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MOLECULAR ANALYSIS
OF
THE LACTOSE METABOLISING GENES
FROM
LACTOCOCCUS LACTIS

*A thesis presented in partial fulfilment of the
requirement for the degree of
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ABSTRACT

Two lactococcal strains possessing different lactose metabolism systems were chosen for the molecular analysis of lactose metabolising genes from *Lactococcus lactis*. These were *Lactococcus lactis* ssp. *cremoris* strain H2 and *Lactococcus lactis* ssp. *lactis* strain ATCC 7962. An attempt was made to sequence a previously cloned 4.4 kb *EcoRI* fragment of pDI21 reported to encode D-tagatose 1,6-bisphosphate aldolase. A comparison of sequence data generated from this fragment with DNA sequences in the GenEMBL data base revealed that the clones provided for this study were not lactococcal DNA but were chromosomal DNA of *E. coli* which encoded genes involved in purine biosynthesis. This part of the research programme was therefore abandoned.

The aim of the second part of this study programme was to clone and characterize the β -galactosidase gene from plasmid pDI3, an uncharacterized plasmid of *Lactococcus lactis* ssp. *lactis* strain ATCC 7962. This was of interest as most lactococcal bacterial strains metabolize lactose by way of the Lac-PEP:PTS system and possess high phospho- β -galactosidase activity, whereas strain 7962 metabolizes lactose by way of the lactose permease system and possesses high β -galactosidase activity and low phospho- β -galactosidase activity. Previous plasmid curing experiments indicated that the β -galactosidase activity of strain 7962 is associated with pDI3. DNA hybridization work between pDI3 and a previously cloned DNA fragment containing the β -galactosidase gene of another Gram-positive genus, *Clostridium acetobutylicum* (*cbgA*) showed that pDI3 contains DNA sequence that is to some extent homologous to β -galactosidase sequence.

Initial experiments were carried out to confirm the involvement of pDI3 on strain 7962's β -galactosidase activity. Strain ATCC 7962 wildtype containing four plasmids and the strain cured of three other plasmids (i.e. derivative strain of 7962 containing only pDI3) exhibited a Lac⁺ phenotype, while the strain cured of all four plasmids exhibited a Lac⁻ phenotype.

As part of the mapping strategy and the attempt to clone the β -galactosidase gene from strain 7962, various fragments of pDI3 were cloned. Hybridization experiments using the cloned pDI3 fragments as DNA probes were also carried out to confirm the arrangement of pDI3 fragments. A physical map of pDI3 was constructed using the restriction enzymes *Bam*HI, *Pst*I, and *Sal*I. The size of pDI3 was confirmed to be 70 kb and it may contain some small repeat sequences. Some *Eco*RI restriction sites contained in pDI3 were also determined.

Several approaches were used to localize and identify the β -galactosidase gene on pDI3. Southern hybridizations were first carried out using the *cbgA* gene. The *cbgA* gene showed weak homology to a 4.3 kb *Eco*RI doublet from pDI3. Two redundant oligonucleotide probes were designed from the highly conserved domain of deduced amino acid sequences of the available β -galactosidase sequences from other closely related Gram-positive bacteria as well as some deduced amino acid sequences derived from β -galactosidase sequences of Gram-negative bacteria. The 4.3 kb *Eco*RI doublet and a 4.3 kb *Hind*III doublet exhibited weak homology to these probes.

On the basis of these results one of the 4.3 kb *Eco*RI fragments was subsequently cloned and transformed into a *Lac*⁻ *E. coli* strain, MC1022. The cloned 4.3 kb *Eco*RI fragment (the 4.3a fragment) was shown to cross hybridize to one of the oligonucleotide probes, but did not show β -galactosidase activity. The 4.3a kb *Eco*RI fragment was also cloned into pBR322 and transformed into another *Lac*⁻ *E. coli* host (JM109) and no β -galactosidase activity was detected.

Based on the pDI3 physical map constructed, the 4.3a kb *Eco*RI fragment was shown to overlap a 13.6 kb *Sal*I fragment. This fragment was cloned into pBR322 and the plasmid designated pSY303, and this was transformed into *E. coli* JM109. Introduction of pSY303 into *E. coli* JM109 gave a *Lac*⁺ phenotype. *Lac*⁺ phenotypes were also found for other *Lac*⁻ *E. coli* host strains including *E. coli* PB2959. It was found that pSY303 expressed β -galactosidase constitutively, however the addition of 0.1% (w/v) lactose into the medium gave a higher level of expression. An inducer for β -galactosidase, IPTG, was not required for expression. Glucose had no repression effect on β -galactosidase.

Some instability of the Lac⁺ phenotype was observed. Transformants that were initially Lac⁺ manifested phenotypic segregation into Lac⁺ and Lac⁻ colonies. Both were found to retain the intact pSY303 plasmid and there was no difference in pSY303 DNA isolated from representative Lac⁺ colonies and from representative Lac⁻ colonies. Some stable Lac⁺ colonies were observed that became dark blue. Analysis of the plasmid from these colonies showed that pSY303 had undergone a deletion. The generation of these deletion derivatives may be a consequence of small repeat sequences.

In conclusion, a physical map of pDI3 has been constructed and the β -galactosidase gene from pDI3 of *Lactococcus lactis* ssp. *lactis* strain ATCC 7962 was cloned and was found to express constitutively in *E. coli*, and at a higher level in the presence of lactose.

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ABBREVIATIONS

kb	kilobases
bp	basepairs
kDa	kilodaltons
MDa	megadaltons
nm	nanometres
g	gravity
rpm	revolution per minute
cfu	colony forming units
Ap	ampicillin
Cm	chloramphenicol
Em	erythromycin
Tc	Tetracycline
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
IPTG	Isopropyl thiogalactopyranoside
X-gal	5-bromo-4-chloro-3-indoyl galactopyranoside
ONPG	o-nitro-phenyl- β -D-galactoside
ONP	o-nitro-phenyl
ORF	open reading frame
MW	molecular weight
PCR	polymerase chain reaction
PTS	phosphotransferase system
PS	permease system
Lac-PEP:PTS	lactose phosphoenolpyruvate dependent phosphotransferase system
P- β -gal	phospho- β -galactosidase
β -gal	β -galactosidase
strain 7962	a plasmid cured derivative of <i>Lactococcus lactis</i> ssp. <i>lactis</i> strain ATCC 7962 containing only one plasmid, pDI3. (is also referred to as strain NZDRI 5191)
SDS	sodium dodecyl sulphate
OD	optical density
A	absorbance

CHAPTER 1: LITERATURE REVIEW

1.1 General Introduction

Fermented milk products have been associated with human civilization for centuries and these foods continue to occupy an important position in the human diet. In the past, the fermentation process was carried out using traditional home style procedures. However, recent science and technology has allowed the development of various kinds of dairy products using carefully controlled and managed fermentation processes. These biological processes utilise the so-called "lactic acid fermentation" initiated by lactic acid bacteria.

