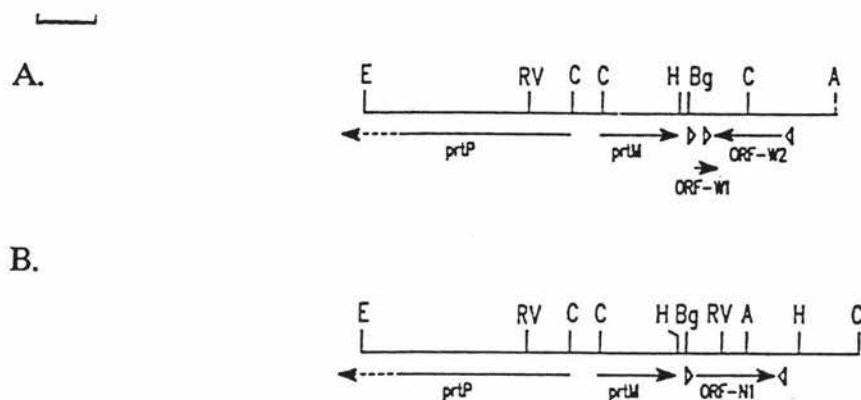
Figure 1.4 The genetic organization in and around the *prt* gene regions of pWV05 (A) and pSK111 (B). Arrows indicate the direction of transcription of *prt*, *prtP* and insertion elements [ORF-W1, ORF-W2 (IS*1*W), ORF-N1 (I*1*N)]. The 18 bp repeats are indicated by arrowheads. The small bar represents 1kb scale of DNA. A:*Acc*I; Bg:*Bg*lII; C: *Cla*I; E: *Eco*RI; H: *Hin*dIII; RV: *Eco*RV.

PART IV: LACTOCOCCAL PLASMID REPLICATION

1.10 Plasmid Replication in Gram-positive Bacteria

Many plasmids from Gram-positive bacteria including *Staphylococcus* [Novick, 1989], *Bacillus* [Gruss and Ehrlich, 1989], *Streptococcus* [del Solar et al., 1989], *Lactobacillus* [Bates and Gilbert, 1989], *Streptomyces* [Kendall and Cohen, 1988] and *Clostridium* [Gruss and Ehrlich, 1989] have been isolated, their properties studied, and used as cloning vectors. Although the range of species from which plasmids have been isolated is broad, there is considerable homology in their sequences. The regions of homology involve genes essential for replication as well as nonessential genes (e.g. plasmid recombination sites). The DNA sequences of a number of these plasmids indicate that most Gram-positive plasmids replicate via a single-stranded DNA (ssDNA) intermediate, probably by the rolling circle replication (RCR) mode (Table 1.7). The RCR mechanism has been extensively studied with ssDNA *E. coli* bacteriophages [Baas and Jansz, 1988]. A detail review on Gram-positive plasmid ssDNA replication was presented recently [Gruss and Ehrlich, 1989].

The RCR replication cycle is schematically shown in Fig. 1.5. The replication initiation protein, Rep (shown for pT181 to have topoisomerase, i.e., nicking-closing activity [Koepsel et al. 1985]) recognizes plus origin sequences and produces a nick to initiate replication (step 1). This event triggers displacement of the plus strand and polymerization of a new plus strand by 3'-OH extension from the nick (step 2). The Rep recognizes a termination sequence which overlaps the origin sequence, and produces a second nick to generate one fully replicated strand and a ssDNA monomer of the displaced strand (step 3). Finally, Rep ligates the ends of the ssDNA to form a circle, which is detectable as a free molecule (step 4). The nick that initiates and terminates a round of plus-strand synthesis occur at the same site. A minus origin (M-O) serves as an efficient initiation site, recognized by host factors, for the conversion of circular plus-strand ssDNA to double-stranded DNA (dsDNA) (step 5). The formation of the dsDNA plasmid product (step 6) marks the completion
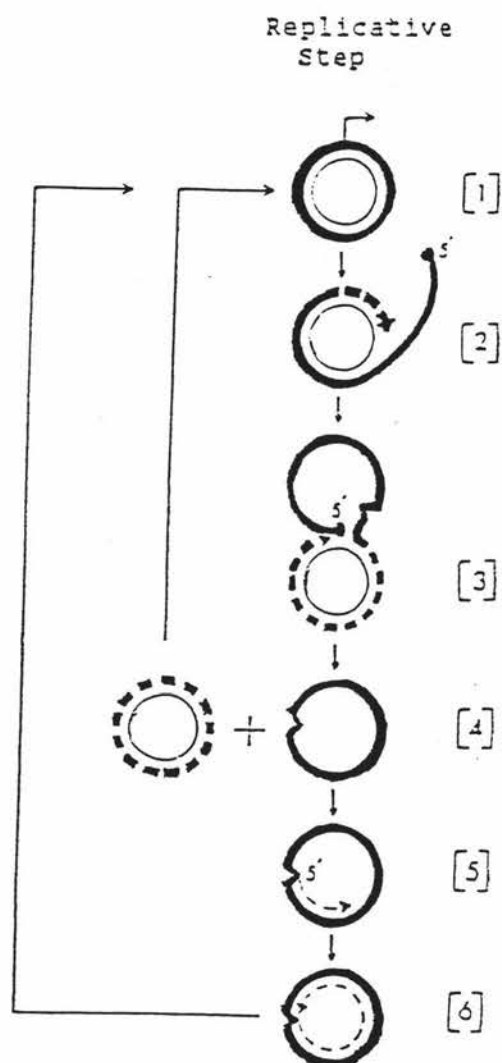
Figure 1.5 Stages of normal RCR, Symbols: ▬▬, ▬▬▬ , plus strand; ----, ——— , minus strand; ▬▬▬,———, parental DNA; ▬▬ ▬, ——— , newly synthesized DNA. --->, direction of replication; >, secondary structure at the M-O.

productive cycle of replication, in which two plasmids are generated from a single parent [Gruss and Ehrlich, 1989].

Table 1.7 Gram-positive ssDNA plasmids and their original hosts

ssDNA plasmids	Original hosts	References
pT181	*S.aureus*	Khan & Novick 1983
pC221	*S.aureus*	Projan et al. 1985
pS194	*S.aureus*	Projan et al. 1987
pC194	*S.aureus*	Gros et al. 1987
pUB110	*S.aureus*	McKenzie et al. 1986
pBAA1	*B.subtilis*	Devine et al. 1989
pCB101	*C.butyricum*	Gruss & Ehrlich 1989
pBC16	*B.cereus*	Perkins & Youngman 1983
pLP1	*L.plantarum*	Gruss & Ehrlich 1989
pIJ101	*S.lividans*	Kendall & Cohen 1988
pE194	*S.aureus*	Horinouchi & Weisblum 1982
pMK158	*S.agalactiae*	Lacks et al. 1986
pLB4	*L.plantarum*	Bates & Gilbert 1989
pSH71	*L. lactis*	Gruss & Ehrlich 1989
pSN2	*S.aureus*	Khan & Novick 1982
pIM13	*B.subtilis*	Projan et al. 1987
pNE131	*S.epidermidis*	Lampson and Parisi 1986

Throughout the Gram-positive bacteria, plasmids replicating by the ssDNA mechanism also show substantial homology in their plus origins, initiation proteins and minus origins (Figs. 1.6, 1.7, 1.8).

```
pT181      105 AGACAATTTTTCTAAAACCGGCTACTCTAATAGCCGGTTgg 145
pC221     1246 AGACAATTTTTCTAAAACCGGCTACTCTAATAGCCGGTTAA 1286
pS194     3404 ctcaAATTTTTCTAAAACCGGaTACTCTAATAGCCGGTTAA 3444


φX174     4280 aTg TgC TccCccAaCTTGATA tTA 4303
pC194     1428 CTT TTC TTTCTTATCTTGATA ATA 1451
pUB110    4313 CTTGTTC TTTCTTATCTTGATACATA 4288
pBAA1      811 tcgGgTCtTTTCTTATCTTGATAC TA 836
pCB101         TT TTC TTTCTTATCTTGATA ATA
pLP1      2045 gTT TTC  TTCTTATCTTGATAC TA 2067


pE194      870 AAAAcATaGGGGGG    TACTACGA  CCtcCCCCCTAG GTGtCCatTGT CCAT 918
pLS1       427 AAAAtATgGGGGGGC   TACTACGA  CC  CCCCCTAtAGTG CC GAGTGCCAA 474
pADB201     57 tcacgAccaGGGGGC   TACTACGAtagC  CCCCAatGAGTGatt  TGTGaCAT 106
pSH71          AAAAaAT  GGGGG & TACTACGA   C  CCCCATTaAGTG CC GAGTGCCAA
```

Figure 1.6 Consensus sequences (left to right, 5' to 3') within the plus origins of ssDNA plasmid sequences. Nucleotides in capital letters are homologous; those in lowercase letters are nonhomologous. Vertical arrows and dash line indicate replication nick-site and strongly conserved octamer in the pE194 type plasmids, respectively. & represents a 23 bp intervening sequence aaaggaagcgaattttgcttccg, not present in the other origins [from Gruss and Ehrlich, 1989].

A.

```
pLB4      27  G R N W G I V V Y P E S L P E N W K  44
pLS1       9          F T F L L Y P E S I P S D W E  23
pE194     14  G R N W T F V L Y P E S A K A E W K  31
pADB201    8    R N W T L L V Y P D S A P E N W K  24

pLB4      45  D I I R Q   E   P I A   V   S P L H  58
pLS1      24  L K L E T L G V P M A   I   S P L H  39
pE194     32  L Y L K E L       H I Q F V V S P L H  47
pADB201   25  E I L D Q N G V E   Y F       G A L H  39

pLB4      59  D K D V N P   D G E K   K K S H Y H  74
pLS1      40  D K D K S S I K G Q K Y K K A H Y H  57
pE194     48  D R D T D T   E G R M   K K E H Y H  63
pADB201   40  D K D V N P   D G T I   K K P H Y H  57

pLB4      75    L V L N Y  79
pLS1      58    V L     Y  60
pE194     64  I L V M   Y  68
pADB201   56  I V L   A Y  60
```

B.

```
φx174    A K Y V N K K S A   (E.coli        51% GC)
pBAA1    S Q Y P V K A T A   (B.subtilis    42% GC)
pC194    A Q Y S G K A S A   (S.aureus      34% GC)
pUB110   A Q Y P V K A T A   (S.aureus      34% GC)
pCB101   F Q Y M T K V T G   (C.butyricum   27% GC)
pIJ101   A Q Y I A K T Q A   (S.lividans    72% GC)
pLP1     A K Y Q V K S A A   (L.plantarum   nd     )
```

Figure 1.7 A. Comparison of Rep protein sequences from pE194 group plasmids. B. Rep proteins of pC194-like origins have a conserved region related to the enzymatic active site of φx174 Rep protein. The percent G+C contents of the hosts are listed to indicate genetic distance between the species. nd, not determined [from Gruss and Ehrlich, 1989].

pC194 M-O (palA)

2209   1
GCATTTATGCCGAGAAAATTTATTGTGCGTTGAGAAGAACCCTTAACTAAACTTGCAGACGAATGTCGGCATAGCGTGAGCTATTAAGCCGACCATTCGA

2109   1   2050
CAAGTTTTGGGATTGTTAAGGGGTTCCGAGGCTCAACGTCAATAAAGCAATTGGAATAAAG

pUB110 M-O

1523   1   1
ACCTCTCTTGTATCTTTTTTATTTTGAGTGGTTTTGTCCGTTACACTAGAAAACCGAAAGACAATAAAAATTTTATTCTTGCTGAGTCTGGCTTTCGGTA

1423   2   2
AGCTAGACAAAACGGACAAAATAAAAATTGGCAAGGGTTTAAAGGTGGAGATTTTTTGAGTGATCTTCTCAAAAAATACTACCTGTCCCTTGCTGATTTT

1323   3   3   1246
TAAACGAGCACGAGAGCAAAACCCCCCTTTGCTGAGGTGGCAGAGGGCAGGTTTTTTTGTTTCTTTTTTCTCGTAAA

pBAA1 M-O

186   1   1   2   2
TGGCGTGAGTCAACGGTAACCGGACCGTAGGGAGGATTAAGGAGTTGACTCGCTCAGCGCCACCCGAACCCTTTCAGCACTCAAACAAACCCGTTTGTTT

286   3   3   355
GACGCCAACCGGCGAGGGAGCCCCCCGAAGATGCGGGGGGGTTGGGGGGATTGAATGCTGGCATCCAACGG

Figure 1.8 DNA sequences of three minus origin (M-O). Palindromes are indicated by arrows above the DNA sequences; dots show positions of nonpalindromic bases. Arrows with the same numbers above form a single palindrome. Variants of the first M-O described. *palA*, have been found on many staphylococcal plasmids. The second is the M-O of pUB110 and is only one thus far known to function in at least two hosts *S. aureus* and *B. subtilis*. The third has been found in *Bacillus* plasmids pBAA1, pLS11 and pTA1060 [Gruss and Ehrlich].

1.11 Lactococcal Plasmid Replication

While many lactococcal plasmid vectors have been constructed and used for gene cloning, a detailed study of the complete genetic organization of a lactococcal plasmid replicon has not yet been reported. This is in contrast to other common Gram-positive plasmids [Gruss and Ehrlich, 1989]. In the *L.lactis* subsp. *lactis* plasmid pSH71, only a plus origin sequence has so far been published [Gruss and Ehrlich, 1989]. In *L. lactis* subsp. *lactis* pCI305, a 1.6 kb minimal replicon was identified by a combination of Tn5 mutagenesis allied to subcloning in replication probe vectors. This region was separable into *trans repB* (Rep protein) and *cis repA* (plus origin) segments [Hayes et al., 1990].

PART V AIM OF THIS THESIS

1.12 Aim of This Thesis

Starter cultures used for the manufacture of cheese and casein mainly comprise *L. lactis* subsp. *lactis* and *cremoris*. Currently, 120,000 tonnes of cheese and 35,000 tonnes of casein are produced annually in New Zealand with approx. 80% of the products being exported. Their value to the New Zealand economy has been conservatively estimated at $NZ 700 million export dollars in the 1989-1990 dairying season.

The application of recombinant DNA technology to the lactococci has greatly increased our understanding of these bacteria [de Vos, 1987]. Construction of stable vectors with high transformation efficiency is essential for the use of gene cloning to analyse genes for industrially important traits such as lactose utilization, proteinase activity and certain types of phage resistance.

The most useful lactococcal vectors are now being constructed solely based on lactococcal plasmid replicons, that is, to provide 100% food grade vectors. There are both theoretical and practical reasons for identifying various lactococcal plasmid replicons suitable for construction of such vectors and for studying their genetic organization.

Lactococcal cell-bound proteinases, usually encoded by plasmid DNA, are essential for starter cultures growing in milk. Proteolysis of milk protein also ultimately contributes significantly to the production of good quality cheese and casein. As such, studies on proteinase genes are of considerable fundamental and industrial importance [Thomas and Pritchard, 1987].

The objectives of this study were to, 1) develop a set of lactococcal vectors. 2) use them for cloning lactococcal genes such as proteinase and tagatose pathway genes. 3) analyze the genetic organization of the vector plasmid by DNA sequencing.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacteria Strains, Plasmids and Phages

These are described in Table 2.1.

Table 2.1 Bacterial strains, plasmids and phages used in this project.

Bacterial strains, plasmids and phages	Relevant features	References
Bacterial strains		
E. coli		
HB101	hsdS20 ($r_B^- m_B^-$) recA13	Maniatis et al. 1982
JM109	recAl hsdR17 relAl end A1	Yanisch-Perron et al. 1983
POP13	hsdR $r_k^- m_k^+$ supE	Murray 1983
Q358	hsdR$_k^-$ hsdM$_k^-$ supE	Maniatis et al. 1982
L. lactis subsp. _lactis_		
5136	Prt$^+$Lac$^+$, containing 4 plasmids (81, 51, 32 and 5.5 kb)	NZDRI[a]
4125	Prt$^-$Lac$^-$, plasmid-free derivative of strain 5136	Crow et al. 1983
LM0230	Prt$^-$Lac$^-$, plasmid-free	Efstathiou & McKay 1977
MG1363	Prt$^-$Lac$^-$, plasmid-free	Gasson 1983
L. lactis subsp. _cremoris_		
H2	Prt$^+$Lac$^+$, containing pDI21 and other plasmids	Crow et al. 1983
4873	Prt$^-$Lac$^-$, plasmid-free	NZDRI[a]
Plasmids		
pC194	Cm^r	Horinouchi & Weisblaum 1982
pBH201	Ap^r, carried the 2.0kb isomerase gene fragment from pDI21	Yu[b]
pCK17	Cm^{r}Em^r	Gasson & Anderson 1985
pDI21(63 kb)	Prt$^+$Lac$^+$	Davey et al. 1984
pDI1(32 kb)	Prt$^+$Lac$^+$	Limsowtin et al. 1986
pFX1	Cm^r, broad host-range vector, constructed from the 5.5 kb plasmid of strain 5136	this work
pFX101	Cm^r, 6.5 kb HindIII prt gene fragment of pDI21 in pFX1	this work
pFX2	Cm^r, pFX1 derivative with a 1.9 kb ClaI region deletion	this work
pFX201	Cm^{r}Ap^r, cointegrated plasmid of pFX2/HpaII-pGEM3Z/SmaI	this work
pFX202	Cm^{r}Ap^r, cointegrated plasmid of pFX2/HpaII-pGEM4Z/SmaI	this work
pFX202-01	Cm^{r}Ap^r, pFX202 derivative with a deletion in pFX2 part (positions 1-1214 in DNA sequence)	this work

Table 2.1 (Contd.)

Bacterial strains, plasmids and phages	Relevant features	References or sources
pFX202-62	Cm^{r}Ap^r, pFX202 derivative with a deletion in pFX2 part (positions 1-962 in DNA sequence)	this work
pFX203	Cm^{r}Ap^r, cointegrated plasmid of pFX2/ThaI-pUBS/SspI	this work
pFX3	Cm^r, pFX2 plus 0.9 kb region of lacZ' & polylinker from pUBS	this work
pFX301	Cm^r, 4.4 kb EcoRI aldolase gene fragment of pDI1 in pFX3	this work
pFX4,5,6	Cm^r, pFX2 plus 3.1 kb regions of lacZ & polylinker from pNM480,481 & 482 respectively	this work
pFX401	Cm^{r}lacZ$^+$, pFX4 carried a prt promoter	this work
pFX501	Cm^{r}lacZ$^+$, 2.0 kb EcoRI isomerase gene promoter fragment in pFX5	this work
pFX601	Cm^{r}lacZ$^+$, pFX6 carried a prt promoter with opposite direction from that in pFX401	this work
pFX7	Cm^r, pFX2 derivative with 0.7 kb CfoI-ClaI-CfoI region deletion	this work
pGEM3Z	Ap^r, sequencing vector	Promega Co.[c]
pGEM4Z	Ap^r, sequencing vector	Promega Co.[c]
pGKV500	Cm^{r}Em^r, proteinase gene probe	Kok et al. 1985
pUBS	Ap^r	Forster[d]
pNM480,481,482	Ap^r, translational fusion vectors	Minton, 1987
pNZ12	Cm^{r}Em^r	de Vos, 1987
Phages		
λNM1149	λb538 srIλ3° imm434 srIλ4° shndIII λ6° srI λ5°	Murray, 1983
λ114965	6.5 kb HindIII prt fragment in λNM1149	this work

NZDRI[a], Culuture stock of New Zealand Dairy Research Institute.
Yu[b], obtained from P.-L. Yu (Massey University).
Promega Co.[c], purchased from Promega Co. WI, USA.
Forster[d], obtained from R.L.S. Forster (Auckland University).

2.2 Culture Media

Citrate milk agar [Brown and Howe, 1922] Composition (g/l): 10% skim milk, trisodium citrate.5H$_2$O, 2; agar, 1.5%; pH 6.8.