Many countries with well developed scientific and technological infrastructures have expanded the original "cottage fermentation" process into industrial scale operations. Dairy products have become important export earners in nations such as Australia, Denmark, France, Germany, The Netherlands, New Zealand, Switzerland, U.K. and U.S.A. In Denmark for example the export value for dairy products in 1985 amounted to 9,300 million kr. of which 6,000 million kr was export generated (Danish Dairy Economical Statistical Data, No 1., 1986). In 1993 New Zealand produced about 143,000 tonnes of cheese alone of its 907,000 tonnes total of manufactured dairy products. During the current season cheese manufacture will exceed 170,000 tonnes of which about 75% will be exported (N.Z. Dairy Board, Annual Report 1993).

These dairy fermentations rely significantly on the activities of carefully selected microorganisms. Although the exploitation of fermentative processes existed long before anyone was aware of the involvement of the indigenous lactic bacteria it was not until about 1850 that there developed an increasing interest in their role. The modern dairy industry utilizes lactococci yeasts and lactobacilli as starter cultures in milk fermentation plants producing cheese, cultured butter, yoghurt, fermented milks (Yakult, Acidophilus Milk, Bulgarian Buttermilk, Kefir) or in whey fermentation for ethanol, animal feeds and supplements, methane (biogas), fermented whey beverages and lactic acid (Prentice and Neaves, 1986).

Most of the earlier investigatory work was concerned with explanations of those processes already in use rather than attempting to improve production technology and the microbial activities (Prentice and Neaves, 1986). The understanding and control of unwanted microbes in milk and fermented milk products as well as the production of quality food products and harmless by-products using the controlled activities of microbes has evolved steadily in the modern dairy industry.

A major factor that affects dairy product quality is the nature of the lactic starters (see 1.2). In the modern dairy industry the availability of well-defined cultures exhibiting acceptable activity, consistency and stable characteristics has become crucial. These properties are now known to be governed by the genetic determinants of the cell (McKay *et al.*, 1970). Thus the development of a background knowledge of genetics and of the genetic transfer systems of the dairy starters has provided useful information about the instability and loss of the important characteristics of the starters. Therefore a study of the genetically linked properties is also necessary in the development of new applications which will contribute to the industry. One of the useful tools from recent developments in molecular biology is recombinant DNA technology which offers considerable potential for the manipulation of the desired performance characteristics of the starters.

1.2 Lactic starters

Starter cultures of lactic acid bacteria are preparations of especially selected cultures used to initiate the fermentation. They are of fundamental importance in the manufacture of dairy products. Before lactic cultures were fully characterized, "self-acidified" milk was used in the cottage industries for cheese making. About 1890, the first lactic acid starters cultures were isolated and purified by V. Storch at the Royal Veterinary and Agricultural University in Denmark (Nielsen *et al.*, 1987). The first attempt to use pure cultures of *Lactococcus lactis* ssp. *lactis* (*L. lactis* ssp. *lactis*) or *Lactococcus lactis* ssp. *cremoris* (*L. lactis* ssp. *cremoris*) to make ripened cream butter was reported by Van Neil in 1929 employing, as it was then named, a *Streptococcus* species.

Lactic starters are predominantly mesophilic strains of Lancefield's group N lactococci. These are *L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *L. lactis* ssp. *lactis* var *diacetylactis* and *Leuconostoc cremoris*. The thermophiles *Streptococcus thermophilus* and *Lactobacillus helveticus*, *Lactobacillus lactis*, *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* are also used. In some other manufactured fermented milk products, the bifidobacteria, propionibacteria, yeasts and moulds are also used in combination with lactic acid bacteria (Prentice and Neaves, 1986; Nielsen *et al.*, 1987).

Currently it is common practice in the industry to use carefully designated starter cultures that come from appropriate suppliers, e.g. commercial companies, research institutes or commercial starter dairies (West, 1983). They are available as liquid, deep frozen or freeze dried cultures with specified viable cell counts (about 10^7 bacteria/ml of milk). On propagation they give a sufficient number of cells for production purposes, or alternatively if available as concentrated deep frozen cultures they may be inoculated directly into the bulk milk vat (Gilliland and Speck 1974; Wigley, 1977).

The overall functions of the starter cultures for use in the manufacture of dairy products can be summarized as follows:

1. The fermentation of lactose to lactic acid. Lactic acid imparts a fresh acidulous taste and contributes to the development of the texture of fermented dairy products through the precipitation of casein (Daly, 1983; Nielsen *et al.*, 1987).
2. *L. lactis* ssp. *lactis* var *diacetylactis* and *Leuconostoc cremoris* also ferment citric acid to produce CO₂ and aroma compounds such as diacetyl and acetaldehyde. The CO₂ contributes to the "freshness" of sour milk and is responsible for eye formation in cheese (Prentice and Neaves, 1986; Nielsen *et al.*, 1987).
3. The proteolytic enzymes of the starters contribute to the ripening of cheese by liberation of peptides and amino acids from the caseins (Nielsen *et al.*, 1987).
4. The reduction of the pH value within the fermentation vessel inhibits pathogens and specific spoilage organisms (Daly, 1983; Nielsen *et al.*, 1987).
5. Some lactic acid bacteria are considered to be of dietetic value by influencing the intestinal microflora (Nielsen *et al.*, 1987).

There are various types of dairy products which can be developed from different combinations of starter bacteria. Cheddar and Feta cheese manufacture, for example, does not use those starters that produce unwanted diacetyl flavour and CO₂ (Nielsen *et al.*, 1987). In addition, the lactic starters can be divided into three industrial categories:

1. "Single strain starters" contain only one strain of the species but are often used in pairs or triplets to reduce phage inhibition. Triplets usually consist of strains of *L. lactis* ssp. *cremoris* (Crawford, 1972; Prentice and Neaves, 1986).
2. "Multiple strain starters" consist of known numbers of single strains, and allow an extended time of use throughout the manufacturing season (Limsowtin *et al.*, 1977).
3. "Mixed strains starters" are combinations of all the common three subspecies of lactococci as well as *Lactobacillus cremoris* and/or *Lactobacillus dextranicum* (Sharpe, 1979) and are used without any exactitude of composition.