<u>Luria broth</u> (LB) [Miller, 1972] Composition (g/l): tryptone, 10; yeast extract, 5; NaCl, 5; pH 7.2.

<u>M17 broth</u> [Terzaghi and Sandine, 1975]. Composition (g/l): lactose, 5; polypeptone, 5; beef extract, 5; soy peptone, 5; yeast extract, 2.5; ascorbic acid, 0.5; sodium glycerophosphate, 19; 1 M $MgCl_2$, 1 ml. pH 7.2.

<u>M17-glu</u> Composition: M17, 0.5% glucose (g/l).

<u>RSMG</u> Composition (g/l): 10% reconstituted skim milk, 0.5% glucose.

<u>T5 broth</u> [Thomas et al., 1974] Composition (g/l): Beef extract, 2; polypeptone, 5; phytone peptone, 2; yeast extract, 2; Na_2HPO_4, 8.5; KH_2PO_4, 2; ascorbic acid, 0.5; 1 M $MgCl_2$, 1 ml; pH 7.2.

Solid media contain 1.5% agar. Soft media contain 0.7% agar for phage growth.

2.3 Growth and Maintenance of Bacteria and Phages

E. coli and *B. subtilis* were cultured aerobically in LB broth at 37° C. *E. coli* Q358 was used to propagate phage λNM1149, and recombinant phages were selected in *E. coli* POP13 [Hohn and Murray, 1977]. Lactococci were grown at 30° C in M17 or M17-glu as required. Exponential stage cells were harvested at an absorbance (600 nm) of 0.3-0.7 (10 mm light path). Solid or liquid medium was supplemented as required with the appropriate antibiotics: chloramphenicol (Cm, 25 μg/ml for *E. coli* and *B. subtilis*, 5 μg/ml for lactococci), ampicillin (Ap, 100 μg for *E. coli*). All antibiotics were supplied by Sigma Chemical Co. (St Louis, MO, USA).

E. coli and *B. subtilis* cultures were maintained in LB broth or on LB plates at 4° C. Lactococci were maintained in M17 or M17-glu broth at 4° C. Bacteriophages were stored in SM buffer [Maniatis et al., 1986] [g/l; NaCl, 5.8 g; $MgSO_4.7H_2O$, 2 g; 1 M Tris (pH 7.5), 50 ml; 2% gelatin, 5 ml]. For long term maintenance, all cultures and phage suspensions were stored in 50% (v/v) glycerol at -70° C.

2.4 Isolation of DNA

2.4.1 Isolation of plasmids from <u>E. coli</u> and <u>B. subtilis</u>

The method of Birnboim and Doly [1979] was used for mini-prep and large-scale preparation of *E. coli* and *B. subtilis* plasmid DNA.

The large-scale preparation steps were as follows. (1) The bacterial pellet from a 500 ml culture was resuspended in 10 ml solution I (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8.0) containing 5 mg/ml lysozyme and incubated for 10 min at room temperature. (2) 20 ml of freshly made solution II (0.2 N NaOH, 1% SDS) was added and mixed gently for 10 min. (3) 15 ml ice-cold solution III (5 M potassium acetate, pH 4.8) was added and the tube shaken sharply several times. The tube was held on ice for 10 min. (4) The debris was removed by centrifugation (6000 g, 10 min, 4° C). (5). The supernatant was transferred to a clean tube in which the DNA was precipitated with 0.6 volume isopropanol for 20 min at 4° C. (6) The DNA was recovered by centrifugation (12000 g, 20 min) and the pellet was washed once with 70% ethanol, dried in a vacuum desiccator and resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer.

For mini-prep, (1) The pellet from a 1.5 ml culture was suspended in an eppendorf tube with 100 μl solution I and then 200 μl solution II was added. The mixture was shaken gently for 5 min. (2) 150 μl solution III was added and the tube vortexed for 1 min and then spun for 5 min in an eppendorf centrifuge. (3) The supernatant was extracted once with phenol/chloroform and the DNA was precipitated with 2.5 volume of ethanol for 20 min at -20° C. (4) The DNA pellet was recovered by centrifugation (10 min). (5) The pellet was washed twice with 70% ethanol, dried and the DNA resuspend in 50 μl TE containing DNase-free pancreatic RNase (20 μg/ml).

2.4.2 Isolation of lactococcal plasmids

A modification of the method of Birnboim and Doly [1979] was used for lactococcal

plasmid DNA isolation with sizes smaller than 15 kb. The protoplasting step was modified as follows. Fresh overnight cultures were resuspended in protoplasting solution (50 mM Tris, pH 8.0, 1 mM EDTA, 8% sucrose, 10 mg/ml lysozyme) and incubated at 37° C for 10 min. Subsequent alkaline lysis and extraction steps were the same as described above.

The procedure of Anderson and McKay [1983] was used for isolation of large size (> 15 kb) lactococcal plasmids (pDI1, 32 kb; pDI21, 63 kb) (Table 2.2)

Table 2.2 Large size lactococcal plasmid isolation protocol [Anderson and McKay, 1983]

Step	Details of following protocol:	
	Screening (1.5–10 ml)[a]	Preparative (600 ml)[a]
Resuspend pelleted cells in 6.7% sucrose–50 mM Tris–1 mM EDTA, pH 8.0 ....379 μl Warm to 37°C		30 ml
Add lysozyme (10 mg/ml in 25 mM Tris, pH 8.0)..................96.5 μl Incubate for 5 min at 37°C		7.5 ml
Add 0.25 M EDTA–50 mM Tris. pH 8.0.........................48.2 μl Add sodium dodecyl sulfate (20% [wt/vol] in 50 mM Tris–20 mM		3.75 ml
EDTA, pH 8.0)..27.6 μl Mix immediately Incubate for 5 to 10 min at 37°C to complete lysis		2.25 ml
Vortex at highest setting for 30 s in an appropriate tube...........1.5-ml Eppendorf		15 ml per tube (25 by 150 mm)
Add fresh 3.0 N NaOH..27.6 μl Mix gently by intermittent inversion or swirling for 10 min ....Inversion		2.40 ml Swirl in 250-ml centrifuge bottle
Add 2.0 M Tris-hydrochloride, pH 7.0 ....49.6 μl Continue gentle mixing for 3 min		3.90 ml
Add 5.0 M NaCl..71.7 μl Add phenol saturated with 3% NaCl; mix thoroughly ....700 μl Centrifuge ....5 min		5.7 ml 55.8 ml 5.000 rpm in GSA rotor, 10 min
Remove upper phase and extract with chloroform-isoamyl alcohol (24:1)..700 μl Remove upper phase. precipitate with 1 vol of isopropanol		55.8 ml
Incubate at 0°C...>30 min Centrifuge ....5 min		>60 min 8.000 rpm in GSA rotor, 20 min
Remove excess isopropanol and resuspend in 10 mM Tris–1 mM EDTA. pH 7.5 ....20 μl Examine 5 to 10 μl by agarose gel electrophoresis		1.200 μl

[a] The culture volume used in each protocol is indicated in parentheses.

2.4.3 Isolation of phage DNA

(1) Fifty fresh confluently lysed phage plates (titre ca. 10^{10}/per plate) were prepared, 5 ml SM buffer [per liter: NaCl, 5.8 g; $MgSO_4.7H_2O$, 2 g; 1 M Tris (pH 7.5), 50 ml; 2% gelatin 5 ml] was added to each plate to suspend the phage. (2) The lysate were pooled and spun to remove the debris (7000 g, 15 min). (3) The phage in the supernatant was precipitated overnight with PEG6000 (final concen. 10%, w/v) and NaCl (adjusted to 1 M). (4) The phage pellet was recovered by centrifugation (11000 g, 10 min) and dissolved in SM buffer. (5) The phage was purified by CsCl gradient by the procedure of Maniatis et al. [1982].

2.4.4 DNA purity and concentration

All plasmid DNA from large-scale preparations was purified by CsCl-ethidium bromide density gradient in a Beckman L8-80 ultracentrifuge (VTi80 rotor, 75000 rpm, 5 h). DNA purity was assessed by restriction enzyme digestion, followed by analysis on agarose gels. If the DNA was not digested satisfactorily, the sample was further purified by phenol/chloroform (phenol:chloroform:isoamyl alcohol, 25:24:1) extracted once, or alternatively by twice precipitating (using ethanol) the DNA in 2.5 M ammonium acetate solution.

DNA concentration was measured by comparing its fluorescent intensity with those of a dilution series of standard DNA on agarose gels incorporating ethidium bromide.

2.5 DNA Manipulations

Restriction enzymes, Klenow-large fragment polymerase and T4 DNA ligase were purchased from commercial companies (BRL Inc., MD, USA, Promega Co. WI, USA and Boehringer Mannheim GmBH, Mannheim, W. Germany) and reaction conditions followed the manufacturers' recommendations. Restriction maps were constructed by analyses of single and double digests after electrophoresis on 0.5-1.2% agarose gels.

2.6 DNA-DNA Hybridization

Phage and plasmid DNAs were labelled with [^{32}P]dCTP using a nick translation kit (BRL Inc., MD. USA). The free nucleotide was removed on a Sephadex G-50 column equilibrated with TE buffer. DNA probes were usually labelled to a specific activity of $>1\times10^8$ cpm/μg DNA.

Procedures for DNA transfer to nitrocellulose filters varied according to the DNA sources (see below). Hybridization steps for all DNAs were generally the same.

2.6.1 DNA transfer from agarose gels

Gels were stained and photographed. DNA was depurinated by shaking the gels in 0.25 M HCl for 15 min, then the DNA was denatured by shaking for 30 min in denaturation buffer (0.5 M NaOH, 0.5 M NaCl), followed by shaking for 30 min in neutralizing buffer (0.5 M Tris, 2.0 M NaCl, pH 7.2). The gel was placed on a blotting stand for DNA transfer to a nitrocellulose filter. The nitrocellulose filter was removed after 16 h and baked in a vacuum at 80° C for 2 h.

2.6.2 DNA transfer from bacterial colonies

Nitrocellulose filters were peeled off from fresh colony plates (absorption time, 5 min) and placed, colony side up, on 10% SDS-impreganated 3MM paper for 3 min. The filters were transferred to a second 3MM paper saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 min, and then neutralized on new 3MM papers saturated with solution 1.5 M NaCl, 0.5 M Tris, pH 8.0. The filters were dried, colony side up, at room temperature for 30 min and then baked at 80° C.

2.6.3 DNA transfer from phage plaques

Nitrocellulose filters were peeled off from fresh plates with well isolated plaques

(absorption time, 8 min), and denatured and neutralized as described for " DNA transfer from bacterial colonies ". Filters were rinsed with chloroform, air dried and then baked.

For DNA hybridization, a filter was sealed in a plastic bag containing 20 ml prehybridization buffer [10 ml: 4.9 ml H_2O, 2.5 ml 20xSSC (3 M NaCl, 0.3 M sodium citrate), 2.5 ml 20xDehardt solution (1 M Hepes buffer pH 7.0; 20xSSC, 75 ml; 20% SDS, 2.5 ml; Ficoll, 1 g; bovine serum albumin, 1 g; polyvinnylpyrrolidone 5 g; H_2O 400 ml], denatured salmon sperm DNA (10 mg/ml) 0.1 ml] and prehybridized at 65° C for at least 5 h. Most of the liquid in the bag was then drained off and 20 ml hybridization buffer (10 ml: 6.4 ml, H_2O; 2.5 ml, 20xSSC; 1 ml, 20xDehardt solution; 0.1 ml denatured salmon sperm DNA) plus the boiled probe ($> 10^5$ cpm) were added. Hybridization proceeded at 65° C overnight. Filters were washed at room temperature once with 2.0xSSC, 0.1% SDS and once with 0.1xSSC, 0.1% SDS), the blotted filter was dried, covered in "Gladwrap" and exposed to X-ray film (Cronex, Du Pont Ltd, Australia) in the presence of an intensifying screen at -70° C. Exposure time ranged from 6 h to 3 days depending on the radioactivity.

2.7 Elution of DNA from Agarose Gels

DNA was separated on agarose gels (SeaKem, GTG grade, FMC Co. Rockland, ME, USA). The band of interest was cut out under long-wave U.V. light with a scapel. The gel block was trimmed into tiny pieces and frozen at -70° C for 30 min in an eppendorf tube. The gel debris was spun down for 10 min and the supernatant was transferred into a clean tube where DNA was precipated with ethanol.

2.8 Electroporation, Transformation and Infection

Electroporation of lactococci was performed using a Gene Pulser apparatus (Bio-Rad Lab., Richmond, CA, USA). The procedure was according to the method of Powell et al. [1988] with modifications (Powell, personal communication). Initially, overnight

lactococcal cultures were diluted in M17-glu and grown at 30°C to A_{600} 0.3-0.7 (10 mm light path). The cells were washed with ice-cold EPB1 (0.5 M sucrose, 1 mM $MgCl_2$, 7 mM Na_2HPO_4-NaH_2PO_4, pH 7.4) buffer, resuspended in 0.1 culture volume of EPB1 plus 250 U/ml lysozyme (Sigma, St. Louis, MO, USA), and incubated at 37°C for 20 min. The treated cells were further washed with EPB2 (0.5 M sucrose, 1 mM HEPES, pH 7.4) buffer and resuspended in 0.005 culture volume of EPB2. In later experiments, cells were grown in 40 mM DL-threonine, and the protoplasting step was omitted. Volumes of 80 μl concentrated cells plus 1-5 μl DNA (10 μg/ml-200 μg/ml) were pulsed in a 2 mm Gene Pulser cuvettes at 12500 V/cm and 25 μF with a Pulse Controller set at 200Ω. The electroporated cells were growed in expression broth (0.5M sucrose, M17-glu) for 1 h and spread on agar plates with the appropriate antibiotics.

Competent cells of *E. coli* were transformed using the $CaCl_2$ method [Cohen et al., 1972]. 25 ml log-phase cells were spun down, resuspended gently in 10 ml 60 mM $CaCl_2$ buffer and incubated on ice for 20 min. Cells were washed once with $CaCl_2$ buffer and resuspended in 100 μl of the same buffer. 50 μl competent cell suspension was mixed with 5 μl DNA (1-10 μg) and 45 μl TEC buffer (10 mM Tris, 0.25 mM EDTA, 30 mM $CaCl_2$, pH 8.0). The mixture was incubated first on ice (5 min) and then heat shocked at 42° C (2.5 min). The treated cells were incubated on ice (5 min), growed in LB for 1 h and then spread on agar plates with antibiotics.

Electroporation was also used for *E. coli* transformation with the above procedure except that the cells were grown in LB media and not treated with lysozyme.

Lambda DNA was packaged using the Packagene system (Promega Co., WI, USA) to provide viable phage. (1) 50 μl frozen packaging extract in a eppendorf tube was held in ice to thaw. (2). 10 μl λ DNA was added and gently mixed. (3). The tube was incubated at 22° C for 2 h. (3). 0.5 ml SM buffer and 25 μl chloroform were added and mix gently, and then the tube was spun for 2 min to remove the debris. (4). The phage supernatant was diluted appropriately to infect *E. coli* POP13 (ratio,10:1). The infected bacteria were mixed with 3 ml of soft agar and poured on LB agar plates.

2.9 Evaluation of Strains Growing in Milk

Milk coagulating ability was evaluated as follows. Cultures were grown overnight at 30°C in autoclaved reconstituted skim milk containing 0.5% glucose (RSMG). Ten ml volumes of RSMG were inoculated (1%) with the overnight cultures and incubated at 22°C. Cultures were examined for coagulation at 18 and 30 h. Growth rates in RSMG were examined using water-washed cells from M17-glu broth cultures inoculated at 5% into RSMG. Cell numbers were determined by plating on M17-glu at 1 h intervals, and acid production was measured by titration with 0.1 M NaOH. Citrate-milk agar was used as a medium to differentiate Prt^+ and Prt^- strains.

2.10 Proteinase Isolation and Activity Test

Casein proteolysis by lactococci and recombinant phage lysates was detected using a modification of the procedure of Hill and Gasson [1986]. Lactococcal cells grown on citrated milk agar were resuspended in 5 ml 0.1 M NaH_2PO_4 buffer (pH 7.2) to a density of about 10^{10} cells/ ml. After 3 h incubation at 30° C the released proteinase was recovered in the supernatant by centrifugation (13800 g, 30 min). The supernatant was dialysed against 4 changes of distilled water over a 24 h period. The preparations were lyophilized, and as required, reconstituted in 0.5 ml NaH_2PO_4 buffer for proteinase assay.

Phage obtained from two confluently lysed plates was suspended in 10 ml 0.1 M NaH_2PO_4 buffer, dialysed against four changes of the same buffer and the suspension was directly lyophilized. The lyophilized phage lysate was reconstituted in 0.5 ml 0.1 M NaH_2PO_4 (pH 7.2).

Proteolysis reaction mixtures containing 150 μl of reconstituted cell extract or phage lysate and 10 μl ß-casein or whole casein (15 mg/ml in 0.1 M NaH_2PO_4, pH 7.2) were incubated at 30°C. Products of proteolysis were separated on 15% SDS-polyacrylamide gels [Laemmli, 1970]. ß-casein was purchased from Sigma Chemical Co., St. Louis, MO, USA. Whole casein was obtained from the

Protein Chemistry Section of the New Zealand Dairy Research Institute.

2.11 ß-galactosidase Assay

For agar plating assay, 40 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-ß-galactopyranoside) was incorporated into solid media.

Quantitative analysis of ß-galactosidase was according to Crow et al. [1983]. 100 ml late log phase cells growing in T5 were recovered by centrifugation, washed once with the assay buffer (50 mM NaCl, 10 mM MgCl$_2$, 1 mM dithioerythritol) and disrupted in the same buffer (3.5 ml) by shaking 3x2 min at 4° C with glass beads (3 ml volume) in a Mickle disintegrator. Debris was removed by centrifugation (27000 g, 15 min). Enzyme assays were completed within 2 h from preparation of extracts. ß-galactosidase activity was assayed in a 1 ml reaction mixture containing 50 mM sodium-potassium phosphate buffer (pH 7.2), 1 mM o-nitrophenyl-ß-galactopyranoside (ONPG) and limiting amounts of ß-galactosidase. The reaction was followed at 410 nm at 37° C using a spectrophotometer. Enzyme activity (nanomoles ONP produced/min/ml) was calculated by the formula (A_{change}/min x 1/2.45 x dilution x 10^3). Quantitation of the extract proteins was according to Bradford [1976]. Specific activity was expressed as nanomoles of o-nitrophenyl (ONP) released from ONPG/min/mg of the total extract protein.

2.12 Evaluation of Plasmid Stability

A single bacterial colony on a selective plate was inoculated in broth with appropriate antibiotics and grown to stationary phase. An 0.1% inoculum was made into the same medium but without antibiotic selection and again grown to stationary phase. The cycle of dilution and growth was performed 10 times. Each cycle constituted approx. 10 generations. The final grown culture was streaked for single colony isolation on nonselective plates. Individual colonies from nonselective plates were further tested on selective plates with appropriate antibiotics. The stability was measured as the

percentage of resistant colonies in the total number of colonies tested.

2.13 Agarose and Polyacrylamide Gel Electrophoresis

Horizontal slab agarose gels were used for separating DNA using TBE electrophoresis buffer (89 mM Tris, 1 mM Na_2EDTA, 89 mM boric acid, pH 8.3). Electrophoresis was usually carried at below 5.0 V/cm for good separation and 10.0 V/cm for checking. Gels were stained with ethidium bromide (0.5 μg/ml) and photographed. Various DNA molecular weight marker ladders (1 kb linear fragment ladder, *Hin*dIII fragments and 1 kb supercoiled plasmid ladder) were purchased from BRL Inc., MD, USA.

Proteins were separated in vertical slab SDS-polyacrylamide gels according to Laemmli's method [1970] by using a Bio-Rad Mini-Protein II apparatus [Bio-Rad, CA, USA]. The separating gel was 15% polyacrylamide with a buffer system (1.5 M Tris, pH 8.8. 0.4% SDS). The stacking gel was 5% polyacrylamide with a different buffer system (0.5 M Tris, 0.4% SDS, pH 6.8). The gels were stained with Coomassie Brilliant Blue R250 solution (0.2%) in methanol/water/acetic acid (4:5:1) overnight and destained in methanol/acetic acid/water (2:1:7). The destaining buffer was changed every 3 h for 3-5 times. Protein molecular weight markers were purchased from Sigma Co., St. Louis, MO, USA.

DNA was sequenced using a S2 model gel apparatus (BRL Inc., MD, USA) with 6% Urea-SDS-polyacrylamide (BRL "Ultrapure" grade chemicals) gels. The running systems, gel preparation and processing procedures followed the manufacturer's instructions. Gels were exposed to X-ray films (Cronex 4, Du Pont Ltd., Australia) for 2-4 days.

2.14 Preparation of ExoIII Deletions

Plasmid pFX2 DNA was linerized with *Hpa*II and cloned into *Sma*I sites of

sequencing vectors pGEM3Z and pGEM4Z to obtain plasmids pFX201 and pFX202, respectively (Fig. 2.1). Overlapping templates were created by ExoIII digestion with the Erase-a-Base system (Progema Co., WI. USA). Double digestion of pFX201 and pFX202 with *Pst*I and *Sal*I produced linear fragments with 5' overhang and 3' overhang ends, allowing subsequent ExoIII unidirectional digestion of the pFX2 DNA sequence.

The ExoIII deletion protocol was as follows. (1). 10 μg supercoiled DNA was digested with *Pst*I and *Sal*I in the same buffer system. (2). The digested DNA was purified as described in 2.4.4. (3). The linear DNA was suspended in a tube with 60 μl 1xExoIII buffer (60 mM Tris pH 8.0; 0.66 mM MgCl$_2$). Meanwhile, 25 eppendorf tubes were prepared, each containing 7.5 μl of S1 mix [172 μl H$_2$O, 27 μl 7.4xS1 buffer (0.3 M potassium acetate, pH 4.6, 2.5 M NaCl; 10 mM ZnSO$_4$; 50% glycerol); 60 units S1 nuclease] and left on ice. (4). 60 units of ExoIII were added to the DNA tube which was then incubated at 38° C for 30 seconds. (5). Subsequently, 2.5 μl samples were removed every 40 seconds into the S1 tubes on ice. (6). After all samples had been taken, the tubes were removed for reaction at room temperature for 30 min. (6). 1 μl of S1 stop buffer was added and the tubes were heated at 70° C for 10 min to inactivate the S1 enzyme. (7). The time point tubes were then transferred to 37° C and 1 μl Klenow mix [30 μl 1xKlenow buffer (20 mM Tris pH 8.0; 10 mM MgCl$_2$), 3-5 units Klenow DNA polymerase] were added for 3 min. 1 μl dNTP (0.125 mM each of dATP, dCTP, dGTP and dTTP) were added and the tubes were incubated for another 5 min. (8). The tubes were moved to room temperature and 40 μl ligase mix [790 μl H$_2$O, 100 μl 10xligase buffer (500 mM Tris, pH 7.6, 100 mM MgCl$_2$, 10 mM ATP); 100 μl 50% PEG; 10 μl 100 mM DTT; 5 units T4 DNA ligase] was added and the tubes were then incubated at room temperature for 1 h. (9). The ligation mixtures were transformed into *E. coli* JM109 cells as described in 2.8. (10). A number of recombinants from each time point were screened to select appropriate deletions. (11). The selected deletion plasmids were further digested with *Eco*RI and *Hin*dIII to examine the progressive sizes of the deleted inserts.

pFX201

Ssp I 2142 Aat II 2260

Xmn I 1937

Sca I 1818

Nde I 2509
Nar I
2561

Amp^r

pGEM®-3Z
plasmid
(2743 bp)

lacZ

T7 ▼	1	start
EcoR I	5	
Sac I	15	
Kpn I	21	
Ava I	21	
Sma I	23	
BamH I	26	
Xba I	32	
Sal I	38	
Acc I	39	
Hinc II	40	
Pst I	48	
Sph I	54	
Hind III	56	
	69	
▲SP6		

pFX2

Cm^r

ori

pFX202

Ssp I 2144 Aat II 2262

Xmn I 1939

Sca I 1820

Nde I 2511
Nar I
2563

Amp^r

pGEM®-4Z
plasmid
(2746 bp)

lacZ

SP6 ▼	1	start
EcoR I	7	
Sac I	17	
Kpn I	23	
Ava I	23	
Sma I	25	
BamH I	28	
Xba I	34	
Sal I	40	
Acc I	41	
Hinc II	42	
Pst I	50	
Sph I	56	
Hind III	58	
	70	
▲ T7		

Cm^r

pFX2

ori

Figure 2.1 Cloning of plasmid pFX2 in pGEM3Z and pGEM4Z, resulting in pFX201
and pFX202, respectively.

Figure 2.2 Progressive deletion plasmids derived from pFX201 (A) and pFX202 (B) digested with *Hin*dIII and *Eco*RI.

2.15 DNA Sequencing

SP6 and T7 promoter primers were used for sequencing DNA templates from pFX201 and pFX202 respectively, according to the dideoxynucleotide chain termination method [Sanger et al., 1977]. In this manner, both strands of pFX2 DNA were sequenced. The Sequenase kit was obtained from USB, OH, USA. $(\alpha^{35}S)ATP$ was purchased from Amersham, UK. Both were used according to the manufacturer's instructions. DNA used for sequencing was purified by CsCl-ethidium bromide centrifugation. DNA sequences were analyzed with a sequence analysis software package developed by the University of Wisconsin Genetics Computer Group.
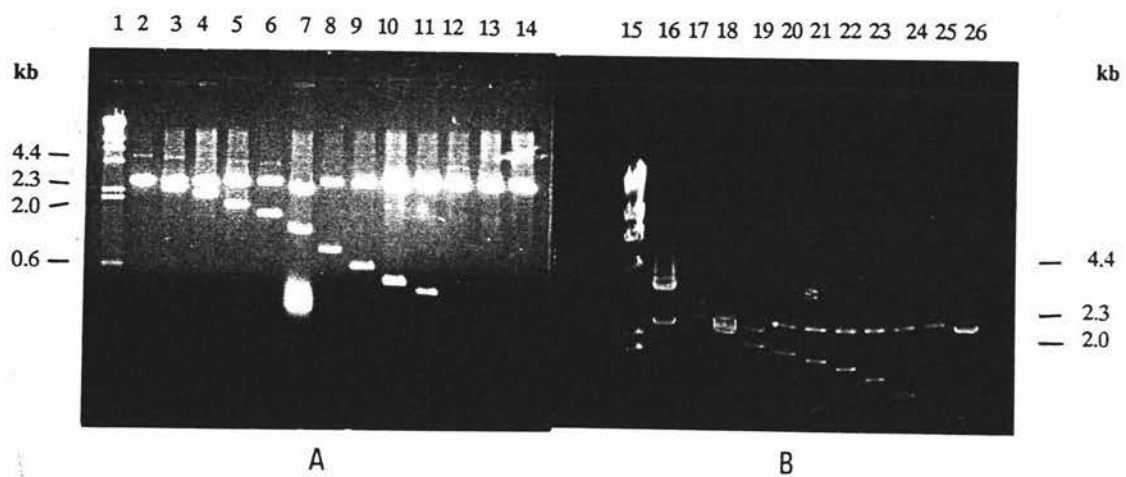
CHAPTER 3: CLONING AND EXPRESSION OF A PROTEINASE GENE OF L. LACTIS SUBSP. CREMORIS H2 IN E. COLI

3.1 Background and Aim

L. lactis subsp. *cremoris* H2 carries plasmid pDI21 (63 kb) which encodes genes for both lactose metabolism (Lac) and the proteinase positive phenotype (Prt). This plasmid had been previously transferred by conjugation into *L. lactis* subsp. *lactis* 4125, a plasmid-free recipient [Davey et al., 1984]. The plasmid pDI21 was also named pJI70 by Inamine et al. [1986] who cloned genes for the lactose phosphotransferase system and phospho-ß-galactosidase into *E. coli* and *S. sanguis*. More recently, Yu et al. [1989] cloned tagatose-6-phosphate pathway genes from pDI21 into *E. coli*, and mapped the Lac and Prt regions of this plasmid. To further our understanding of the pDI21 proteinase, its specificity was determined and the *prt* gene was cloned in *E. coli*.

3.2 Results

3.2.1 pDI21 proteinase resembles a PI type proteinase

A Prt+ transconjugant *L. lactis* subsp. *lactis* 4760 (4125 carrying pDI21), was used to characterize pDI21 proteinase specificity. An extracelluar extract from 4760 was prepared, reconstituted and incubated with whole casein. 4125 was used as a control. The proteolysis products were separated on 15% SDS-polyacrylamide gels. The ß-casein was clearly preferentially degraded by 4125 (pDI21) (Fig. 3.1, A), showing that the pDI21 proteinase resembled a PI type proteinase [Visser et al., 1986].

Figure 3.1 Casein degradation patterns by lactococci and *E. coli* clones. (A). Whole casein degradation patterns by lactococci (2 h, 30° C). Lane 1, whole casein control; lane 2, 4125(pDI21); lane 3, 4125; lane 4, M.W. standards. (B). ß-casein degradation by the recombinant phage lysate (16 h, 30° C). Lane 1, λNM114965; lane 2, λNM1149; lane 3, *E. coli* Q358; lane 4, ß-casein control.

Da				
66 000				
45 000				
36 000				
29 000				
α_{S1}				
24 000 β				
20 100 κ				
14 200				

A   B

59
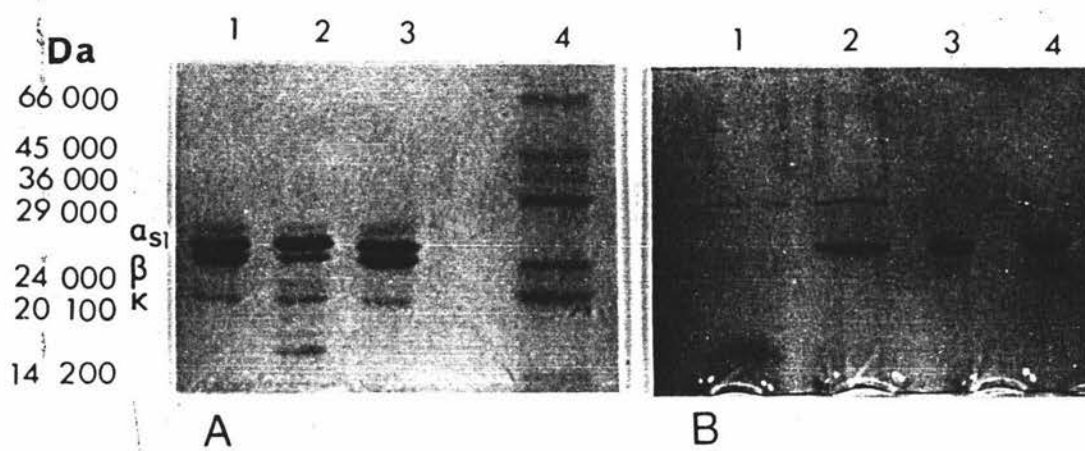
Figure 3.2 DNA Hybridization of a *prt* gene probe pGKV500 to pDI21 *Hind*III digest. (A). Lane 1, positive control (pGKV500/*Hind*III); lane 2, λDNA *Hind*III digest as M.W. standard; lane 3, pDI21 DNA/*Hind*III. (B). Hybridization autoradiogram result (pGKV500 as a probe).

kb

23.1
9.4
6.6
4.4

A

B
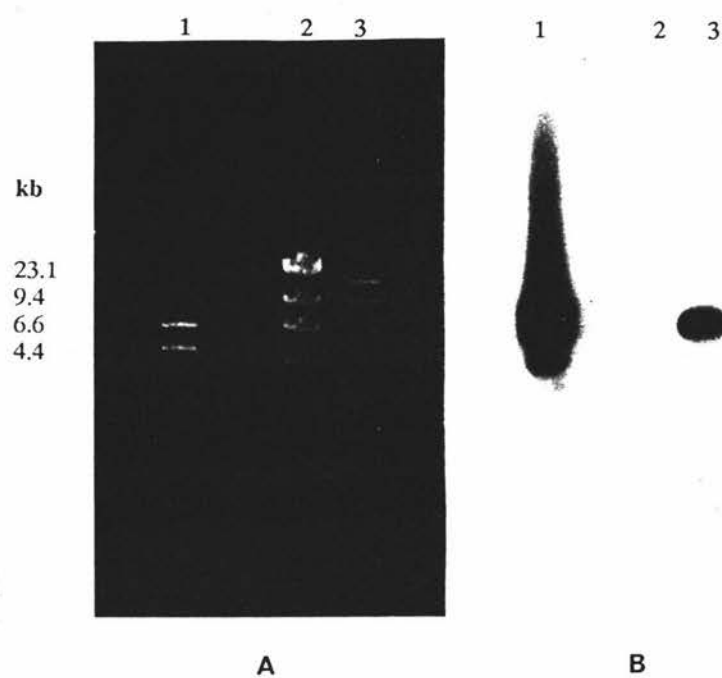
3.2.2 Cloning and expression of pDI21 proteinase in <u>E. coli</u>

Since the cell wall bound proteinase activity was encoded on plasmid pDI21 in *L. lactis* subsp. *cremoris* H2, the *prt* gene of this plasmid was cloned for further genetic studies.

Plasmid DNA was isolated from strain 4760, digested with *Hin*dIII and only a 6.5 kb fragment was found to hybridize pGKV500, a plasmid carrying the *prt* gene fragment from *L. lactis* subsp. *cremoris* Wg2 [Kok et al., 1985] (Fig. 3.2). Because expression of the lactococcal proteinase gene was found to be lethal in *E. coli* [Kok et al., 1985; Gasson et al., 1987; von Wright et al., 1987], the 6.5 kb *Hin*dIII fragment from pDI21 was cloned into the *Hin*dIII site of the λNM1149 vector. The ligation mixture was packaged with an *in vitro* packaging system and used to infect *E. coli* POP13. Positive plaques were identified by *in situ* plaque hybridization and the phage DNA (λNM114965) was digested with *Hin*dIII to characterize the cloned inserts (Fig. 3.3). The lyophilized recombinant phage lysate degraded ß-casein, showing that the cloned proteinase gene was expressed in *E. coli* (Fig. 3.1, B).

3.3 Discussion

The cell wall bound proteinase activity of *L. lactis* subsp. *cremoris* H2 was shown by conjugation experiments to be encoded on plasmid pDI21 [Davey et al., 1984]. In this study, the proteolytic activity of pDI21 was found to resemble a PI type proteinase, preferentially degrading ß-casein.

Gene cloning techniques have made it possible to study the individual genes of the complex lactococcal proteinase systems [Kok and Venema, 1988]. The problem of lethal expression of proteinase in *E. coli* observed by earlier workers [Kok et al. 1985; Gasson et al., 1987; von Wright et al., 1987] was circumvented by cloning the pDI21

Figure 3.3 Identification of the 6.5 kb *prt* gene fragment in λNM1149. (A). Lane 1, λDNA *Hin*dIII digest as M.W. standard; lane 2, λNM114965 DNA/*Hin*dIII; lane 3, λNM1149 DNA/*Hin*dIII; lane 4, positive control (pGKV500). (B). Hybridization autoradiogram result (pGKV500 as a probe).

kb

23.1
9.4
6.6
4.4

1   2   3   4   1   2   3   4

A   B
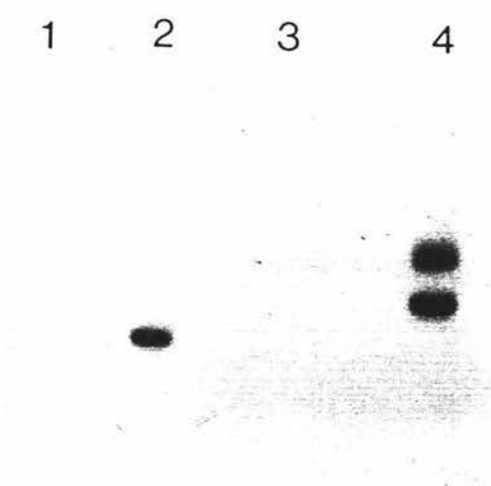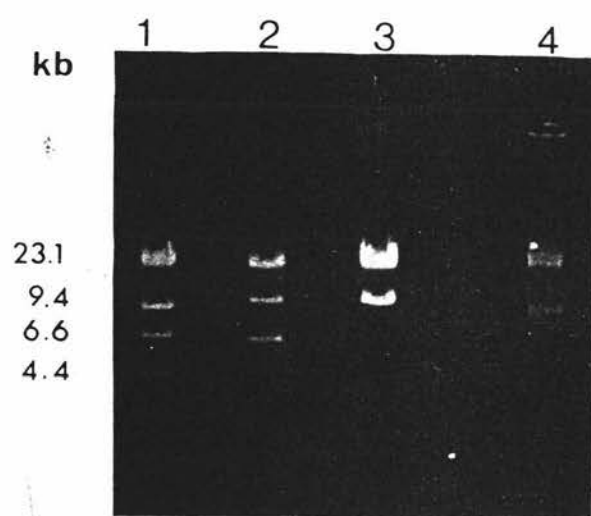
prt gene into the phage insertion vector λNM1149. Lytic infection led to active enzyme being produced in the cell lysate, thus avoiding gene expression in normal cells. A similar strategy was recently reported where the pSK111 proteinase was cloned and expressed in *E. coli* using λEMBL3 vector [de Vos et al., 1989]. λ phage vectors therefore offer a satisfactory mechanism for the general cloning of these and other genes whose synthesis may be detrimental to the *E. coli* host.

Compared with restriction maps of other *prt* genes [de Vos, 1987], that of the pDI21 6.5 kb *Hin*dIII *prt* gene fragment showed only minor differences (Fig. 3.4). Based on the published DNA sequences of the lactococcal *prt* genes [Kok et al., 1987; Kiwaki et al., 1989; Vos et al., 1989a], it is likely, however, that the 6.5 kb *Hin*dIII fragment does not encode the complete proteinase gene. While most plasmid *prt* genes cross-hybridize and presumably share a close evolutionary relationship [Kok and Venema, 1988], there are important specificity differences between them. Proteinases from strains 763, HP and H2, though all being PI type, show some differences in their degradation patterns for ß-casein [Monet et al. 1986; Visser et al. 1988; Ng, 1988]. The PIII type proteinase from strain SK11, which degrades κ- and α_{s1}-casein as well as ß-casein, has some amino acid substitutions and sequence duplications compared with strain Wg2 proteinase (PI type). [Visser et al., 1986; Vos et al., 1989a]. Proteinase specificity variations can thus arise from only small changes in the DNA sequences.