The advantage with single strain starters is that technological and organoleptic properties can be defined exactly. However, the strains are susceptible to bacteriophage attack and can cause considerable economic loss because the starters may fail completely (Prentice and Neaves, 1986). Multiple strain and mixed strain starters have an advantage in that they diminish the risk of sudden bacteriophage attack. However, the balance between the strains may change during culture transfers and inoculum preparation (Nielsen *et al.*, 1987). Cheese manufacture in New Zealand makes use of a rotational system involving multiple strains of *L. lactis* ssp. *cremoris* that differ in their susceptibility and resistance to bacteriophage (Prentice and Neaves, 1986). Trials on a multiple starter containing up to six strains of *L. lactis* ssp. *cremoris* in which rotation is not required have yielded promising results.

The search for good quality starters is still at an active stage because improved technology and better dairy products will come from the use of superior starters. The following characteristics are considered essential to any lactic starter (Nielsen *et al.*, 1987): high cell numbers in starter concentrates; rapid growth and lactose fermentation; maximum resistance to bacteriophages; stability and reliability of performance; formation of firm milk coagulum; no bitterness in cheese and sour milk; antioxidative

properties; rapid cheese ripening; high metabolic activity at elevated temperatures; increased salt tolerance and improved dietetic properties.

1.3. Characteristics of lactococci

Lactococci are non-spore forming Gram-positive coccal bacteria. They are members of a wider group of microorganisms known by the collective name of "lactic acid bacteria" because of their typical characteristic of fermenting lactose into lactic acid. Their cell shape is spherical (cocci) or ovoid. The arrangement of cells is in a chain (streptococcus) or in pairs. A few may grow as short rods under certain cultural conditions, especially on solid media. They are non-motile and non-haemolytic (non-pathogenic). They can grow at ambient temperature (i.e. mesophilic, most exhibit a range of 22°C-30°C but some can grow at 5°C and 45°C). With respect to growth pH, some can grow at pH 3.2 and some at pH 9.6 but most can grow in the pH range of 4.0-4.5. They are considered to be food associates and harmless to humans and animals. Their natural habitat is plant matter, however the main source of isolation is milk, dairy products and fermented foods and were among the first bacteria studied by microbiologists (Sandine, 1985). They are facultative anaerobes and catalase-negative. Lactococci are particularly fastidious, requiring growth factors such as amino acids, B vitamins, purine and pyrimidine bases and hence complex media are required for laboratory cultivation (Terzaghi and Sandine, 1975).

Some of their characteristics are beneficial to the fermented milk products industry, such as their ability to rapidly lower the pH during the fermentation process and the ability of certain strains to produce inhibitory (lantibiotic) substances (Mattick and Hirsch, 1944; Dobrzanski *et al.*, 1982). The antagonistic properties of the lactococci have proved useful to the food industry. Nisin, for example, is a food-grade polypeptide lantibiotic which is used widely as a food preservative (Rayman *et al.*, 1981; Scott and Taylor, 1981; Hurst, 1981; Lipinska, 1977).

1.4 Taxonomy of lactococci

1.4.1 Classification

The lactococci are members of a broader group, the lactic acid bacteria, the taxonomy of which is based on physiology, morphology and until recently, serology.

As presently constituted, the lactic acid bacteria consist of the following genera: *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Pediococcus* and *Streptococcus* (Sandine, 1988). These genera are classified together because of their typical ability to produce lactic acid from glucose either via the Embden-Meyerhof-Parnas (EM) pathway or by a combination of the hexose monophosphate pathway (HMP) and the phospho ketolase pathway, (see Table 1).

With the discovery of specific group antigens by Lancefield in 1933 [Lancefield grouping] the genus *Streptococcus* was classified serologically according to unique cell-wall components. The lactococci were thus originally known in Lancefield's scheme as Group N streptococci. However, this serological grouping is now considered inadequate for distinguishing the streptococcal bacteria because there are many strains of the same species that belong to different serological groups (Schleifer and Kilpper-Bälz, 1987).

Using physiological and biochemical tests Sherman (1937) subdivided the streptococci into four groups: pyogenic, viridans, lactic and enteric. Jones (1978) developed these ideas further and sub-divided the pyogenic species into pyogenic, parapyogenic and the pneumococci. The thermophilic streptococci were presented as a separate group as were the anaerobic streptococci. Numerical analytical taxonomy for these bacteria was presented by Bridges and Sneath (1982) and confirmed the decisions of Jones (1978). However, it was from DNA hybridization studies that the most radical restructuring of the streptococcal group was to arise.

Table 1 : Metabolic pathways in lactic acid bacteria^a.

Genus	Sub-genus	Pathway used in glucose metabolism		
		Embden-Myerhof	Hexose monophosphate	phospho-ketolase
<i>Streptococcus</i> ¹	All species	+	-	-
<i>Leuconostoc</i>	All species	-	+	+
<i>Pediococcus</i>	All species	+(probably)	-	-
<i>Lactobacillus</i>	Thermobacteria	+	+	-
	Streptobacteria	+	(+)	(+)
	Betabacteria	-	+	+

^a = According to Garvie, 1984.

+ = present; (+) = present but used only under special conditions;

- = not present.

¹ = including *Lactococcus*.

Comparative oligonucleotide cataloguing of the 16S rRNA (Ludwig *et al.*, 1985) and extensive nucleic acid hybridization studies (Kilpper-Bälz and Schleifer, 1981; 1984; Schleifer and Kilpper-Bälz, 1984; Kilpper-Bälz *et al.*, 1982; Schleifer *et al.*, 1985) have shown that the Gram-positive facultatively anaerobic cocci known as the "streptococci" consist of three genetically distinct groups. The first group includes the pyogenic and the oral streptococci, the second group includes the faecal streptococci, which have been ascribed a new genus name of *Enterococcus* (Schleifer and Kilpper-Bälz, 1984; Collins *et al.*, 1984), while the third group includes the group N lactic streptococci which clearly form a single taxon name *Lactococcus*.