3.4 Summary

The cell wall bound proteinase encoded by plasmid pDI21 of *L. lactis* subsp. *cremoris* H2 was characterized to resemble a PI type, since it preferentially degraded ß-casein. The 6.5 kb *Hin*dIII fragment of pDI21 was cloned into *E. coli* with vector λNM1149 and the proteinase acitivity was detected in the recombinant phage lysate, indicating expression of the lactococcal proteinase gene.
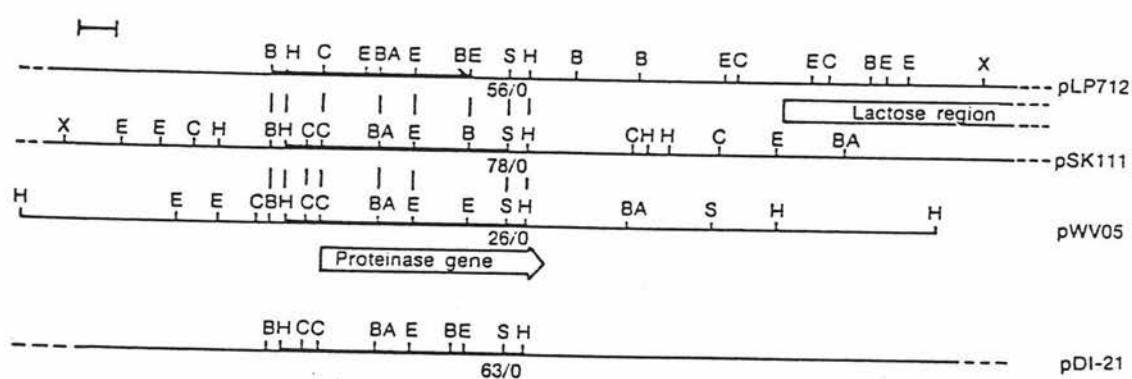
Figure 3.5 Restriction maps of the *prt* genes from *L. lactis* subsp. *lactis* plasmid pLP712 (56 kb) and *L. lactis*. subsp. *cremoris* plasmids pSK111 (78 kb), pWVO5 (26 kb) and pDI21 (63 kb) and. Heavy lines indicated the restriction fragments cloned and specifying proteinases. The genes for proteinase and lactose utilization were marked. The small bar represents 1 kb scale of DNA. BA: *Bam*HI; B: *Bgl*II; C: *Cla*I; E: *Eco*RI; H: *Hind*III; S: *Sal*I; X: *Xho*I.

CHAPTER 4: CONSTRUCTION OF A NEW LACTOCOCCAL VECTOR pFX1 AND EXPRESSION OF THE pDI21 PROTEINASE GENE IN LACTOCOCCI

4.1 Background and Aim

Cloning vectors for Gram-positive organisms were initially constructed using replicons from both Gram-positive and -negative organisms in order to shuttle them between these two genetically divergent groups of bacteria. Recently, however, lactococcal vectors have been reported solely based on replicons from two homologous small cryptic lactococcal plasmids pWV01 and pSH71. These vectors can replicate in Gram-positive organisms as well as in the Gram-negative bacterium *E. coli* [de Vos, 1987].

Currently, vectors for use in lactococci are limited to unstable shuttle plasmids based on non-lactococcal replicons, and to constructs derived only from the above-mentioned lactococcal plasmids pWV01 and pSH71. In this study, we constructed a new lactococcal vector based on the small and high-copy cryptic plasmid pDI25 from *L. lactis*. subsp. *lactis* 5136, and used it for cloning the pDI21 *prt* gene in lactococci.

4.2 Results

4.2.1 Construction of a new lactococcal vector pFX1

The high copy number cryptic 5.5 kb plasmid, designated pDI25, of *L. lactis* subsp. *lactis* 5136 [Crow et al., 1983] was double digested by *Hpa*II and *Mbo*I to produce three fragments (4.5, 0.6, 0.4 kb). These were ligated individually to the 1 kb *Hpa*II-*Mbo*I fragment of the staphylococcal plasmid pC194 which carries the promotorless chloramphenicol acetyl transferase (*cat*) structural gene [Horinouchi and Weisblaum, 1982]. The three ligation mixtures were separately transformed into *E. coli* HB101. Transformants were only obtained when the largest DNA fragment
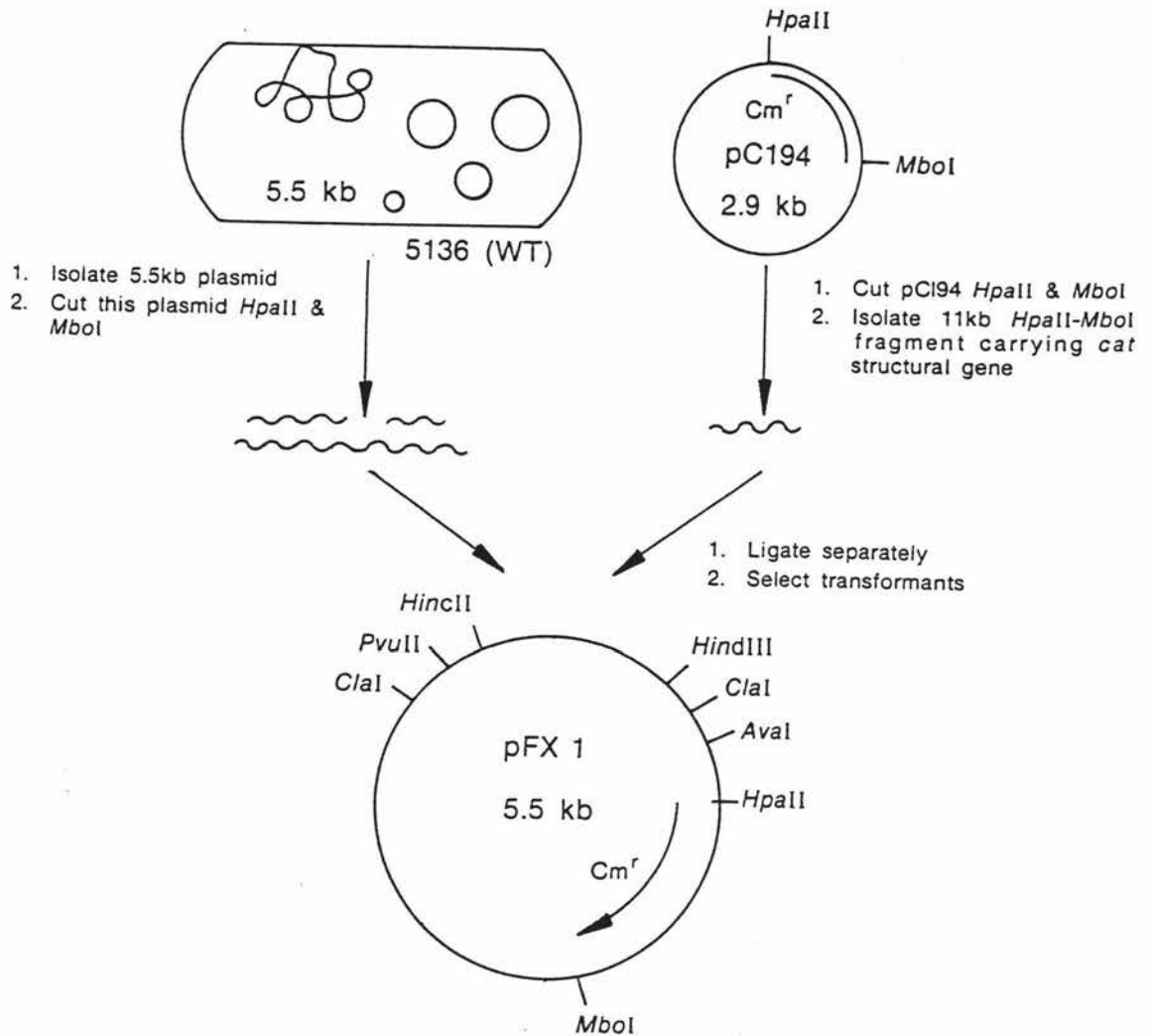
Fig. 4.1 Construction of the new lactococcal vector pFX1 and its restriction map.

(4.5 kb) was ligated to the *cat* fragment, indicating that this fragment carries the pDI25 replicon region. Recombinant plasmids from these transformants were able to replicate in both *E. coli* and lactococci. This plasmid vector was designated pFX1 and a restriction map was determined (Fig. 4.1).

The vector pFX1 could transform at high efficiency into *E. coli* HB101, JM109, *L. lactis* subsp. *lactis* LM0230, MG1363, 4125 and *L. lactis* subsp. *cremoris* 4873 (Table 4.1). Transformation efficiencies were the same whether the vector was isolated from *E. coli* or lactococci. The stability of pFX1 in *E. coli* JM109 and HB101 after approximately 100 generations of growth without Cm selection was 95 and 97% respectively, and was >99% in *L. lactis* subsp. *lactis* MG1363 and 4125 under the same conditions.

Table 4.1. Transformation frequency of pFX1 by electroporation

Strain	Transformation frequency (cfu/μg DNA)
E.coli	
HB101	1.2×10^5
JM109	2.9×10^5
L. lactis subsp. *lactis*	
MG1363	1.4×10^5
LM0230	5.5×10^5
4125	1.5×10^6
L. lactis subsp. *cremoris*	
4873	3.2×10^4

4.2.2 Direct cloning and expression of the pDI21 proteinase gene in L. lactis subsp. lactis 4125

The 1.9 kb *Cla*I region in pFX1 could be deleted without affecting plasmid replication in either *E. coli* or lactococci. As this region was not essential for pFX1 replication, the single *Hin*dIII site in the region was used for cloning the 6.5 kb *Hin*dIII *prt* gene fragment from pDI21.

After digestion with *Hin*dIII and treatment with calf intestinal alkaline phosphatase, pFX1 was ligated to the 6.5 kb *Hin*dIII *prt* gene fragment of pDI21. The ligation mixture was directly transformed into *L. lactis* subsp. *lactis* 4125 by electroporation (10^3 transformants/µg ligated DNA). Eight out of 10 transformants tested were Prt$^+$ by the milk-coagulation test. The orientation of the inserts in recombinant plasmids was determined by comparing double digests with *Hpa*II and *Bgl*II. Recombinants with inserts in either orientation were obtained. These had the same milk-coagulation time, suggesting that the proteinase gene was expressed from its own promoter. One recombinant plasmid was selected and designated pFX101. Both the insert and the vector DNA were unchanged in size after transformation (Fig. 4.2). Plasmid pFX101 showed the same stability in strain 4125 as did pFX1. Following approximately 100 generations of growth in Cm-free broth, 100 colonies all showed the presence of the proteinase gene by colony hybridization. Proteinase released from strain 4125 (pFX101) degraded ß-casein in a manner similar to that observed with the intact pDI21 in strains H2 and 4125 (Fig. 4.3).

4.2.3 Growth of 4125 (pFX101) in milk

L. lactis subsp. *lactis* 4125 (pDI21) showed the same rates of growth and acid production in RSMG as the parent strain H2 (Fig. 4.4). The controls, plasmid free strain 4125 without or with the vector pFX1, produced < 15 µmol/ml titratable acid,

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Figure 4.2 Cloning the 6.5kb *Hin*dIII *prt* gene fragment of pDI21 in pFX1. (A). Lane 1, pFX101/*Hin*dIII; lane 2, pFX1/*Hin*dIII; lane 3, λDNA/*Hin*dIII as M.W. standard; lane 4, λNM114965 DNA (positive control). (B). Autoradiogram result (λNM114965 as a probe).
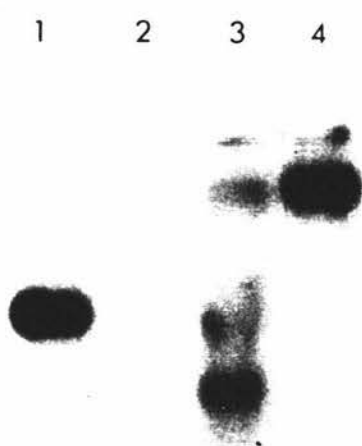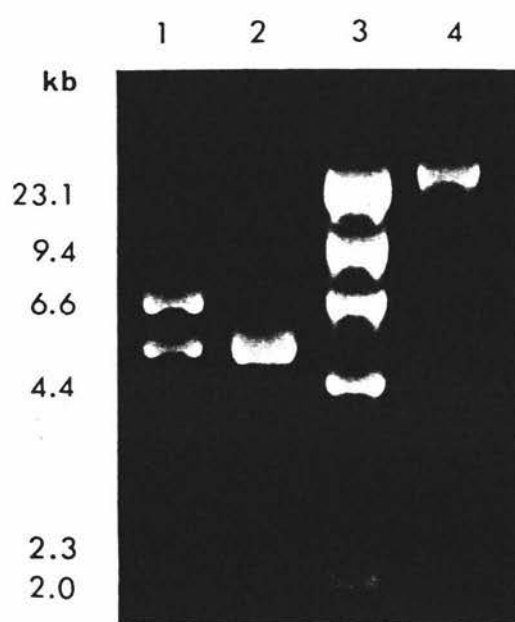
Figure 4.3 ß-casein degradation by different strains of lactococci (16 h, 30° C). Lane 1, 4125 (pFX101); lane 2, 4125 (pFX1); lane 3, 4125; lane 4 (pDI21); lane 5; H2; lane 6, ß-casein control; lane 7, M.W. standards.

1	2	3	4	5	6	7	Da
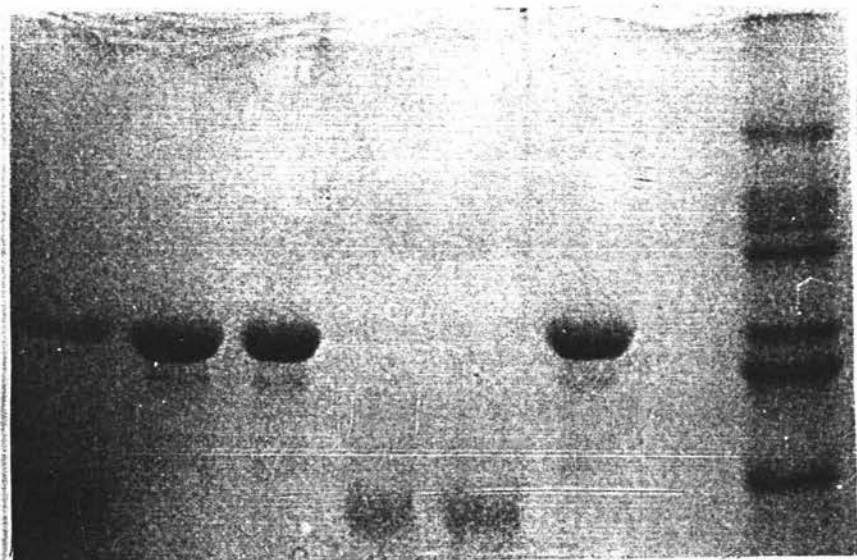
66 000

45 000
36 000
29 000

24 000
20 000

14 200
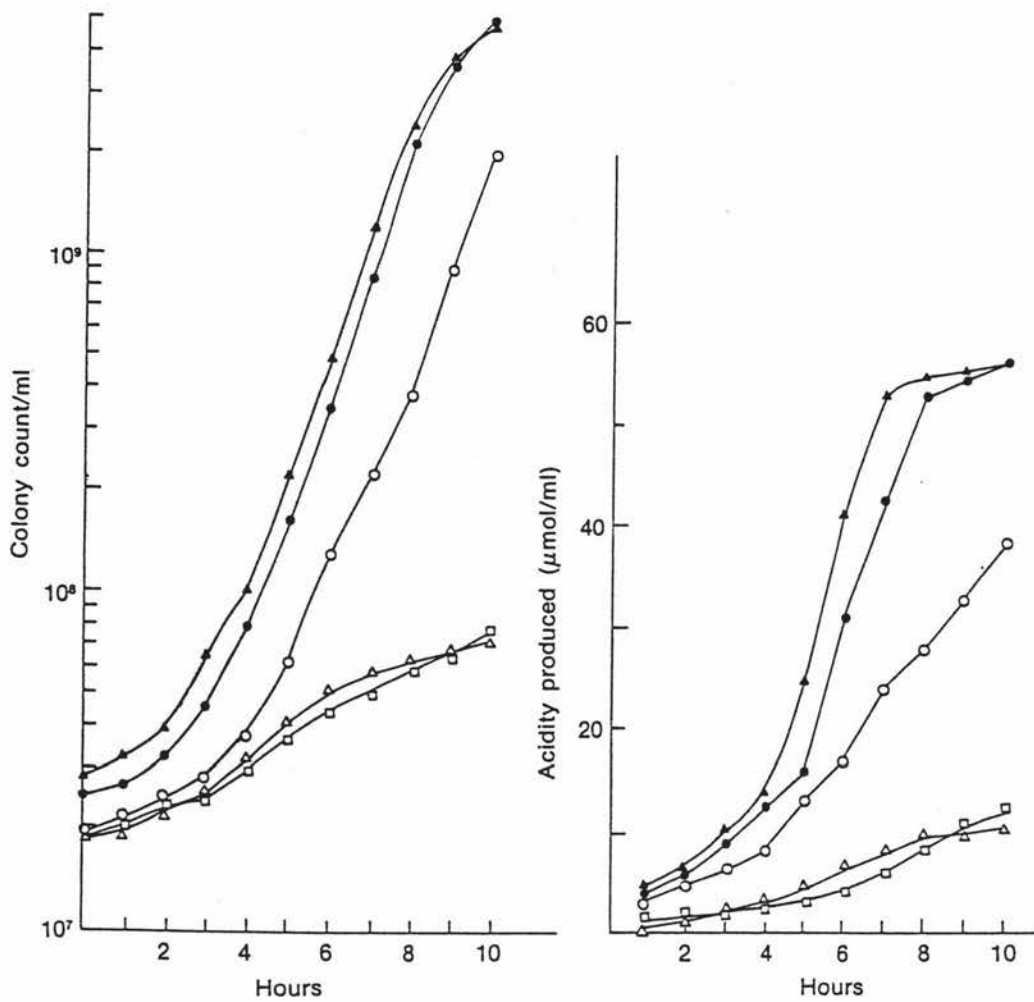
Figure 4.4 Growth and acid production in RSMG by lactococci. H2 [●], 4125 (pDI21) [▲], 4125 (pFX101) [○], 4125 (pFX1) [□] and 4125 [Δ].

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Figure 4.5 Characterization of Prt$^+$ and Prt$^-$ strains on citrate milk agar media. (A). 4125 (pFX101), Prt$^+$; (B). 4125 (pFX1), Prt$^-$.
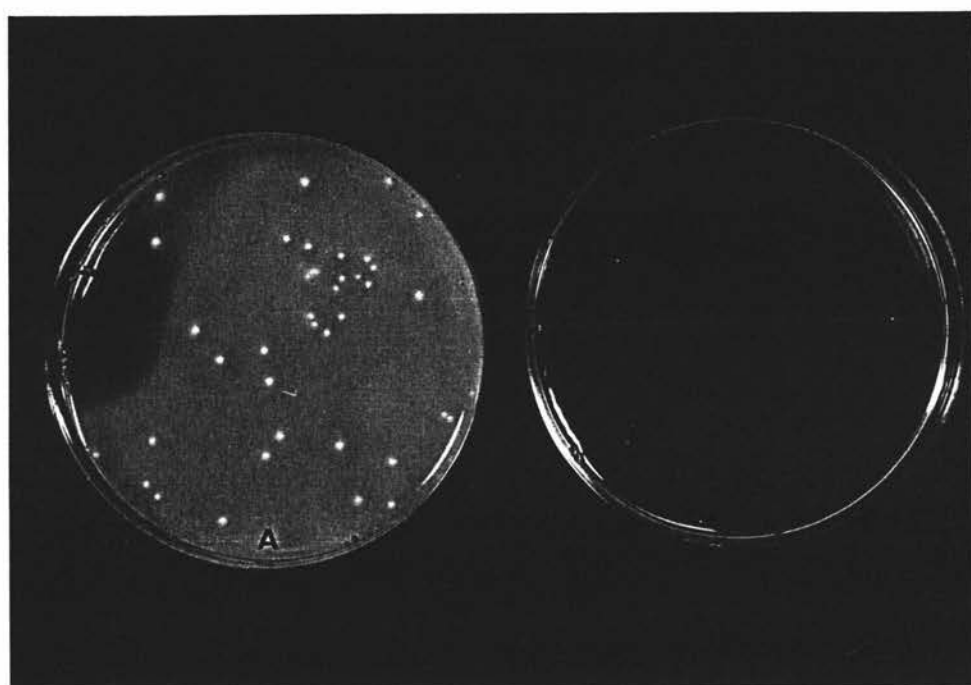
even after 7 days at 30°C [Pearce, L.E., personal communication]. The proteinase gene fragment cloned in pFX101 led to stimulated growth and acid production, but to a lower level than did pDI21 in the same 4125 background (Fig. 4.4).

The Prt$^+$ phenotype of 4125 (pDI21) and 4125 (pFX101) could be distinguished from the Prt$^-$ strains 4125 and 4125 (pFX1) on citrate milk agar plates. After 2 days incubation at 30°C, a white casein precipitate had formed around the Prt$^+$ but not the smaller Prt$^-$ colonies (Fig. 4.5).

4.2.4 Expression of pFX101 (prt) in E. coli and other lactococcal strains

Since the lactococcal proteinase was expressed in a lambda-$E.coli$ lysate, pFX101 was tested for expression of the proteinase in $E. coli$. pFX101 transformed at lower efficiency into $E.coli$ JM109 (10^3 cfu/μg DNA) than did pFX1 (10^5 cfu/μg DNA). The pFX101 transformants grew slower than those of pFX1. There were no obvious size changes of the cloned insert or vector on agarose gels when plasmid DNA from pFX101 transformants were digested with $Hind$III. The plasmid DNA from pFX101 $E. coli$ transformants, however, could not change 4125 to a Prt$^+$ phenotype after transformation. Plasmid pGKV500 was found to act in the same way as pFX101 when transformed into $E. coli$ and subsequently into lactococci.

Plasmid pFX101 was also electroporated into other plasmid-free lactococcal recipients $L. lactis$ subsp. $lactis$ LM0230 and MG1363. Both LM0230 (pFX101) and MG1363 (pFX101) coagulated milk in 18 h at 22° C, the same rate as the Prt$^+$ strains H2 and 4125 (pDI21). As noted above, 4125 (pFX101) was significantly slower, requiring 30 h at 22° C to coagulate milk.

4.3 Discussion

The newly-constructed broad host-range vector pFX1 was transformed efficiently into lactococci and *E. coli*, and stable in both hosts. It has cloning sites for *Hind*III, *Hinc*II, *Pvu*II, *Hpa*II, *Mbo*I and *Cla*I, though several other commonly used restriction sites were not found including *Eco*RI, *Bam*HI, *Sal*I, *Pst*I, *Sma*I, *Xba*I, *Xho*I, *Sph*I, *Bgl*II and *Eco*RV. By using this vector in conjunction with electroporation, DNA fragments can be cloned directly into lactococci, eliminating the necessity for intermediate hosts such as *E. coli*, *B. subtilis* or *S. sanguis* [de Vos, 1987].

Plasmid linkage of the lactococcal proteinase genes had previously been demonstrated by a number of gene transfer systems including transduction, conjugation and protoplast transformation [Kok and Venema, 1988]. In this study, linkage was also demonstrated by direct cloning using electroporation.

The *E. coli* JM109 host enabled both pFX101 as well as pGKV500 to establish following transformation. The plasmid pFX101 was however altered in *E. coli* rendering the *prt* gene(s) non-functional when pFX101 was subsequently electroporated into lactococci, although no detectable size changes in the pFX101 vector or insert were found. This observation is consistent with the earlier reports that lactococcal *prt* genes are unstable and undergo deletions in *E. coli* [Gasson et al., 1987; Kok et al., 1985].

Strain 4125 (pFX101) was Prt⁺ but was significantly slower in growth and acid production than strains H2, 4125 (pDI21), LM0230 (pFX101) and MG1363 (pFX101). The plasmid pGKV500 which expressed full proteinase activity in LM0230 and MG1363 behaved in an identical manner to pFX101 when electroporated into 4125. This phenomenon suggests that DNA sequences are present on the pDI21 plasmid and the chromosomes of LM0230 and MG1363 which enhance cell growth by increasing proteinase expression, stimulating the uptake of peptides or by other mechanisms.

Identification of such sequences would assist our further understanding the lactococcal proteolytic systems.

4.4 Summary

A new lactococal vector pFX1 was constructed using the lactococcal plasmid replicon pDI25 from *L. lactis* subsp. *lactis* 5136 and incorporating a chloramphenicol resistance gene. This vector was transformed efficiently into lactococci and *E. coli* and maintained stably in both hosts. Using this vector, the 6.5 kb *Hin*dIII *prt* gene fragment was directly cloned into lactococci where the proteinase was expressed.

CHAPTER 5: CONSTRUCTION OF VECTORS pFX3, pFX4, pFX5 AND pFX6 FOR LACTOCOCCAL GENE CLONING AND TRANSLATIONAL FUSION

5.1 Background and Aim

A number of cloning and promoter-detecting vectors have recently been constructed to study the structure and function of lactococcal genes [de Vos, 1987]. One important strategy has been to incorporate selectable markers, eg. antibiotic-resistance genes into small multicopy cryptic plasmids from lactococci. With the use of the promoterless *cat* gene, several lactococcal promoter probe vectors were constructed including pGK110 [van der Vossen et al., 1985], pNZ220 [de Vos, 1987], pCK509 [Gasson, 1988] and pMU1358 [Achen et al., 1986]. Promoter sequences could then be isolated from the recombinant plasmids that were resistant to chloramphenicol. One particularly useful feature of these vectors is that they can replicate in both Gram-positive bacteria and *E. coli* [de Vos, 1987; Lakshmidevi et al., 1990]. Thus constructs could be made in both organisms in case instability was a problem in one one of them. In Chapter 4, construction of a new lactococcal vector pFX1 was described. This vector could efficiently transform *E. coli* and lactococci, and was maintained stably in both organisms.

The *E. coli lacZ* gene has been widely used as a marker to construct vectors for both procaryotic and eucaryotic systems [Casadaban et al., 1980; Minton, 1984; Miyazaki et al., 1989]. The *lacZ* gene and its product ß-galactosidase are well characterized genetically and biochemically [Beckwith and Zipser, 1970]. There are sensitive assays for the enzyme that utilizes commercially available substrates. In this study, a family of lactococcal vectors was constructed based on the pFX1 vector using *lacZ* as a reporter gene.

5.2 Results

5.2.1 Construction of pFX3 vector

The cloning vector pFX3 was constructed as outlined in Fig. 5.1. The 3.6 kb plasmid pFX2 was derived from pFX1 by deletion of the 1.9 kb non-essential *Cla*I region (Chapter 4). Plasmid pFX2 was linearized with *Cla*I and filled in with Klenow fragment of DNA polymerase I. Plasmid pUBS is a derivative of plasmid pUC19 with a different polylinker carrying additional cloning sites and T3 and T7 promoters. The pUBS DNA was digested to completion with *Ssp*I and partially with *Pvu*II, and the 0.9 kb *Pvu*II-*Ssp*I fragment carrying the α fragment of the *lacZ* gene (designated as *lacZ'*) and the multiple cloning region was recovered from an agarose gel by freeze-thawing. This fragment was then blunt-end ligated to the linearized pFX2 fragment and the ligation mixture was transformed into *E. coli* JM109 by electroporation. Transformants were selected as blue colonies on LB plates containing X-gal and chloramphenicol. The *lacZ'* orientation in the recombinat plasmids was determined by analysis of single and double restriction digests (*Eco*RI, *Xho*I, *Cla*I, *Pvu*II, *Pvu*II/*Cla*I and *Cla*I/*Ava*I). A construct pFX3 was obtained as shown in Fig 5.1. It was found that a *Cla*I site was restored in the region upstream of the T7 promoter in pFX3 after ligation between the DNA fragments with *Ssp*I and filled-in *Cla*I ends. There are therefore two *Cla*I sites (there is one in the polylinker region) in pFX3, yet they still can be used for cloning by replacing the smaller *Cla*I region. Vector pFX3 prepared from *E. coli* JM109 could be transformed into lactococci.

Besides having multiple cloning sites (the DNA sequence analysis in Chapter 6 showed that all of these sites can be used), pFX3 can be used for direct screening of recombinant plasmids in *E. coli* by inactivation of the *lacZ* function. Recombinant plasmids from white colonies on X-gal plates can then be electroporated into lactococci. If a target gene is unstable in *E. coli*, the construct can be transformed directly into lactococci. In addition, the T3 and T7 promoters flanking the multiple cloning region of pFX3 facilitate direct transcriptional studies or DNA sequencing without recloning.
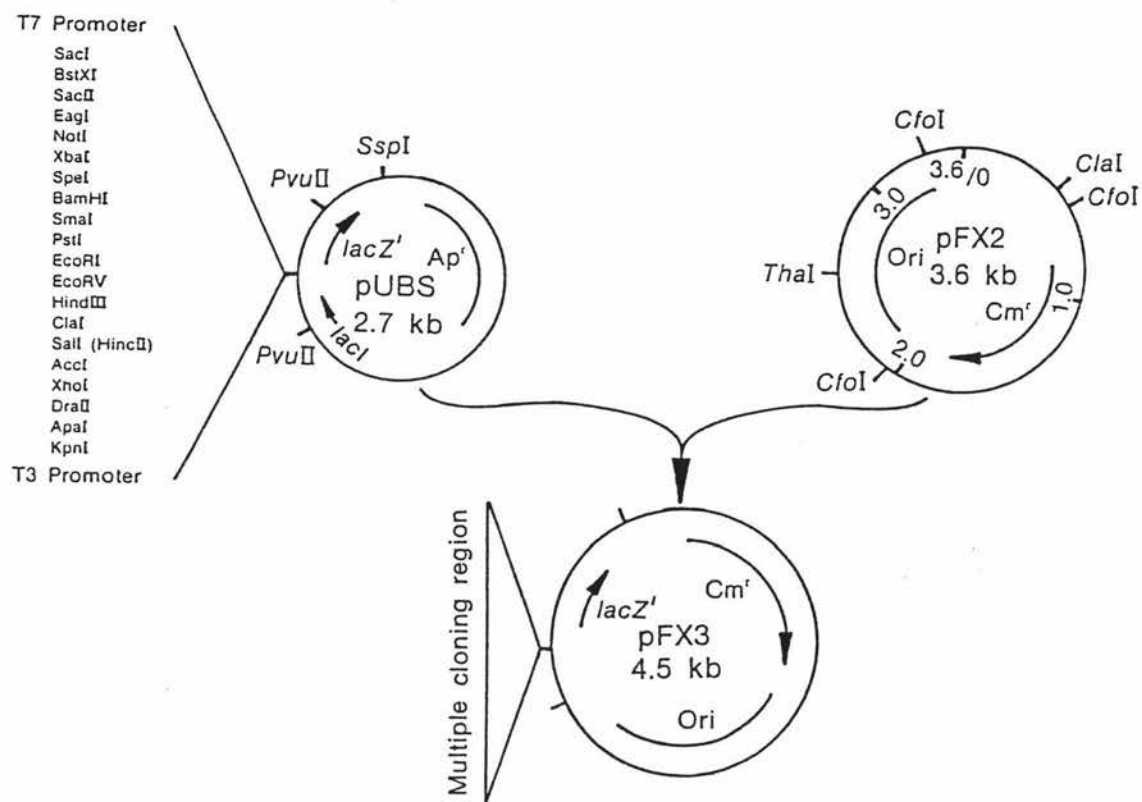
Figure 5.1 Construction of lactococcal vector pFX3. The essential regions for pFX plasmid replication were determined in Chapter 6.

5.2.2 Cloning of the lactococcal tagatose 1,6-bisphosphate aldolase gene into lactococci using pFX3

The tagatose 1,6-bisphophate aldolase gene of *L. lactis* subsp. *lactis* 4560 is located on the 4.4 kb *Eco*RI fragment of plasmid pDI1 and is expressed in *E. coli*[Limsowtin et al., 1986; Yu et al., 1988]. Using pFX3, this 4.4 kb *Eco*RI fragment was cloned into pFX3 and transformed into *E. coli* (Fig. 5.2). The recombinant plasmid pFX301 was then electroporated into *L. lactis* subsp. *lactis* 4125. Plasmid pFX301 was stable in both hosts and no size change of the vector or insert was detected by restriction analysis (Fig. 5.3). The expression of this gene in the two hosts was not examined.

5.2.3. Contruction of lactococcal translational fusion vectors pFX4, pFX5 and pFX6

Lactococcal translational gene-fusion vectors were constructed using an *E. coli lacZ* fusion system (Fig. 5.4). Plasmids pNM480, pNM481 and pNM482 differ from each other by the presence of 3-, 4- and 5-bp inserts respectively between the multiple cloning region and the complete structural *lacZ* gene [Minton, 1984]. The 3.1 kb *Eco*RI-*Dra*I fragment carrying the multiple cloning site and *lacZ* gene from each of these plasmids was isolated individually from agarose gels and the recessed ends filled in. Plasmid pFX2 was linearized with *Cla*I, filled in and blunt-end ligated to each of the above three fragments. The ligated molecules was electroporated into *L. lactis* subsp. *lactis* 4125. Recombinant plasmids with the *lacZ* gene orientations were characterized by restriction analysis. One set of vectors pFX4, pFX5 and pFX6 with the same *lacZ* gene orientation was obtained (Fig. 5.4). The *Eco*RI site in the polylinker was restored in pFX4, 5 and 6 vectors.

The constructed vectors pFX4, pFX5 and pFX6 permit the fusion of cloned genes to *lacZ* in all three translational reading frames in lactococci. Gene expression can be quantitatively monitored by measuring ß-galactosidase activity.

Figure 5.2 Cloning the 4.4 kb *Eco*RI aldolase gene fragment of pDI1 in pFX3. (A).
Lane 1, 1 kb DNA ladder as M.W. standard; lane 2, pDI1/*Eco*RI; lane 3,
pFX3/*Eco*RI; lane 4, pFX301/*Eco*RI; lane 5, pFX301 supercoiled DNA isolated from
E. coli. (B). Hybridization autoradiogram result (pDI1 as a probe).
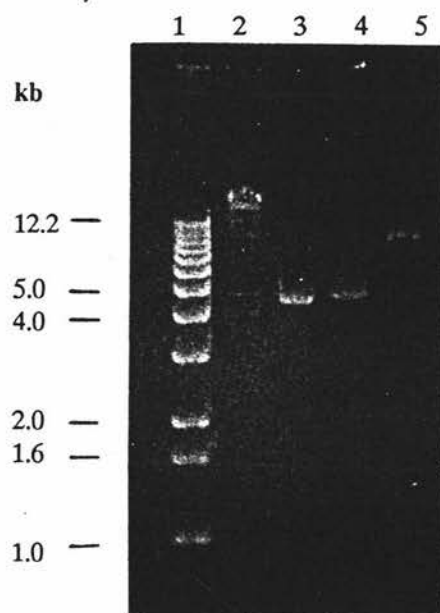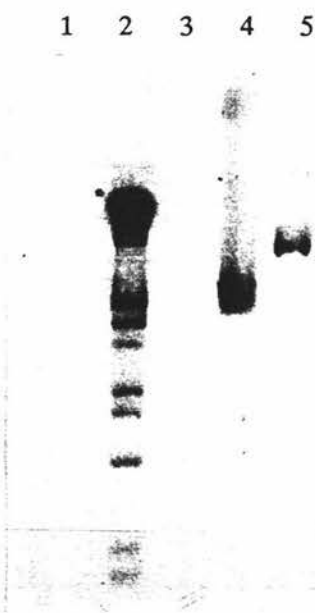
A   B

Figure 5.3 Restriction analysis of pFX301 plasmid DNA isolated from *E. coli* and lactococci. Lane 1, λ DNA *Hin*dIII fragment as M.W. standard; lane 2, pDI1/*Eco*RI; lane 3, pFX301 isolated from *E. coli*/*Eco*RI; lane 4, pFX301 isolated from lactococci/*Eco*RI; lane 5, pFX3 supercoiled DNA isolated from lactococci.
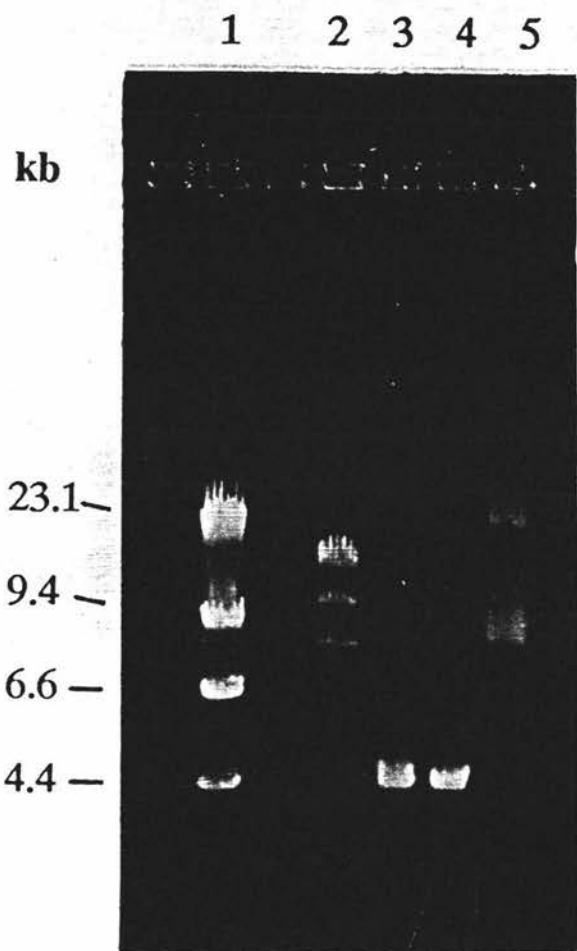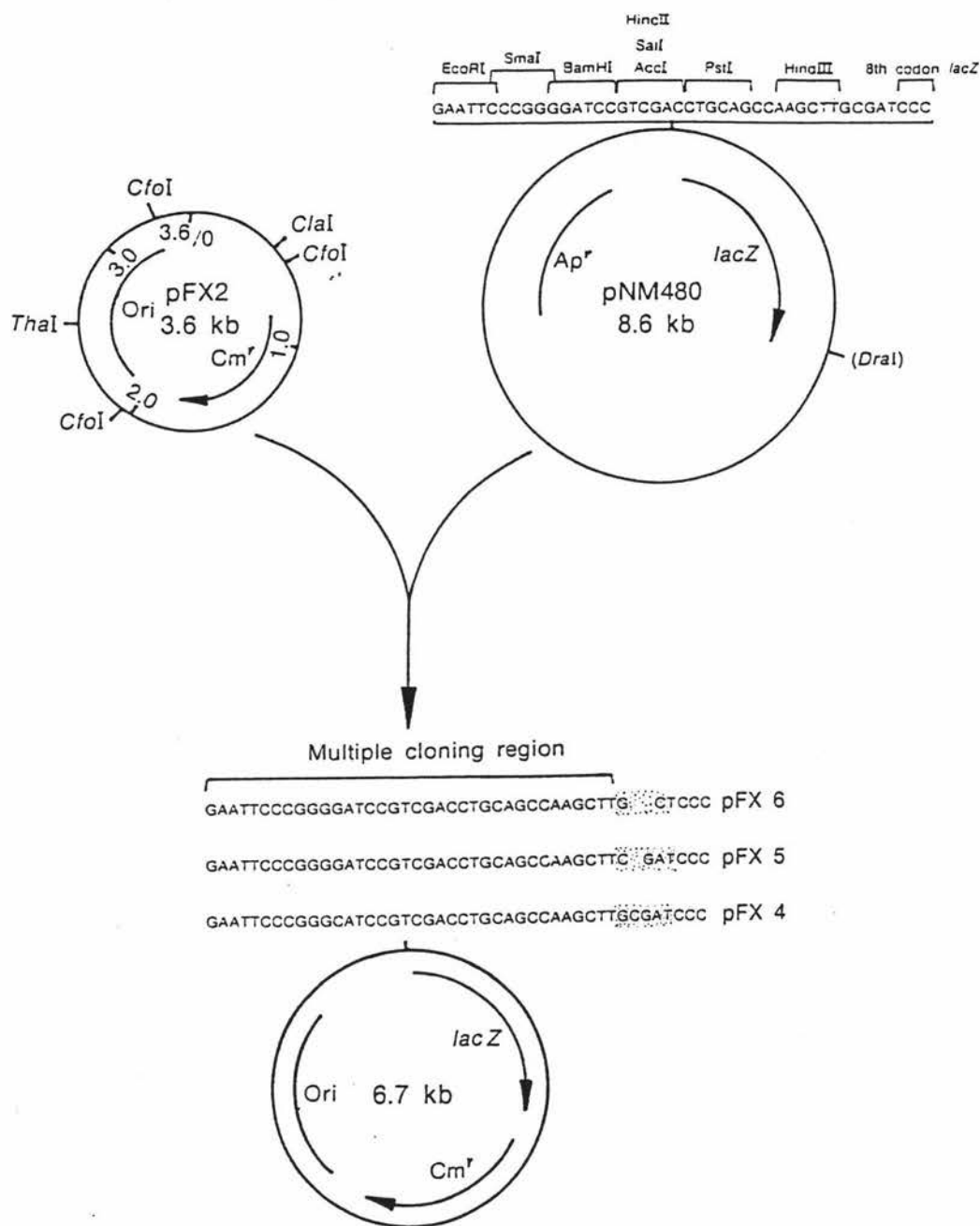
Figure 5.4 Construction of lactococcal translational fusion vectors pFX4, pFX5 and pFX6. Restriction sites are specified in the multiple cloning regions. The shaded nucleotides mark the only differences between pFX4, pFX5 and pFX6. The essential regions for pFX plasmid replication were determined in Chapter 6.