1.4.2 Nomenclature

With respect to lactic streptococci nomenclature the new genus name *Lactococcus* has been proposed and accepted as a valid generic name (Schleifer, 1987). The nomenclature of lactococci together with their classification has also involved a number of other changes. In the eighth edition of Bergey's Manual of Determinative Bacteriology, (1984) *S. cremoris* and *S. lactis* were recognized as two distinct species and *S. lactis* ssp. *diacetylactis* was a subspecies of *S. lactis*. This nomenclature was accepted until the 9th edition of Bergey's Manual of Systematic Bacteriology was published in 1986. This 9th edition combined *S. cremoris* with *S. lactis* as a single extended taxon *S. lactis* (Mundt, 1986). In renaming the genus of the important dairy cocci as *Lactococcus* the International Union of Microbiological Societies in 1986 (Anonymous, 1986) approved the retaining of two subspecies i.e. *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*. Others however, favoured three subspecies (Schleifer and Kilpper-Bälz, 1987). The major biochemical and chemical differences between the subspecies of *L. lactis* are summarized in Table 2. Garvie and Farrow (1982) had also argued the value of having three distinct subspecies for *S. lactis*, the third being *S. lactis* ssp. *diacetylactis*. It exhibited a unique citrate fermenting ability mediated by citrate permease which was plasmid-encoded (Kempler and McKay, 1981). Moreover, because this diacetylactis mode of citrate fermentation was considered commercially important, a new name *L. lactis* ssp. *lactis* var. *diacetylous* (sic) was proposed (Sandine, 1988). Subspecies nomenclature is presented in Table 3.

Table 2 : Biochemical and chemical characteristics differentiating species and subspecies of the genus *Lactococcus*.^a

Species and subspecies	Murein type ^b	Major Menaquinones ^c	Acid production from							Hyd.of arg
			G-	L-	M-	Me-	Mez-	Rf-	R-	
<i>L. lactis</i> ssp. <i>lactis</i>	Lys-D-Asp	MK-9,MK-8	+	+	+	-	-	-	+	+
<i>L. lactis</i> ssp. <i>cremoris</i>	Lys-D-Asp	Mk-9,MK-8	+	+	-	-	-	-	-	-
<i>L. lactis</i> ssp. <i>hordniae</i>	Lys-D-Asp	MK-8,MK-9	-	-	-	-	-	-	-	+
<i>L. garviae</i>	Lys-Ala-Gly-Ala	MK-9,MK-8	+	+	v	v	-	-	+	+
<i>L. plantarum</i>	Lys-Ser-Ala	-	-	-	+	-	+	-	-	-
<i>L. raffinolactis</i>	Lys-Thr-Ala	-	+	+	+	+	v	+	v	v

^a According to Schleifer and Kilpper-Bälz (1987)

^b Abbreviations according to Schleifer and Kandler (1972): Lys-D-Asp=L-Lysine-D-isoasparagine; Ser=serine; Thr=threonine; Ala=alanine; Gly=glycine

^c Abbreviations according to Collin and Jones (1981): MK=menaquinones; G=galactose; L=lactose; M=maltose; Me=melibiose; Mez=melizitose; Rf=raffinose; R=ribose; Hyd. of arg.=Hydrolysis of arginine; + = positive; - = negative; v=variable

Table 3 : Current *Lactococcus* species designations and their former streptococcal species names (Sandine, 1988)

Current nomenclature	Corresponding former nomenclature
<i>L. lactis</i> ssp. <i>lactis</i>	<i>S. lactis</i> ssp. <i>lactis</i>
<i>L. lactis</i> ssp. <i>lactis</i>	<i>S. lactis</i> ssp. <i>diacetylactis</i>
<i>L. lactis</i> ssp. <i>cremoris</i>	<i>S. lactis</i> ssp. <i>cremoris</i>
<i>L. lactis</i> ssp. <i>hordinae</i>	<i>L. hordinae</i>
<i>L. garviae</i>	<i>S. garviae</i>
<i>L. plantarum</i> ^a	<i>S. plantarum</i>
<i>L. raffinolactis</i>	<i>S. raffinolactis</i>

^a Not to be confused with *Lactobacillus plantarum*

1.5 Genetics of lactococci

Lactococcal genetics continues to be one of a number of active areas of research and development in the dairy industry. Since the review by Lawrence *et al.*, (1976) significant progress has been made towards understanding the genetics of lactic starters. Consequently contemporary information explains phenomena associated with these bacteria during the fermentation process. Furthermore, continued improvements in lactic starter culture qualities will offer the dairy industry not only strains matched ideally to a particular fermentation but also the possibility of creating novel strains for new commercial uses thus strengthening the potential to manufacture more and diverse fermented dairy products (Gasson and Davies, 1984).

1.5.1 Genetic material arrangement

In lactococci plasmids are frequently associated with important fermentation characteristics and may be lost with a concomitant loss of particular phenotypic traits coded for by that plasmid (Davies and Gasson, 1981, 1983; Gasson and Davies, 1984; McKay, 1982, 1983). It is suggested that the plasmid instability of lactococci would appear to be genetically consistent with the hypothesis that, with the assistance of genes provided by plasmid DNA, these bacteria have invaded milk as a growth environment comparatively recently in evolutionary terms and they are still undergoing relatively rapid genetic reorganization of the necessary additional enzymes. These organisms are therefore regarded as highly variable and their cultures potentially non-homogeneous even after normal strain purification procedures (Hall and Franks, 1985).

1.5.2 Plasmid biology

Lactococcal plasmid DNA was first resolved experimentally as a satellite peak after equilibrium density gradient centrifugation of radioactively labelled DNA in cesium chloride and ethidium bromide (Cords *et al.*, 1974). Also from electron microscopy evidence, plasmids appeared as circular DNA molecules of various sizes as estimated by contour length measurement (Cords *et al.*, 1974; McKay and Baldwin, 1975;

Anderson and McKay, 1977). The more rapid and convenient technique of agarose gel electrophoresis (Meyers *et al.*, 1976) is used currently to visualize the lactococcal plasmids (Klaenhammer *et al.*, 1978). Before the detection of their plasmids becomes possible the cells have to be lysed and the substantial amount of chromosomal DNA removed. Plasmid detection and visualization employs various protocols based upon two different approaches. The first is a simple cleared lysate method in which the cell membrane with attached chromosomal DNA is precipitated and removed by centrifugation (Klaenhammer *et al.*, 1978; Gasson and Davies, 1980a). The second approach involves the denaturation of chromosomal DNA at alkaline pH. The plasmid DNA is left intact and can be separated and purified (Walsh and McKay, 1981; Anderson and McKay, 1983).