5.2.4 Expression of lactococcal genes fused to <u>lacZ</u>

The vectors pFX4, pFX5 and pFX6 were used to generate functional ß-gal fusions with fragments containing the galactose-6-phosphate isomerase gene [Yu, et al., 1989] and the *prt* gene from the lactococcal plasmid pDI21. The 2.0 kb *Eco*RI fragment of pDI21 which hybridized with a galactose-6-phosphate isomerase gene probe expressed no enzymatic activity in *E. coli* [Yu, et al., 1989]. When this fragment was cloned into pFX4, 5 and 6, and transformed into *E. coli*, blue colonies were found only with the pFX5 construct, indicating fusion of the gene fragment to *lacZ* and the presence of a promoter in the correct reading frame to initiate transcription of the *lacZ*. The orientation of the fragment in the transformant pFX501 was determined by mapping the *Bgl*II site in the cloned insert with *Bgl*II and *Pst*I. All blue transformants with the 2.0 kb *Eco*RI fragment in pFX5 were in the same orientation. Gene expression was directed towards the D-tagatose-6-phosphate kinase gene, and was likely controlled by the isomerase promoter [b in Fig. 5.6]. An *in vitro* expression study using *E. coli* S-30 extract showed a single protein of 13 kDa produced by this fragment (Yu P.-L., personal communication).

Attempts were made to demonstrate expression of this fusion product in lactococci but were unsuccessful, probably due to the instability of the recombinant plasmid (pFX501) or the lethality of the fused protein to the lactococci.

The proteinase genes of different lactococcal plasmids shared a high degree of homology [Kok and Venema, 1988; Chapter 4]. Recent studies of cloned proteinase gene fragments from *L. lactis* subsp. *cremoris* Wg2 and SK11 showed that an open reading frame for a maturation protein was adjacent to and upstream of the proteinase gene coding region [Haandrikman et al., 1989; Vos et al., 1989b]. These two genes are transcribed in opposite orientations with their promoters about 300 bp apart. We used gene fusion to establish whether the 6.5 kb *Hin*dIII proteinase gene fragment from plasmid pDI21 [Chapter 5] also had two promoters in opposite orientations. The 6.5 kb *Hin*dIII fragment was cloned in pFX4, 5 and 6, and electroporated into *L. lactis* subsp. *lactis* 4125. All blue transformants were found to

Figure 5.5 Cloning of the 2.0 kb *Eco*RI isomerase gene fragment in pFX5. Lane 1. 1 kb DNA ladder as M.W. standard; lane 2, pFX5/*Eco*RI; lane 3, pBH201/*Eco*RI; lane 4, pFX501/*Eco*RI.
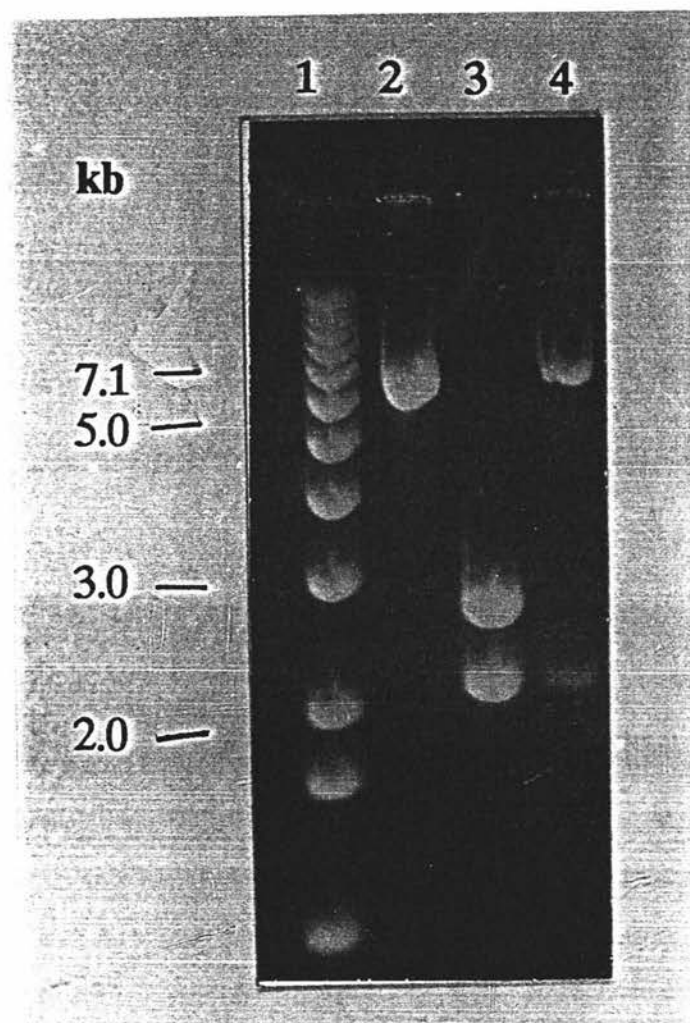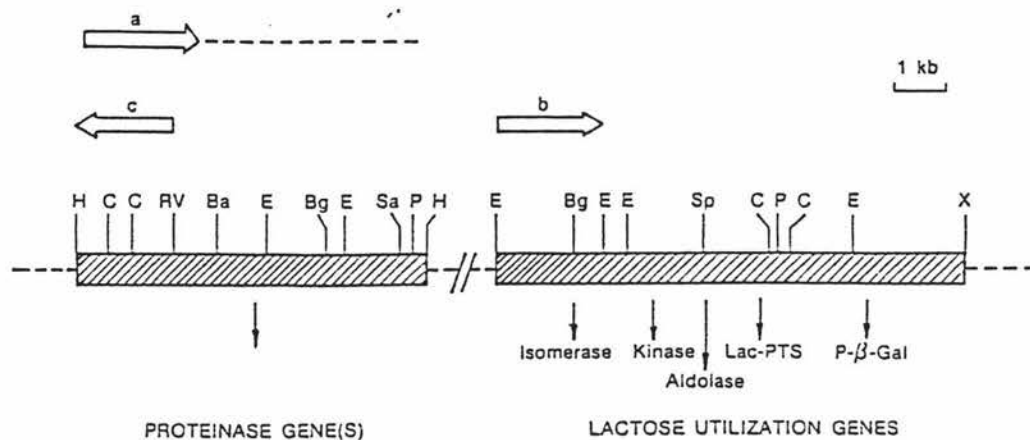
Figure 5.6. Portion of the restriction map of plasmid pDI21 from *L. lactis* subsp. *cremoris* H2, showing proteinase and lactose-utilizing genes. a, b and c are fragments cloned into vectors pFX4, pFX5 and pFX6 to give pFX401, pFX501 and pFX601 respectively. Directions of transcription are arrowed. Ba: *Bam*HI; Bg: *Bgl*II; C: *Cla*I; E: *Eco*RI; EV: *Eco*RV; H: *Hin*dIII; P: *Pst*I; Sa: *Sal*I; Sp: *Sph*I; X: *Xho*I.

Figure 5.7 Cloning of the 1.8 kb *Hin*dIII-*Eco*RV internal region of the pDI21 6.5 kb *Hin*dIII *prt* gene fragment in pFX6. Lane 1, pFX6/*Sma*I; lane 2, pFX601 supercoiled DNA; lane 3, pFX601/*Hin*dIII, *Eco*RI; lane 4, 1 kb DNA ladder as M.W. standard.
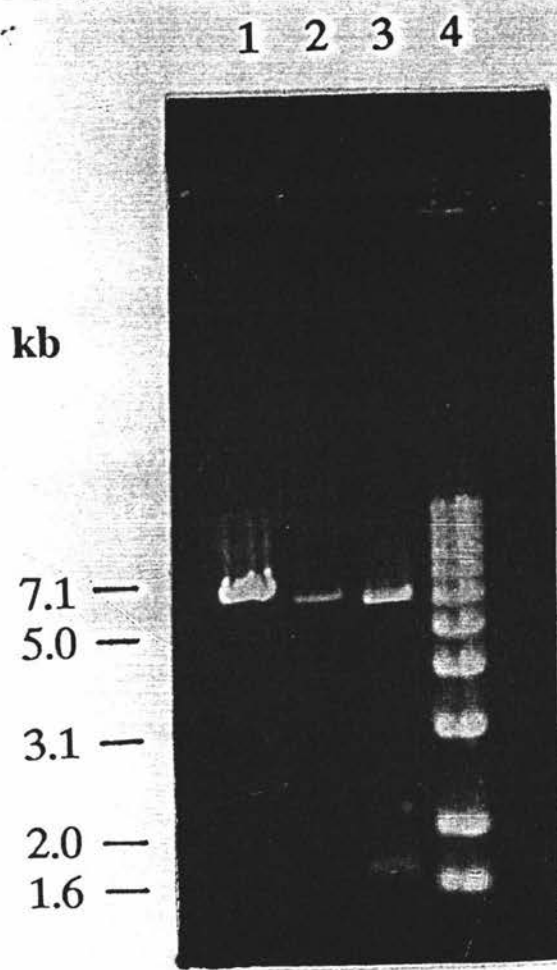
carry deletions. One transformant pFX401 (8.7 kb) had a single *Hin*dIII site and the cloned insert had an *Eco*RV site but not *Bam*HI and *Eco*RI *sites*. These observations suggested that the deletion was in the right hand region starting between the *Eco*RV and *Bam*HI sites to the right hand *Hin*dIII site (a in Fig. 5.6). The 1.8 kb *Hin*dIII-*Eco*RV left hand region from the 6.5 kb *Hin*dIII fragment was filled in and cloned intact into the *Sma*I site of pFX4, 5 and 6. Blue colonies were found only with the pFX6 construct (pFX601) (Fig. 5.7). Restriction analyses of the insert orientations in pFX401 (using *Bam*HI, *Hin*dIII, *Eco*RI and *Cla*I) and pFX601 (using *Cla*I) indicated the *lacZ* was expressed by two different promoters acting in opposite directions (a, c respectively in Fig. 5.6), confirming that the pDI21 proteinase gene was organized similarly to those previously described.

The two fusions pFX401 and pFX601 showed different levels of ß-gal by specific activity assay (7.2 and 20.1 nmol/min/mg protein, respectively), indicating differences in their ability to promote *lacZ* expression in lactococci. The control 4125 with the vector pFX4 only showed no ß-gal activity.

5.2.5 Transformation efficiency and stability of pFX vectors

Plasmids pFX3,4,5 and 6 could be efficiently transformed into lactococci and *E. coli* by electroporation (10^4-10^5 cfu/µg DNA in each host). More than 99% of the pFX3, pFX6, pFX601 cells retained the antibiotic or *lacZ* gene after approximately 100 generations of growth in non-selective media. There were no changes in plasmid size.

5.3 Discussion

The pFX series vectors described above permit efficient gene cloning and the study of gene expression in lactococci. Cloning can be conducted in either *E. coli* or lactococci. The translational fusion vectors constructed provide a straightforward approach to characterizing promoters or other regulatory sequences required for gene expression in lactococci. These vectors are stable and relatively small in size

(Fig. 5.8), enabling them to be used for cloning large inserts. Vector pFX3 has been subsequently used successfully in the cloning of several lactococcal genes in this laboratory, including an 11 kb Lac gene fragment [Yu P.-L. and Ward L.J.H., personal communications].

5.4 Summary

A family of stable lactococcal vectors have been constructed based on the plasmid pFX2 using either the *lacZ'* fragment or the complete *E. coli lacZ* as a selective marker. These vectors also contain multiple cloning sites and examples are given of their use for lactococcal gene cloning and translational fusion studies.

Figure 5.8 The size of pFX series constructs after restriction digest. Lane 1, 1 kb DNA fragment laddar as M.W. standard; lane 2, pFX1/*Hind*III; lane 3, pFX1/*Cla*I; lane 4; pFX2/*Cla*I; lane 5, pFX2/*Cfo*I; lane 6, pFX7 [pFX2 with 0.7 kb *Cfo*I-*Cla*I-*Cfo*I region deletion, Chapter 6); lane 7, pFX7/*Hpa*II; lane 8, pFX4/*Eco*RI; lanes 9-11, pFX4, 5 and 6/*Sma*I, respectively.
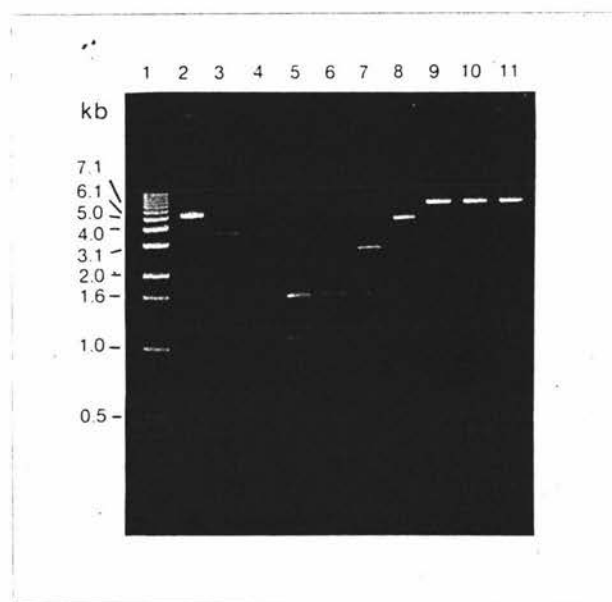
CHAPTER 6: GENETIC ANALYSIS OF A LACTOCOCCAL PLASMID REPLICON

6.1 Background and Aim

The genetic organization of many plasmid replicons from Gram-positive bacteria such as *Staphylococcus* [Novick, 1989], *Bacillus* [Gruss and Ehrlich, 1989], *Streptococcus* [del Solar et al., 1989], *Lactobacillus* [Bates and Gilbert, 1989], *Streptomyces* [Kendall and Cohen, 1988] and *Clostridium* [Garnier and Cole, 1988] is now well established. In contrast, very little is known about lactococcal plasmid replicons at the molecular level. A plus origin (*ori*) site has been found in lactococcal plasmid pSH71 [Gruss and Ehrlich, 1989] and the minimum replicon regions located in two other plasmids from *L. lactis* subsp. *lactis* strains UC317 [Hayes et al., 1990] and SSD207 [von Wright et al., 1990].

In lactococci, several industrially-important genes such as those for lactose utilization, proteinase and citrate utilization are plasmid encoded. The elucidation of plasmid replication mechanisms is fundamental to further vector development and to understanding gene expression in lactococci. In this chapter, the pFX2 DNA sequence and its genetic organization were described.

6.2 Results and Discussion

6.2.1 Localization of the essential replication region for pFX vectors

The essential region for replication of the various pFX vectors was located within the 1.2 kb *Cfo*I region of pFX2 (Fig. 5.4). This identification was based on the following results: (1) *lacZ* gene fragments from either pUBS or pNM480, pNM481 and pNM482 could not be cloned into the *Tha*I site of pFX2 (Fig. 5.4). A cointegrate plasmid of pFX2/*Tha*I and pUBS/*Ssp*I could replicate and express Cm^r, Ap^r and the *lacZ* gene

in *E. coli* but was unable to function in lactococci. The *Tha*I site was therefore in a region essential for replication of the plasmid in lactococci. (2) After deletion of the 0.7 kb *Cfo*I-*Cla*I-*Cfo*I region, the remainder of pFX2 was recircularized and transformed into both *E. coli* and lactococci (Figs. 5.3 and 5.7). (3) The 1.6 kb *Cfo*I fragment covering the Cm^r gene could not replicate in lactococci after self-ligation. (4) The 1.2 kb *Cfo*I fragment and the 1 kb *Hpa*II-*Mbo*I promoterless *cat* fragment of pC194 could be ligated and transformed into lactococci.

6.2.2 General features of the pFX2 DNA sequence

As the *cat* gene DNA sequence has been previously described [Horinouchi and Weisblum, 1982], only the lactococcal portion of pFX2 (2508 bp) was determined (Fig. 6.1). The G+C content of the lactococcal DNA was 35.2%, within the range for lactococci (34.4-36.3%) reported by Garvie et al. [1981]. The complete pFX2 DNA sequence including the *cat* gene sequence was 3536 bp, close to the 3.6 kb estimation obtained by agarose gel analysis [Chapter 5]. The establishment of the pFX2 sequence enables pFX3 [Chapter 5] to be used as a sequencing vector, as the entire pFX3 sequence (4475 bp) comprises the known sequences of pFX2, *lacZ'* and the polylinker regions. All polylinker restriction sites are unique in pFX3 and hence available for cloning; similarly with pFX4,5 and 6 [Chapter 5].

6.2.3 Lactococcal plasmid plus <u>ori</u> site

The essential region for plasmid pFX2 replication has been located within a 1.2 kb *Cfo*I-*Tha*I-*Cfo*I fragment [this chapter, 6.2.1], i.e. positions 1050-2176. By comparing this sequence with three classes of plus origin sequences [Gruss and Ehrlich, 1989], a region homologous to the plus *ori* sites of pE194 group plasmids was identified (Fig. 6.2). Recently, the precise nicking site in pLS1 was located between bases G (448)

Figure 6.1 Nucleotide sequence and inferred amino acid sequences of the lactococcal portion (*Hpa*II-*Mbo*I) of pFX2. Coding regions are RepA (positions 1341-1499) and RepB (positions 1569-2267). Nucleotides in lower case represent the region identical to staphylococcal plasmid pE194. A possible plus *ori* site (solid line) and direct repeat regions (double-dash) are indicated. Within the second direct repeat region are three iterons (stars). The inverted repeat region that could form an attenuator is indicated by facing arrows. Putative promoter region and ribosome binding sites are positioned with markers ∧ and ° respectively. RS_A and important restriction sites are marked.

HpaII
CCGGTTTAGTAAGACTTTTTTTATTGCCCAGTTTTCAGTTGCGTAGGCTGAAAGCTGGGCTTTTTTATTATATCACGATTTCTAGGAACGTGAAACCCTTT 100

AACAATTCCCGTGGGTTTCCACCCACACCCGAGGAGCGTAGGACGCTCCACCCCACCTCCGAAAAGAATAGTGTTTAGATTGATTTTCAATTCTTTTCTT 200
                                                                              CfoI.
ACGGCTTACACAATCTATCACAATCTCAACAACACTGCAAGGGTCGCTTTCGCTTCCTCCCTTTCCGTTTATGTTGCGCTTGTGATTTTTCGATTGTGCC 300
                                  ClaI
GACGAAAAGAATTAAAAATCAATCGCTACAAAGTTATCTCCGACAACATCGATTTCATACATGCCAGCTAATTTTTCAACTTCTACGATGATATGTGAGG 400

TCTGCTATCGCACACCCTCACGCTTGGAACGGAAATAAACGGAGTGTCTTGAAGTCGATTAACCGACATTGTCGCTAATAAACTTTTAGTCACTTGCTTT 500

TCTGCGTCTTTTTGCGTGATATATTTTCCGATTGTCTTCGCCTCgatcgttaaatttatactgcaatcggatgcgattattgaataaaagatatgagaga 600

tttatctaatttctttttttcttgtaaaaaaagaaagttcttaaaggtttttatagttttggtcgtagagcacacggtttaacgacttaattacgaagtaaa 700
                                                    |------------------------------ RS_A --

taagtctagtgtgttagactttatgaaatctatatacgtttatatatatttattatccgATTTTTTATTAAAACGTCTCAAAATCGTTTCTGAGACGTTT 800
-------------------------------|

TAGCGTTTATTTCGTTTAGTTATCGGCATAATCGTTAAAACAGGCGTTATCGTAGCGTAAAAGCCCTTGAGCGTAGCGTGGCTTTGCAGCGAAGATGTTG 900
                                                    CfoI CfoI
TCTGTTAGATTATGAAAGCCGATGACTGAATGAAATAATAAGCGCAGCGCCCTTCTATTTCGGTTGGAGGAGGCTCAAGGGAGTATGAGGGAATGAAATT 1000
                                        CfoI
CCCTCATGGGTTTGATTTTAAAAATTGCTTGCAATTTTGCCGAGCGGTAGCGCTGGAAAATTTTTGAAAAAAATTTGGAATTTGGAAAAAAAATGGGGGGA 1100
                                        ==================================

AAGGAAGCGAATTTTGCTTCCGTACTACGACCCCCCCATTAAGTGCCGAGTGCCAATTTTTGTGCCAAAAACGCTCTATCCCAACTGGCTCAAGGGTTTAA 1200

GGGGTTTTTCAATCGCCAACGAATCGCCAACGTTTTCGCCAACGTTTTTATAAATCTATATTTAAGTAGCTTTATTGTTGTTTTTATGATTACAAAGTGA 1300
              ********* ********* *********====   ^^^^^^

TACACTAACTTTATAAAATTATTTGATTGGAGTTTTTTTAAATGGTGATTTCAGAATCGAAAAAAAGAGTTATGATTTCTCTGACAAAAGAGCAAGATAAA 1400
       ^^^^^^   oooo   MetValIleSerGluSerLysLysArgValMetIleSerLeuThrLysGluGlnAspLys

AAATTAACAGATATGGCGAAACAAAAAGGTTTTTCAAAATCTGCGGTTGCGGCGTTAGCTATAGAAGAATATGCAAGAAAGGAATCAGAACAAAAAAAAT 1500

LysLeuThrAspMetAlaLysGlnLysGlyPheSerLysSerAlaValAlaAlaLeuAlaIleGluGluTyrAlaArgLysGluSerGluGlnLysLys
               ThaI
AAGCGAAAGCTCGCGTTTTTAGAAGGATACGAGTTTTCGCTACTTGTTTTTGATAAGGTAATTATATCATGGCTATTAAAAAATACTAAAGCTAGAAATTT 1600
------------>   <---------------------- oooo   MetAlaIleLysAsnThrLysAlaArgAsnPh

TGGATTTTTATTATATCCTGACTCAATTCCTAATGATTGGAAAGAAAAATTAGAGAGTTTGGGCGTATCTATGGCTGTCAGTCCTTTACACGATATGGAC 1700
eGlyPheLeuLeuTyrProAspSerIleProAsnAspTrpLysGluLysLeuGluSerLeuGlyValSerMetAlaValSerProLeuHisAspMetAsp

GAAAAAAAGATAAAGATACATGGAATAGTAGTGATGTTATACGAAATGGAAATGCACGTCATCAAAAATCCACACTATCACGTATATATATTGCACGGAA 1800
GluLysLysIleLysIleHisGlyIleValValMetLeuTyrGluMetGluMetHisValIleLysAsnProHisTyrHisValTyrIleLeuHisGlyA

ATCCTGTAACAATAGAAAGCGTTAGGAACAAGATTAAGCGAAAATTGGGGAATAGTTCAGTTGCTCATGTTGAGATACTTGATTATATCAAAGGTTCATA 1900
snProValThrIleGluSerValArgAsnLysIleLysArgLysLeuGlyAsnSerSerValAlaHisValGluIleLeuAspTyrIleLysGlySerTy

TGAATATTTGACTCATGAATCAAAGGACGCTATTGCTAAGAATAAACATATATACGACAAAAAAGATATTTTGAACATTAATGATTTTGATATTGACCGC 2000
rGluTyrLeuThrHisGluSerLysAspAlaIleAlaLysAsnLysHisIleTyrAspLysLysAspIleLeuAsnIleAsnAspPheAspIleAspArg

TATATAACACTTGATGAAAGCCAAAAAAGAGAATTGAAGAATTTACTTTTAGATATAGTGGATGACTATAATTTGGTAAATACAAAAGATTTAATGGCTT 2100
TyrIleThrLeuAspGluSerGlnLysArgGluLeuLysAsnLeuLeuLeuAspIleValAspAspTyrAsnLeuValAsnThrLysAspLeuMetAlaP
                                                                            CfoI.
TTATTCGCCTTAGGGGAGCGGAGTTTGGAATTTTAAATACGAATGATGTAAAAGATATTGTTTCAACAAACTCTAGCGCCTTTAGATTATGGTTTGAGGG 2200
heIleArgLeuArgGlyAlaGluPheGlyIleLeuAsnThrAsnAspValLysAspIleValSerThrAsnSerSerAlaPheArgLeuTrpPheGluGl

CAATTATCAGTGTGGATATAGAGCAAGTTATGCAAAGGTTCTTGATGCTGAAACGGGGGAAATAAAATGACAAACAAAGAAAAAGAGTTATTTGCTGAAA 2300
yAsnTyrGlnCysGlyTyrArgAlaSerTyrAlaLysValLeuAspAlaGluThrGlyGluIleLys

ATGAGGAATTAAAAAAAAGAAATTAAGGACTTAAAAGAGCGTATTGAAAGATACAGAGAAATGGAAGTTGAATTAAGTACAACAATAGATTTATTGAGAGG 2400

AGGGATTATTGAATAAATAAAAGCCCCCTGACGAAAGTCGAAGGGGGGTTTTTATTTTGGTTTGATGTTGCGATTAATAGCAATACAATTGCAATAAACCA 2500
   MboI
AAATGATC

and A (449) in the *ori* site (Fig. 6.2) [de la Campa et al., 1990]. The pFX2 *ori* sequence differed slightly from that of lactococcal plasmid pSH71 with additional bases G(1099) and C(1131). The location of the *ori* site in pFX2 was confirmed by deleting the region between positions 1 and 1151 with ExoIII, making the plasmid non-functional in lactococci. In contrast with the *ori* sequences of other plasmids in this group, lactococcal plasmids pFX2 and pSH71 have an additional conserved 23 bp inverted sequence, forming an extended stem-loop structure with a possible nicking site on the side of the stem (Fig. 6.2). A stem loop structure in the pLS1 *ori* site has been found to be the signal for plasmid replication initiation [de la Campa et al., 1990]. Two regions carrying direct repeats were found flanking the *ori* site, positions 1054-1093 and 1211-1248. The latter direct repeat region was similar to the direct repeat region (iterons) of pLS1 (positions 534-566) [del Solar et al. 1989] in GC-content (50% G+C), number of repeats (3 repeats; 11 bp for pLS1 and 9 bp for pFX2) and in the repeat position relative to the *ori* and promoter regions for the replication proteins. In pLS1, the region with the three direct repeats was shown to be involved in the initiation protein and repressor binding [Puyet et al., 1987; del Solar et al., 1989; de la Campa et al., 1990].

6.2.4 Lactococcal plasmid replication proteins

Downstream of the *ori* site, two open reading frames ORF1 and ORF2 with 54 and 234 codons respectively were located using Mapping programme (Fig. 6.1). Eight bp upstream from the ATG site of ORF1 (positions 1341-1343), there was a potential ribosome binding site (RBS) GGAG (G° =-9.4 kcal/mole), preceded 11 bp upstream by a lactococcal promoter sequence TATAAA (-10) and ATTACA (-35) separated by 17 bp. The spacing of the promoter elements is similar to that published for lactococci [Lakshmidevi et al., 1990; van der Vossen et al., 1987]. The region from position 1241 to 1328 including the promoter and upstream DNA was very AT rich (82%), similar to the strong promoter regions in *B. subtilis* [Doi et al., 1984].

(A)

```
S    427  AAAAtATgGGGGGGc    TACTACGA  cCCCCCCtaT AGTG CC GAGTGCCAA   474
B   2004  AAAAcAT GGGGG      TACTACGAcacCCCCCCAT    GTGtCC attgtCCAt  2049
E    870  AAAAcATaGGGGGGG    TACTACGAcctCCCCCCtag   GTGtCCattGT CCAt    918
A     57  tcAcgAccaGGGGGGc   TACTACGAtag CCCCCAaTgAGTGatt  tGTGaCAt    106
H         AAAAAAT GGGGG  & TACTACGA    CCCCCATTAAGTG CC GAGTGCCAA
F   1087  AAAAAAT GGGGGG & TACTACGA    CCCCCATTAAGTG CC GAGTGCCAA  1155
                  -------->               <--------

(&: 23 bp, aaaggaagcgaatttttgcttccg)
                  --------> <--------
```

(B)

```
                    T
                  A · T
                  A   T
                  G   T
                  CG
                  GC
                  AT ·
                  AT
                  GC
                  GC
              A A   GTAC T
                A   AGCA
                  GC ↑
                  GC
                  GC
                  GC
                  GC
                  GC
                  TA
           .      AT            1155.
        (1087)AAAAATAAGTGCCGAGTGCCAA
```

Figure 6.2 The plus *ori* site of lactococcal plasmid pFX2. (A) Comparison of plasmid plus *ori* sites. S, pLS1; B, pLB4; E, pE194; A, pADB201; H, pSH71; F, pFX2;. Inverted repeat sequences are indicated by facing arrows and the nicking site in pLS1 is arrowed. (B) A possible loop structure in the plus *ori* locus of lactococcal plasmid pFX2 (ΔG°= -22 kcal/mole). The possible nicking site is arrowed.

(A)

(B)
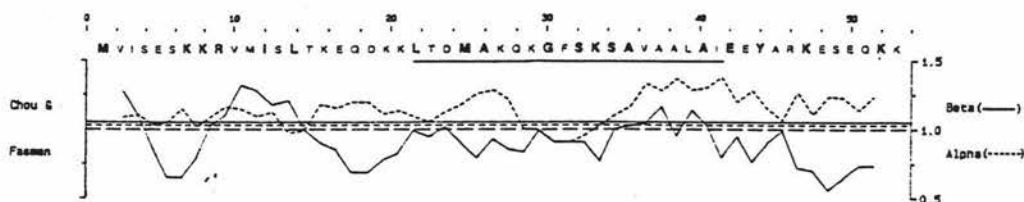
```
F    MAI------------KNTKA-------RNFGFLLYPDSIPNDWKEKLESLGVSMAVSPLHDMDEK----KIKIHGIVVMLYEMEMHVIKNPHYHVYILHG
     ::                 ::        : : ::::: ::: :: ::: ::: :: :::::: :       ::              : ::::
S    MA------------KEKA-------RYFTFLLYPESIPSDWELKLETLGVPMAISPLHDKDKS----SIK-----------GQKYKKAHYHVLYIAK
     :                :          :::: : : :    : : :::::: :               :
B    MKSESKIDWTVPRPNKNPKTKQPYKRGRNWGIVVYPESLPENW--KDIIRQEPIAVSPLHDKDVNPDGEKKKSHYHLVLNYKGNKSFEQIDEIARSLRAP
     :               ::         ::: : :::: :         :::::: :
E    MSENVIKETENK---KNSRG-------RNWTFVLYPESAKAEWLEYLKELHIQFVVSPLHDRD----------------TDTEGRMKKEHYH--ILVM
     :                :         :::: :: :: ::: :   ::: :             : :: :::
A    M-S------------TNIKK-------RNWTLLVYPDSAPENWKEILDQNGV-EYFGALHDKD-----------------VNPDGTIKKPHYHIVLAYS


F    NPVTIESVRNKIKRKLGNSSVAHVEILDYIKGSYEYLTHESKDAIAKNKHIYDKKDILNINDFDIDRYITLDESQKRELKNLLLDIVDDYNLVNTKDLM-
     ::::  ::: ::: :: : : :                : ::::::::::: :: ::: :: ::::: ::: : : :          : : :::
S    NPVTADSVRKKIKLLLGEKSLAMVQVVLNVENMYLYLTHESKDAIAKKKHVYDKADIKLINNFDIDRYVTLDVEEKTELFNVVVSLIRAYTLQNIFDLY-
     : :                                   ::::    : :: :::  ::    :        :            :
B    APQRISS-------------------LTGAVRYLTH----MDNPEKYQYDNADIETFGGFDLESCLALSTGDKRQALRDMLAFISENEIMHLKDFA-
     :                          : : :       ::::::  : : ::   ::         : :::        : ::::
E    YEGNKSYEQIKIITEELN------ATIPQIAGSVKGLVRYMLHMDDPNKFKYQKEDMIVYGGVDVDELLKKTTTDRYKLIKEMIEFIDEQGIVEFKSLMD
     :                         : :         ::: :     :: :         :
A    GPTT----FNNVKTLCNTLNSPKPLPLDGVGGMWRYMTH----KDNPEKYQYDDSIIFTGNGFDISNYKELTKKEISDIKLGLIDI-------IKNKQI-


F    -AFIRLRGAEFGILNTNDVKDIVSTNSSAFRLWFEGNYQCGYRASYAKVLDAETGEI----------------------K          (233)
     ::   :: : : : :          :: : :: :  .                         
S    -DFIDENG-ETYGLTINLVNEVIAGKTGFMKLLFDGAYQRSKR--------GTKNEE----------------------R          (210)
     :                        ::
B    -DYCMSEEAPAG----------------WFELLTERN---------------TLFIKEYIKSNWQKQQQYASKNINKMSD          (217)
E    YAMKFKFDDWFPLLCDNSAYVI----------------QEYIKSNRYKSD----------------------R                 (199)
     :: :                        :: : : :
A    -TEYSTFIDVVSNLGNIDVFDVASKNTIFF---------TSYINSFRFKLREEMEQEKYT--------------------K          (196)
```

Figure 6.3. Replication proteins of lactococcal plasmid pFX2. (A) A graph (PEPPLOT) of αhelix and ßsheet probablities in the RepA secondary structure, predicted according to Chou and Fasman. Large letters represent the amino acids homologous to repA of pLS1, and the 20-residue αhelix-turn-αhelix motif showing consensus to the major domain of DNA-binding proteins is underlined. (B) Comparison of RepB(pFX2) with plasmid replication initiation proteins. F, pFX2(RepB); S, pLS1(RepB); B, pLB4(RepB); E, pE194(RepF); A, pADB201(RepA). Total residue numbers of these peptides are indicated in brackets.

The -10 and -35 hexamers were also similar to the *E. coli* consensus promoter (TATAAT and TTGACA, respectively) [Harley and Reynolds, 1987], which probably explains why pFX2 is functional in *E. coli*. Ten bp upstream from the ORF2 start codon ATG (positions 1569-1571), another possible RBS was located, AAGG ($\Delta G^\circ = -8.4$ kcal/mole).

ORF1(*repA*) encoded a predicted 53-residue peptide with molecular mass of 6.0 kDa. The RepA amino acid sequence had high homology with the translated peptides which precede the replication initiation proteins from pLS1(RepA), pADB201(RepB), pLB4(RepA) and pE194(RepG) [Bates and Gilbert, 1989]. Prediction of the secondary structure and characteristics of the RepA peptide of pFX2 was determined using Pepplotting programme. RepA had a predicted isoelectric point of 10.50, and was positively charged due to its 12 strongly basic residues. The secondary structure of RepA predicted by the PEPPLOT program showed an αhelix-turn-αhelix motif, typical of many DNA-binding proteins (Paba and Sauer, 1984) (Fig. 6.3). This motif extended from residues 16-27 (αhelix-1), and from residues 34-52 (αhelix-2). A βsheet structure was observed between residues 10-13. A flexible segment was also found between residues 28-33 (the turn motif). The central αhelix-turn-αhelix 20-residue (22-41) motif of RepA fit well with the geometry requirements proposed for repressor proteins [Pabo and Sauer, 1984; Ohlendorf et al., 1984]. Conserved hydrophobic A, G and V residues were located at relative positions 26, 30 and 36. Residues at relative positions 25(M), 31(F), 39(L), 40(A) were also hydrophobic. Hydrophilic residues were at positions 24(D) and 28(Q). RepA of pFX2 showed strong homology with the RepA repressor of pLS1, especially in the N-terminal and αhelix-turn-αhelix domains (Fig. 6.3). The relatively conserved N-terminal domains in repressors [lambda repressors, repA(pLS1) and TrfB(RK2)] are believed to play a functional role in DNA binding [Jordan and Pabo, 1988; del Solar et al., 1989].

ORF2(*repB*) encoded a 233-residue peptide with molecular mass of 26.9 kDa with no homologous promoter region, suggesting polycistronic transcription with ORF1.

The well-characterized replication initiation proteins of pT181 [Novick et al., 1989] and pLS1 [Puyet et al., 1988] also do not have their own separate promoters. The RepB amino acid sequence showed substantial homology throughout the whole sequence to the pLS1 replication initiation protein (RepB) [de la Campa et al., 1990], but homology only to the N-terminal regions of replication initiation proteins from pE194(RepF) [Villafane et al., 1987], pLB4(RepB) [Bates and Gilbert, 1990] and pADB201(RepA) [Bergemann et al., 1989] (Fig. 6.3). RepB of pLS1 has recently been shown to bind at the three direct repeats region and plasmid replication is initiated at the nicking site 86 bp upstream from the first basepair of the direct repeats [de la Campa et al., 1990]. In pFX2, the putative nicking site is 84 bp upstream from the three iterons. RepB is therefore most likely to be the replication initiation protein of pFX2. It was shown that the 1.2 kb *Cfo*I-*Tha*I-*Cfo*I region (positions 1050-2176) could be ligated to give a functional replicon. In this construct 29 amino acids had been deleted from the C-terminal without affecting replication function. A similar observation was reported with pE194 where 43 C-terminal amino acids of the replication protein were found to be nonessential [Villafane et al., 1987]. A cointegrate plasmid of pFX2/*Tha*I and *E. coli* plasmid pUBS/*Ssp*I was previously shown to replicate in *E. coli* but not in lactococci (this chapter 6.2.1), suggesting that the insertion in the *Tha*I site of pFX2 disrupted RNA transcription through *repB*. The failure of this cointegrate plasmid to replicate in lactococci was unlikely to be due to the *E. coli* insert, because another similar construct (pFX2/*Hpa*II and pGEM3Z/*Sma*I) expressed the Cm^r marker in lactococci.

6.2.5 Possible regulation mechanisms for lactococcal plasmid replication

Regulation systems for plasmid replication have been extensively studied for plasmids ColE1 [Tomizawa, 1986] and IncFII [Praszkier et al., 1989] from Gram-negative bacteria and plasmids pT181 [Novick, 1989], pLS1 [del Solar et al., 1989], pC194 [Alonso and Tailor, 1987] and pE194 [Villafane et al., 1987] from Gram-positive

bacteria. These systems all involved negative regulation of the synthesis of replication initiation proteins by antisense RNA. In pLS1, the repressor protein (*repA*) was shown to specifically bind to the operator/promoter region of the *repAB* polycistron [del Solar et al., 1989]. Considering the similar structure and organization in replication proteins and their promoter regions, RepA of pFX2 might also bind to a putative operator/promoter region and prevent RNA polymerase from binding. This would be a self-regulated feedback mechanism as the *repA* and *repB* of pFX2 appear to be transcribed in the same operon.