Lactococci in nature harbour large complements of plasmids. The number may range from 2 to 14 plasmids but typically it is between 4 and 7 distinct plasmids species. Size varies from 1 to 40 Md or greater (Larsen and McKay, 1978; LeBlanc *et al.*, 1980; Kuhl *et al.*, 1979; Pechmann and Teuber, 1980; Davies and Gasson, 1981; McKay and Baldwin, 1982; McKay, 1983). These plasmid patterns or plasmid profiles of lactococci are strain specific and can be used as "fingerprints" in starter strain identification (Macrina *et al.*, 1980; Pechmann and Teuber, 1980) and to establish strain interrelationships (Davies *et al.*, 1981).

The large complement of plasmids found in lactococci is unique since other related bacteria such as the lactobacilli and strains of *S. thermophilus* often contain fewer plasmids, perhaps only one or even none at all. However, strains of *Lactobacillus acidophilus* are an exception (Vescovo *et al.*, 1981; Somkuti and Steinburg, 1981). Even though some of the plasmids of lactococci may be spontaneously lost or may vary slightly in number in response to different growth environments the overall profiles are still sufficiently unaltered to retain recognizability (Davies *et al.*, 1981). Loss of plasmids can be encouraged either by growing cultures at elevated temperatures (Teuber and Lembke, 1983), by growing in the presence of ethidium bromide, acriflavine or acridine orange (Pearce and Skipper, 1971; Gasson, 1984) or by treating with N-methyl-N'-nitro-N-nitrosoguanidine (McKay *et al.*, 1976). These procedures have been used in

plasmid curing experiments (Davies and Gasson, 1983, Kondo and McKay, 1985). It is thus possible to correlate phenotypic loss with the absence of a particular plasmid and the function of the missing plasmid can be verified. Therefore, it is of immense value to characterize not only plasmid-free strains produced from plasmid curing procedures but also derivative strains of lactococci which continue to carry a specific plasmid molecule. Strains which lack certain capabilities are useful in studies of genetic transfer mechanisms of lactococci (Gasson and Davies, 1984).

1.5.3 Functional properties of lactococcal plasmids

As noted above (1.5.2) lactococci have a diversity of plasmids which vary in size and number. Although many plasmids are cryptic (i.e., of unknown function) some encode known physiological functions (McKay, 1983). Several important properties of lactococci are found to be plasmid-encoded, an observation deduced from the concomitant loss of both specific characteristics and plasmids. Plasmid loss arises through its ability to replicate independently of the bacterial chromosome and any mutation resulting in a failure of plasmid replication also results in a daughter cell that does not receive a copy of this plasmid from the parent cell. Thus the daughter cell will no longer contain this particular plasmid. It is for this reason that plasmid-associated properties may be more unstable than the properties controlled by chromosomal genes (McKay, 1983; Gasson and Davies, 1984).

The relationship between specific plasmids and phenotypic properties is generally implied from the observation that spontaneous or induced curing of the property is accompanied by loss of the individual plasmid (Davies and Gasson, 1984). However, before any conclusion is drawn on this basis more specific evidence must be sought since some plasmids are intrinsically unstable and their disappearance may be unconnected with the simultaneous loss of a particular property (Davies and Gasson, 1981, 1984; Gasson, 1983b; McKay, 1982).

Important properties that are found to be plasmid-encoded are as follows: lactose metabolism (McKay, 1982, 1983; Gasson, 1982); galactose metabolism (McKay *et al.*,

1970; Demko *et al.*, 1972, Cords and McKay, 1974); other carbohydrate metabolic activities rather than lactose and galactose (LeBlanc *et al.*, 1980); proteolytic activity (Pearce, 1970; Thomas *et al.*, 1974; McKay and Baldwin, 1975; Gasson, 1983a); citrate utilization (Kneteman, 1952; Collins and Harvey, 1962; Mostert, 1976; Kempler and McKay, 1981); antagonistic properties (Kozak *et al.*, 1974; McKay and Baldwin, 1982; Davey and Pearce, 1982; Scherwitz *et al.*, 1983; Davey, 1984; Gonzalez and Kunka, 1985); restriction/modification (R/M) systems (Lawrence *et al.*, 1976; Limsowtin *et al.*, 1978; Sanders and Klaenhammer, 1980).

There are some other properties that are also found to be plasmid-involved but they are not necessarily related to the fermentation activities, for example; inorganic ion resistance and arginine hydrolysis (McKay, 1983). More interest has been directed towards the identification and location of those plasmids associated with lactose, protein and citrate metabolism because of their importance in dairy fermentations. Plasmid instability occasionally can be a troublesome phenomenon for the dairy industry (Davies and Gasson, 1983).

1.5.3.1 Lactose Metabolism

Lactose metabolism is a characteristic feature of the lactococci and is known to be plasmid-mediated in many strains (McKay, 1983) as evidenced by the simultaneous loss of both the lactose plasmids (Lac plasmid) and the ability to ferment lactose by the phenotype or Lac⁺ strains. Biochemical aspects of lactose metabolism will be developed in Section 1.7. The conclusion that lactose-fermenting ability is plasmid-mediated has been strengthened by three different lines of evidence namely phenotypic, physical and genetic (McKay, 1983). The phenotypic evidence involves observations of any change of the phenotypic expression after subjecting the organism to plasmid curing procedures (see 1.5.2). The absence of lactose metabolism in these treated cultures was observed by earlier workers (Okulitch and Eagles, 1936; Sherman and Hussong, 1937; Yawger and Sherman, 1937; Hunter, 1939; Okulitch, 1939; Hirsch, 1951). The phenomenon was confirmed by McKay *et al.*, (1972) who discovered more strains that had lost their lactose fermenting abilities (designated Lac⁻) after either treatment with acridine dyes

or cultivation at elevated temperatures. Physical evidence for plasmid involvement was provided by comparing the plasmid profiles of Lac⁺ parental strains with plasmid profiles of their own Lac⁻ derivatives. Consequently, the specific lost plasmid (Lac plasmid) could be identified. For example, a 30 Md plasmid of *L. lactis* ssp. *lactis* strain C2 (McKay *et al.*, 1976; Klaenhammer *et al.*, 1978), a 33, 40 or 45 Md plasmid of *L. lactis* ssp. *lactis* strain ML3, C10 and M18, respectively (Kuhl *et al.*, 1979); a 36 Md plasmid of *L. lactis* ssp. *cremoris* (Anderson and McKay, 1977); and *L. lactis* ssp. *lactis* var. *diacetylactis* 18-16 Lac⁻ variants (Kempler and McKay, 1979b) were all missing a 41 Md plasmid. Genetic evidence was supported by the transfer of the Lac plasmid from Lac⁺ strains to Lac⁻ strains either by transduction, conjugation or transformation. Physical evidence confirmed successful Lac plasmid transfer (McKay, 1983). The conclusion must be drawn based on these results as well as the evidence drawn from curing experiments that lactose metabolism in lactococci is a plasmid-mediated property. The exception to this appears to be in circumstance where lac genes may become integrated into the chromosome (Okamoto *et al.*, 1989; Feirtag *et al.*, 1991). Lac plasmids of some strains of lactococci have been identified and characterized. Their properties are summarized in Table 4.

1.5.3.2 Proteolytic Activity

The second fundamental property of the lactococci is their proteolytic activity and this is also found to be plasmid-encoded (Prt plasmid; see Lawrence *et al.*, 1976; Davies and Gasson, 1981, 1983; McKay, 1983; Gasson and Davies, 1984). Proteolytic activity is required for the growth of lactococci because they are nutritionally fastidious and require significant amounts of nitrogenous compounds from milk proteins (Law *et al.*, 1974; Law, 1978; Law and Kolstad, 1983; McKay, 1983; Gasson and Davies, 1984). Normally the free amino acids and small peptides contained in fresh milk will support *L. lactis* ssp. *cremoris* growth (Thomas and Mills, 1981). These organisms need at least 10 to 13 amino acids for fast growth (Thomas and Pritchard, 1987). Because these amino acids are at growth limiting concentrations in milk, efficient lactic starter growth and acid production critically depend on their proteolytic action on milk protein. Their proteolytic enzymes can hydrolyze casein to produce sufficient small peptides and free

Table 4 : Plasmids associated with lactose and/or protein hydrolysing activities.^a

Genus and Species	strains	Lac (Md)	Prt (Md)	Lac/Prt (Md)	Reference
<i>S. lactis</i> ssp. <i>lactis</i>	C2	30	18 and 25		Kempler and McKay, 1979b
	ML3	33		33*	Walsh and McKay, 1982
	712			33	Gasson, 1982
	C10			40*	Kuhl <i>et al.</i> , 1979
	M18			45*	Kuhl <i>et al.</i> , 1979
	DR1251	32			LeBlanc <i>et al.</i> , 1979
<i>S. lactis</i> ssp. <i>diacetylactis</i>	18-16	41			Kempler and McKay, 1979a
	DRC3	52			McKay <i>et al.</i> , 1980
	11007	32			McKay <i>et al.</i> , 1980
	DRC1			31*	Kempler and McKay, 1979b
<i>S. lactis</i> ssp. <i>cremoris</i>	B1	37			Klaenhammer <i>et al.</i> , 1978
	HP		9		Larsen and McKay, 1978
	ML1			2.2*	Larsen and McKay, 1978
	C3	34/27			Snook and McKay, 1981
	R1	34			Snook and McKay, 1981
	EB7	42			Snook and McKay, 1981
	Wg2		16		Otto <i>et al.</i> , 1982
<i>L. helveticus</i> ssp. <i>jugurti</i>	536-2	8.2			Smilely and Fryder, 1978
<i>L. casei</i> ssp. <i>casei</i>	DR1002	23			Chassy <i>et al.</i> , 1978

^a According to Davies and Gasson, 1983

* Plasmid observed to be absent from lac⁻ prt⁻ strains but on supportive data for allocation of both functions to plasmid.

amino acids which become available for transport across the cell membrane (Gasson and Davies, 1984). Additional to the fundamental role proteolysis plays in the growth of lactococci it also contributes significantly to the production yields and flavour development of both cheese and casein (Thomas and Pritchard, 1987; Monnet *et al.*, 1989). Different aspects of proteolysis affecting cheese production and quality have been reviewed in recent years, e.g. flavour (Law, 1984a), texture (Lawrence *et al.*, 1987), bitterness (Richardson and Creamer, 1973; Visser, 1981; Exterkate, 1987) and accelerated cheese ripening (Law, 1984b).

Loss of proteolytic activity, i.e. the absence of proteinases (Prt⁻ strains) has long been recognized as one of the problems of starter culture technology (e.g., Harriman and Hammer, 1931; Garvie and Mabbitt, 1956; Citti *et al.*, 1965; Westhoff *et al.*, 1971) because it results in slow acid production whereby 48 h or more is required to coagulate milk at 21°C (McKay, 1983; Gasson and Davies, 1984). An increase in the coagulation times was observed to be a consequence of plasmid curing experiments which produced Prt⁻ variants (Pearce, 1970; Molskness *et al.*, 1974; Pearce and Skipper, 1971; Pearce *et al.*, 1974). The Prt⁻ variants were however, able to grow at normal rates in milk supplemented with hydrolysed protein and reach 10 to 25% of the cell density of Prt⁺ strains growing in coagulated milk (Thomas and Mills, 1981). Attempts have been made to identify proteinase plasmids (Prt plasmids) in a number of strains (Davies and Gasson, 1981) although obtaining unequivocal data has been difficult. However, a number of proteinase plasmids have been identified in some strains (see Table 4) following the first physical evidence for them which was obtained from electron micrographs of variants of *L. lactis* ssp. *lactis* strain C2 (McKay and Baldwin, 1975). A 10 Md plasmid was involved. Later, Klaenhammer *et al.* (1978) using the same strain and agarose gel electrophoresis found two plasmids, of 12.5 Md and 18 Md in size and suggested the involvement of one or both of these in proteinase production.