Recently, plasmid replication regulation by transcription attenuation was proposed for plasmid pT181 [Novick et al., 1989]. A similar attenuation model is postulated here as a second regulation mechanism for pFX2 replication. In the presence of the appropriate regulators, a rho-independent terminator structure consisting of a GC rich hairpin followed by a stretch of U-residues [Rosenberg and Court, 1979] could be formed in the inverted repeat region between the *repA* and *repB* (Fig. 6.4). Terminators of this type have been found in several lactococcal genes [de Vos, 1987] but not in the replicons of other pE194 group plasmids. The terminator would prevent RNA polymerase from transcribing through to the *repB*. In the absence of the regulators, a RNA polymerase read-through structure could be formed (Fig. 6.4) in the same inverted repeat region. The regulators in this system might be antisense RNA as was found with pT181 plasmid [Novick et al., 1989], and here could bind to a region upstream from the inverted repeat sequence, preventing pairing of the A-rich (position 1491-1497) and U-rich (position 1546-1552) regions.

6.2.6 A region identical to staphylococcal plasmid pE194

The pFX2 sequence contains a 215 bp region (positions 545-759) with 100% homology to staphylococcal plasmid pE194 (positions 2925-3139) [Horinouchi and Weisblum, 1982]. In pE194, this region carried the complete RS_A (recombination site

```
                                              A G
                                            U     A
                                            U     A
                                             U G
                                             U G
                                             UA
                                            G U
                                            C A
                                             GC
                                             CG
                                             UA
                                             CG
                                            G U
                                             AU
                                             AU
                    A G                       AU
                  U     A                     GC
                  U     A                     CG
                   U G                        GC
                   U G                        AU
                   UA                        A A
                  G U                        U C
                  C A                         AU
                   GC                         AU
                   CG                        A G
                   UA                         AU
                   CG                         AU
                  G U                         AU
                   AU                         AU
                   AU                 1486.   AU     1571.
                   AU                 (5')CAGAACGAUAAGGUAAUUAUAUCAUG(3')
                   GC                      ••••              •••
1498.   GC   1551.                    Read-through (ΔG°=-20.0 kcal/mole)
(5')AAUAAUACUUGUUUUU(3')

Termination (ΔG°=-16.2 kcal/mole)
```
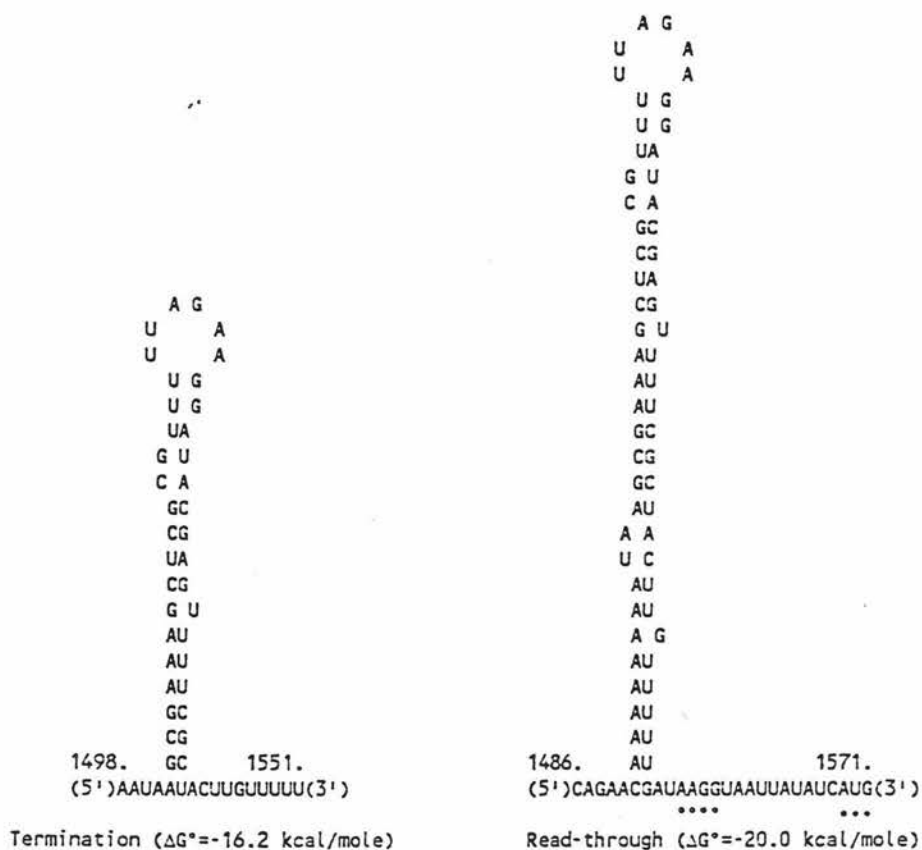
Figure 6.4. A computer generated transcriptional attenuation model for regulating lactococcal plasmid replication. Bases marked with ° represent the possible RBS and . is the start codon for RepB(pFX2).

A) locus and the staphylococcal *pre* (plasmid recombination enzyme) promoter region [Gennaro et al. 1987] (Fig. 6.1). In pFX2, however, there was no analgous ORF for the staphylococcal consensus Pre protein which nicks specifically at RS_A [Gennaro et al., 1987]. Staphylococcal plasmid pE12 has also been reported to carry RS_A without the *pre* [Novick, 1989]. Plasmid recombination/cointegration mediated by the Pre-RS_A process is common in staphylococci and bacilli [Gruss and Ehrlich, 1989; Novick 1989] and is *recA* independent [Gennaro et al., 1987]. RS_A has also been postulated to act as the *oriT* site for conjugative mobilization of streptococcal plasmid pMV158 [Priebe and Lacks, 1989].

In *E. coli*, small plasmids such as ColE1 that cannot mediate their own transfer during conjugation can be mobilized by a conjugative plasmid. A mobilization protein (Mob), encoded by this small plasmid, nicks at a specific site *oriT* on the plasmid. Palindromic sequences are found near the *oriT* sites of six plasmids from Gram-negative bacteria (two conjugative, F and RK2, and four nonconjugative, ColE1, CloDF13, pSC101 and RSF1010) [Willetts and Wilkins, 1984]. Seven plasmids from different Gram-positive bacteria (pUB110, pMV158, pTB913, pT181, pE194, pNE131 and pT48) are also known to carry almost identical palindromic sequences in the putative *oriT* site RS_A [Priebe and Lacks, 1989; van der Lelie et al., 1989]. The RS_A site in pFX2 might also act as a generalized nicking site for the formation of cointegrate plasmids during conjugative mobilization. While no Pre-like proteins to mediate this process are encoded downstream of RS_A in pFX2, it has yet to be established whether such a gene is present elsewhere on plasmids or the chromosome of the original lactococcal host. It has been suggested that although the presence of Pre is a requirement for RS_A-mediate recombination, the host *rec* system may function to stimulate this recombination process [Gennaro et al., 1987].

Recently, a RS_A sequence has also been described in the *Lactobacillus plantarum* plasmid pLB4 [Bates and Gilbert, 1989]. It is interesting to note that plasmids from different Gram-positive genera isolated from different geographic origins share the

same regions involved in genetic exchange, and similarly homologous regions for plasmid replication [de la Campa et al., 1990].

6.2.7 Genetic organization and replication mode of pFX2

As well as the individual components of the replication apparatus of pFX2 which have homology with pE194 group plasmids, the genetic organization of these replicons is also very similar (Fig. 6.5). The region between the *ori* site and the replication initiation protein coding region comprises the negative control system, or the copy number control system (*cop*) [Novick, 1989] in these plasmids.

In the pFX2 DNA sequence, however, no sequence homologous to the published minus origin sequences was detected [Gruss and Ehrlich, 1989]. The putative minus origin sequence of pLB4 is different from those of pE194 and pLS1 [Bates and Gilbert 1989]. The minus origin sequence of plasmid pFX2 was expected to be different and encoded somewhere upstream of the plus *ori* site in pFX2 or in the 1.9 kb *Cla*I non-essential region of pFX1. Further work needs to be done to identify this locus.

Most plasmids so far examined from Gram-positive bacteria replicate by a rolling circle mechanism via a single-stranded DNA (ssDNA) intermediate [Gruss and Ehrlich, 1989]. Plasmids with DNA or amino acid sequence homology to pFX2 are of the ssDNA replication type, suggesting that pFX2 also replicates in this manner. It has been demonstrated that insertion of pBR-type DNA into plasmids of ssDNA type results in the generation of high-molecular-weight plasmid multimers [Gruss and Ehrlich, 1989]: such forms could also be seen with the cointegrate plasmids of pFX2 and pGEM3Z (Fig. 6.6). By analogy with pE194 [Scheer-Abramowitz et al., 1981] and pLS1 [Puyet et al., 1988], pFX2 replication would proceed towards the *Mbo*I site, i.e. anticlockwise on the circular map (Fig. 5.3).
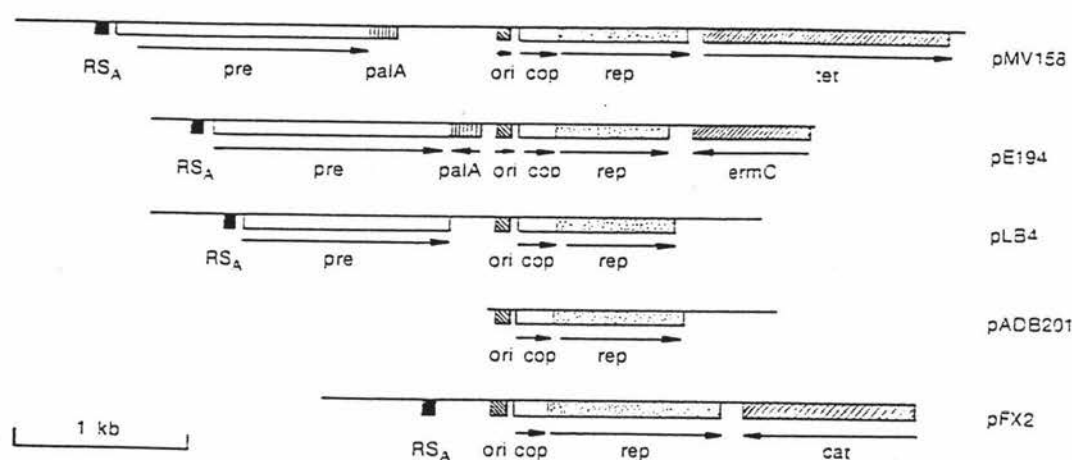
Figure 6.5. Comparison of genome organization of lactococcal plasmid pFX2 with those of other bacterial genera. Plasmid pMV158 is the parent plasmid of pLS1. Arrows represent direction of transcription for major open reading frames and functional orientation for other sequence elements. Abbreviations: RS$_A$, recombination site A; *pre*, plasmid recombination function; *palA*, lagging-strand conversion signal (minus origin); *cop*, replication control system; *rep*, initiation replication protein determinant; *tet*, tetracycline resistance; *ermC*, ribosome methylase determinant; *cat*, chloramphenicol transacetylase determinant.

Figure 6.6 Formation of high molecular weight multimers by the cointegrates of plasmids pGEM3Z and pFX2 (pFX201). Lane 1, 1 kb supercoiled plasmid ladder as M.W. standard; lane 2, pFX201 isolated from *E. coli*; lane 3, pFX201 isolated from lactococci; lane 4, pFX2.

kb

50.1 —

31.5 ⁄

16.2 —

8.1 ⊢

6.0 ⊢

4.0 ⁄
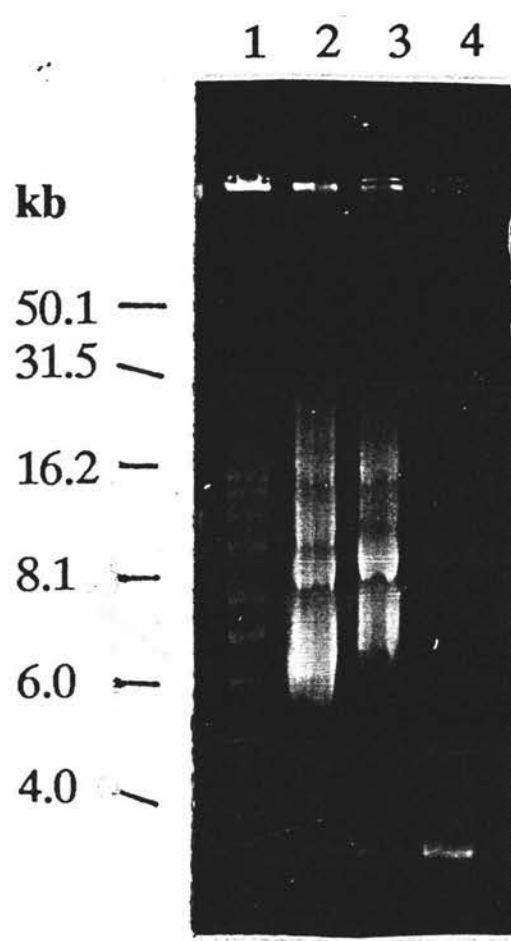
6.3 Summary

The essential region for pFX vectors replication was located by deletion experiments. The 2508 bp lactococcal portion of pFX2 was sequenced and its genetic organization examined. A lactococcal plasmid plus origin and two replication protein coding regions (*repA* and *repB*) were located. RepA had an αhelix-turn-αhelix motif, a geometry typical of DNA-binding proteins. RepB showed high homology to the plasmid replication initiation proteins from other Gram-positive bacteria and *Mycoplasma*. The transcribed inverted repeat sequence between *repA* and *repB* could form an attenuator to regulate pFX2 replication. Upstream of the *ori* site, and in a region nonessential for replication, a 215 bp sequence identical to the staphylococcal plasmid pE194 and carrying the RS_A site was identified. The genetic organization of this lactococcal plasmid replicon shares significant similarity with pE194 group plasmids. The sequencing results have given an insight into the genetic organization of a lactococcal plasmid replicon. This information will assist further vector construction such as food-grade type vectors, and future plasmid studies with these industrially important bacteria.

CHAPTER 7: FINAL DISCUSSION AND CONCLUSIONS

The application of recombinant DNA technology to the lactococci has greatly increased our understanding of these industrially important bacteria. Construction of stable vectors with high transformation efficiency is essential for cloning genes, including those for industrially important traits such as lactose utilization, proteinase activity and phage resistance. As most of the important genes are plasmid-encoded, the genetics of lactococci has currently mainly focused on the plasmids.

The first vectors used in lactococci were unstable shuttle plasmids based on non-lactococcal replicons. More recently, constructs derived from the homologous lactococcal plasmids pWV01 and pSH71 have been described [de Vos, 1987]. These vectors which are based solely on lactococcal plasmid replicons, replicate in many Gram-positive organisms as well as in *E. coli*, and have proved to be most useful in lactococcal gene cloning studies.

Although there is now no difficulty in introducing lactococcal vectors into plasmid-free lactococci by electroporation, transformation of wild-type lactococci which carry an average of 4-5 plasmids has been much less successful in this laboratory. Plasmid incompatability will clearly be an important consideration if cloning vectors are to be used with such strains. For this reason, it will be useful to be able to select from a variety of vectors based on different lactococcal replicons.

Until recently, lactococcal genes were cloned into vectors based on the well-established *E. coli* or other gene transfer systems. Gene cloning was usually initially conducted in these systems. Recombinant plasmids could then be transformed into lactococci. Sometimes, however, the constructs are either unstable, or the gene product is lethal to these hosts. There is thus a need for constructs to be made directly in lactococci. The pFX vectors with their high transformation efficiency and stability can fulfil this requirement. Using pFX1, the pDI21 proteinase gene was

directly cloned and expressed in lactococci where the *prt* recombinant plasmid was stably inherited.

pFX3 vector with the multiple unique cloning sites, enables cloning of DNA fragments created by various restriction enzymes. This vector can also be directly used for transcription studies or DNA sequencing of cloned inserts, greatly facilitating gene structure and expression studies.

Currently, lactococcal promoter-detecting vectors were constructed by incorporating the staphylococcal promoterless *cat* gene as a marker [de Vos, 1987]. In this work, the *E. coli lacZ* gene was successfully used as a marker in lactococcal system. The pFX4, 5 and 6 vectors enable the fusion of cloned genes to *lacZ* in all three translational reading frames. The constructs can be directly electroporated into lactococci.

The lactococcal proteinase is essential for good growth with concomitant rapid acid production, with the production of peptides and amino acids, which influence both cheese and casein flavour. Characterization of the enzyme is of crucial importance in determining and ultimately controlling reliable product quality. The pDI21 proteinase was characterized as a PI type, and lactococcal starters carrying proteinase of this specificity have a tendency to produce bitter peptides in cheese production.

Natural resident plasmids are abundant in lactococci and yet very little is known about their mode of replication, stability, or other functions. No lactococcal plasmid DNA sequence has previously been published. The complete DNA sequence of the lactococcal portion of pFX2 (*Hpa*II-*Mbo*I, 2508 bp) was thus determined and the genetic organization analyzed. A consensus plus *ori* site with strong homology to the pE194 group plasmids and two open reading frames were found within the 1.2-kb *Cfo*I-*Tha*I-*Cfo*I minimum replicon, which had been previously located by deletion experiments.

ORF1 was preceded by a consensus lactococcal promoter and ribosome binding site and encoded a 53-residue peptide (RepA) which had a αhelix-turn-αhelix motif, a geometry typical of proteins which act as DNA-binding repressors. ORF2 encoded a 233-residue peptide (RepB), which showed strong homology to the whole sequence of the pLS1 replication initiation protein and to the N-terminal regions of replication initiation proteins of pE194, pLB4 and pADB210. Although it is likely that all of these Rep proteins are derived from a common ancestor, evolution has resulted in different proteins, each with its own specific target site.

There appear to be two possible mechanisms for controlling the transcription of RepB and therefore maintaining the plasmid copy number. The DNA binding repressor RepA could bind at a putative operator site, a region immediately upstream of RepA with strong homology to the pLS1 operator. In addition, the RNA transcribed from the inverted repeat region between the ORF1 and ORF2 was predicted as being capable of forming either attenuator or terminator type configurations. Further work needs to be done in the future to identify the regulators, which probably are antisense RNA to trigger the conversion of these two structures.

The individual components of the replication apparatus of pFX2 have substantial homology with plasmids from other Gram-positive bacteria and *Mycoplasma*. The genetic organization of these replicons is also very similar. The region between the *ori* site and the replication initiation protein coding region comprises the negative control system (*cop*) in these plasmids. Mutations in the *cop* region are likely to lead to a plasmid copy number change. Suitable mutations would thus be useful in controlling gene expression. Based on the similarity of pFX2 to other plasmids, it can be assumed that this lactococcal plasmid replicates by the rolling circle model via a single-strand intermediate.

A 215 bp region with 100% homology to the staphylococcal plasmid pE194, which included the RS_A sequence (recombination site A) was found in a nonessential region

for plasmid replication. The RS_A locus has also recently been found in several *Lactobacillus* plasmids. The origin of this similarity among the different Gram-positive genera is at present unknown.

In conclusion, plasmid pDI25 has been used to construct the pFX series of vectors which provide an efficient means for gene cloning and expression studies in lactococci. Cloning can be in either *E. coli* or lactococci. The genetic organization of this lactococcal plasmid replicon shows significant homology to that of plasmids from Gram-positive bacteria and *Mycoplasma*. The DNA sequence results have provided the first detailed description of the genetic organization of a lactococcal plasmid replicon. This information has opened up a number of possibilities for future work in both basic and applied areas. Of particular interest will be studies to establish the mechanism(s) of plasmid incompability in the lactococci. This phenomenon is likely to be a major consideration when vectors carrying cloned genes of interest are to be introduced into wild-type (multiple-plasmid containing) cells. The question of plasmid copy number control will be an important consideration, affecting the level of cloned gene expression.

At present, starter cultures derived by recombinant DNA techniques cannot be used commercially either in New Zealand or overseas. In the future, when such constructs are approved for use, they will need to be in 100% food-grade vectors. Such a vector can be readily derived from pFX2 by replacing the Cm resistance gene with a gene marker such as nis^r [Dodd et al., 1990] or *thyA* [Ross et al., 1990a].

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