Genetic interrelationships between the Lac and the Prt plasmids still remain unclear. Some confusion has arisen with the Prt plasmid's identification in *L. lactis* ssp. *lactis* mainly because genetically the Prt activity is incompletely linked to the Lac determinant (Gasson and Davies, 1984). Why strains can become Lac⁺ Prt⁻ or Lac⁻ Prt⁻ and

spontaneously lose different plasmids has not yet been resolved (McKay, 1983). In *L. lactis* ssp. *lactis* strain C2, ML3, 712, C10, M18, the loss of their lac and Prt activities was correlated with the loss of a particular plasmid (see also Table 5) whereas a Lac⁺ Prt⁻ variant of *L. lactis* ssp. *lactis* strain C2 retained the 30 Md but lost the 12.5 and 18 Md plasmids (McKay and Baldwin, 1974; Klaenhammer *et al.*, 1978). The linkage of Lac and Prt plasmids has already been evidenced from gene transfer experiments. Here, Lac gene transfer was sometimes accompanied by co-transfer of *Prt* genes, both in transduction experiments (McKay *et al.*, 1976; Davies and Gasson, 1981) and in conjugation experiments (Gasson and Davies, 1980a; Gasson, 1983b). The simultaneous loss of lactose and proteolytic activities observed in spontaneous or plasmid-cured variants has led to speculation that these properties are genetically linked and may in some cases reside on the same plasmid (Kuhl *et al.*, 1979). Furthermore, the observed proteinase activity (Prt⁺ phenotype) may be controlled by several types of proteinases which are coded for on one or more different plasmids or even on chromosomal DNA [e.g., proteinase activity in *L. lactis* ssp. *cremoris* B₁ appears to be chromosome-mediated (Anderson and McKay, 1977; McKay, 1983)].

1.5.3.3 Citrate Utilization

The ability of *L. lactis* ssp. *lactis* var. *diacetylactis* (previously *L. lactis* ssp. *diacetylactis*) to produce diacetyl from citrate has encouraged its extensive use as a flavour enhancer in some dairy fermentations (McKay, 1983; Davies and Gasson, 1981). However, citrate utilization (Cit⁺) is not a stable character (Mostert, 1976; McKay, 1983). Evidence that citrate utilization is plasmid-encoded has come from several observations. Firstly, the spontaneous loss of this ability to ferment citrate can occur (Kneteman, 1952; Collins and Harvey, 1962; Mostert, 1976). Secondly, strains can become Cit⁻ after treatment with plasmid curing agents (Kempler and McKay, 1979b). Finally, there is a correlation between citrate-fermenting ability and the presence of a 5.5 Md Cit plasmid (Kempler and McKay, 1981). The absence of the Cit plasmid was associated with the loss of citrate permease activity (Davies and Gasson, 1981) but not of citrate lyase activity (i.e., Cit⁻ strains retained low citrate lyase activity).

It is now well established that this 5.5 Md plasmid present in many strains of *L. lactis* ssp. *lactis* var. *diacetylactis* is the citrate plasmid (Kempfer and McKay, 1981; Davies and Gasson, 1981).

1.5.4 Genetic transfer systems

A variety of genetic transfer systems operate in lactococci. Most have been exploited as techniques for studying important genetically controlled starter culture characteristics relevant to the dairy industry (Gasson and Davies, 1984). Earlier lactococcal genetic studies were concerned mainly with the transfer of plasmid DNA by transduction, conjugation and protoplast transformation into and between competent strains. These studies provided both phenotypic and physical evidence for plasmid involvement in many physiological functions of these bacteria. The more recent developments in recombinant DNA technology have been applied to lactococcal genetic transfer systems to allow rapid molecular analyses of lactococcal genetic system. With the development of electroporation techniques, the prospects of successful lactococcal genetic mechanism analyses has become even more promising. The genetic transfer mechanisms most useful in studies of lactococcal genetics are discussed below.

1.5.4.1 Transduction

Transduction is the transferring of DNA from one cell to another via bacteriophages, either virulent or temperate. Transduction with virulent bacteriophages was the first DNA transfer system to be demonstrated in lactococci (Sandine *et al.*, 1962; Allen *et al.*, 1963). The first temperate phage system developed was used to transfer to recipient strains chromosomal mannose and maltose markers as well as plasmid linked lactose genes of *L. lactis* ssp. *lactis* C2 (McKay *et al.*, 1973). Work by McKay and Baldwin (1974) established that approximately 50% frequency of cotransfer existed for *lac* and *pri* genes for the C2 strain. Transduction experimental results with this particular strain C2 actually provided the first evidence for a plasmid controlled metabolic trait, namely lactose metabolism in lactococci, (McKay *et al.*, 1976). When transduction experiments were repeated using bacteriophages induced from a lactose transductant rather than from

the parental strain C2, the frequency of gene transfer was found to be substantially increased (McKay *et al.*, 1973; McKay *et al.*, 1976). This situation is well known in other transduction systems and is referred to as high frequency transduction (HFT) phenomena (McKay *et al.*, 1973; Gasson and Davies, 1984). Two phage bands were produced from the C2 strain lysates by CsCl gradient centrifugation and although two morphological distinct phages were demonstrated, both phage bands had equal transducing ability. The absence of a distinct band of transducing phage in the gradients led other investigators to suggest that the HFT phenomenon resulted from recombination between the temperate phage and an unlinked lactose plasmid rather than by a process analogous to the formation of specialized lambda transducing phage (Klaenhammer and McKay, 1976).

DNA transfer via transduction may be limited due to the bacteriophage packaging capacity and specific requirements of bacterial host strains containing relevant bacteriophage receptors (Gasson and Davies, 1984). It was speculated that the Lac⁺ Prt⁻ and Lac⁺ Prt⁺ transductants of the strain C2, which harboured a plasmid of approximately 20-23 kD in size originating from transductive transfer of the 30 kD plasmid from the parental C2 strain, was a consequence of the limited bacteriophage head packaging capacity (Lawrence *et al.*, 1976; McKay *et al.*, 1976). This process was termed transductional shortening after these two defective transducing phages (Klaenhammer and McKay, 1976; McKay *et al.*, 1976; Gasson and Davies, 1979).

A similar Lac transduction phenomenon has been observed also in *L. lactis* ssp. *lactis* 712, the parental strain of strain C2 (Davies and Gasson, 1981). However, another speculation is that transducing phages may preferentially package pre-formed deleted plasmids which are already present in the population. These plasmids may arise spontaneously, rather than being formed by a transductional shortening process (Davies and Gasson, 1981; Gasson and Warner, 1982). The shortening process associated with limited phage packaging capacity makes transduction very useful for introducing fine structural changes into the genetic material (Gasson and Davies, 1984). Moreover, these deletions are also found to be useful in mapping studies in which plasmid-encoded genes need to be located on a restriction endonuclease cleavage map (Gasson, 1983b).

Transduction studies have also been carried out using other strains of *L. lactis* ssp. *lactis* as well as *L. lactis* ssp. *cremoris* (McKay *et al.*, 1980). An induced temperate phage from *L. lactis* ssp. *cremoris* C3 was used to transduce lactose genes into *L. lactis* ssp. *lactis* ML3 and into a plasmid-free derivative of the strain C2 (Snook *et al.*, 1981). In addition to the lactose plasmid, the 17 Md erythromycin resistance plasmid pAMB1, initially characterized in *Enterococcus faecalis* DS-5 (Clewell *et al.*, 1982), was transduced between lactococci but in this case the HFT phenomenon did not occur (Gasson and Davies, 1981).

Additional to the accumulating experimental evidence for lactococcal plasmid functions, beneficial contributions to the commercial development of improved starter strains have been made and involve transductants integrated into chromosomal DNA (Davies and Gasson, 1983). Consequently the genes become stabilized in their new location but the extent of gene expression may be reduced (McKay and Baldwin, 1978). For example, transduced *lac* genes, usually plasmid-linked, can be integrated into the chromosome in the homologous transduction systems of *L. lactis* ssp. *lactis* C2 (McKay and Baldwin, 1978; Davies and Gasson, 1981; Snook *et al.*, 1981). It was found that a stabilized chromosomal Lac⁺ Prt⁺ transductant of the strain C2 had a weaker proteolytic activity than the wild type and was capable of producing a Cheddar cheese with reduced bitterness (i.e., lower concentrations of bitter peptide). The possibility existed that the transductant had a reduced copy number of the *prt* genes (McKay and Baldwin, 1978; Kempler *et al.*, 1979).

1.5.4.2 Conjugation

Conjugation is the phenomenon by which DNA is transferred from one mating type of bacterium to another during close physical contact (i.e., cell-to-cell contact) and occurs widely in lactococci (Gasson, 1983b; Gasson and Davies, 1979, 1984; Gasson *et al.*, 1992). Conjugation may involve plasmids which encode a transfer system for directing their transmission (conjugative plasmid) to bacterial cells which do not carry them (Gasson and Davies, 1979, 1980b; Kempler and McKay, 1979b; McKay *et al.*, 1980). First reports were of conjugal transfer of plasmid-encoded lactose metabolism genes

between marked strains of *L. lactis* ssp. *lactis* strain 712 (Gasson and Davies, 1979; 1980b) and from *L. lactis* ssp. *lactis* var. *diacetylactis* 18-16 into a plasmid-free derivative of *L. lactis* ssp. *lactis* C2 (Kempler and McKay, 1979a).

The conjugation transfer processes were initially detected at relatively low frequency, i.e. about 10^{-5} - 10^{-9} per donor (Davies and Gasson, 1981, 1983). However, the use of transconjugants (or ex-conjugant, progeny derived from conjugation) as donor strains in subsequent mating experiments (secondary mating) revealed variants able to transfer at an increased frequency (Gasson and Davies, 1979, 1980b). In this way, high frequency donor strains (as high as 10^{-1} per donor) were observed and isolated in *L. lactis* ssp. *lactis* strains 712, C2, ML3, ME2, C20; in *L. lactis* ssp. *cremoris* strain M43 and in *L. lactis* ssp. *lactis* var. *diacetylactis* strains DRC3 and WM4 (Gasson and Davies, 1980b; Anderson and McKay, 1983; Scherwitz *et al.*, 1983; McKay and Baldwin, 1984; Klaenhammer and Sanozky, 1985; Kondo and McKay, 1985; Steenson and Klaenhammer, 1985).

These transconjugants from the above conjugation transfer processes exhibited a high frequency of conjugation (HFC) together with a novel cell aggregation phenomenon which caused striking changes in both colony morphology on plates and broth culture appearances. It has been postulated that this acquisition of high frequency donor ability together with a cell aggregation phenotype involves enlargement of the lactose gene plasmids by insertion of novel DNA coding for these new functions (Walsh and McKay, 1981, 1982; Gasson, 1983b; Anderson and McKay, 1984). However, gene transfer from low frequency conjugation to HFC is not always necessarily synonymous with the formation of an enlarged plasmid. During the conjugal transfer of the lactose plasmid of *L. lactis* ssp. *lactis* ME2, HFC seemed neither to be accompanied by the formation of enlarged plasmids in the recipients nor by the cell aggregation phenomenon (Klaenhammer and Sanozky, 1985).

Conjugation has enabled the introduction of wide host range plasmids into lactococci. Examples are the erythromycin resistance plasmid pAM β 1, initially characterized in *Enterococcus faecalis* DS-5 (Clewell *et al.*, 1974) and the chloramphenicol, erythromy-

cin resistance plasmid pIP501 (Gonzalez and Kunka, 1983). They have also been introduced into lactobacilli and pediococci again by conjugation (Gasson and Davies, 1980b; Gibson *et al.*, 1979; Davies and Gasson, 1981; Vescovo *et al.*, 1983). Plasmid pAMB1 has proved useful for its ability to mobilize nonconjugative or poorly conjugative plasmids by mobilizing proteinase genes from *L. lactis* ssp. *cremoris* strains UC317, UC205 and UC411 to *L. lactis* ssp. *lactis* (Hayes *et al.*, 1990). The wide range of host strains in which they transfer, replicate and express has made pAMB1 and pIP501 potentially very useful for both *in vivo* strain development and as the basis for new vector constructions (Gasson, 1983b; Evans and Macrina, 1983).

In addition to the conjugation efficiency, the type of mating techniques used should also be considered (Gasson and Davies, 1981). Lactose gene transfers were found to be most efficient using a plate or filter technique (Gasson and Davies, 1980a; McKay *et al.*, 1980) whereas pAMB1 transfers were most effective using the latter technique only (Gasson and Davies, 1980a). These techniques are based on immobilization of the cells in a medium which will enhance close physical cellular contact during the conjugation process. The surface contact required with lactococcal conjugation probably reflects the uniqueness of the system involved and emphasizes its difference from the model conjugation system of the *E. coli* sex factor F. While sex pili are generally vital for partner recognition in Gram-negative conjugation process, such organelles do not appear to be present in lactococci (Gasson and Davies, 1984).

Conjugation among lactococci is considered a potentially useful mechanism for the construction of new dairy starter strains (Gasson and Davies, 1984). It has the advantage of technical feasibility. There is no theoretical limit to the amount of DNA that can be transferred and the widely promiscuous nature of the plasmid pAMB1 conjugation makes transfer possible both within and between species. Also, several plasmids have been conjugated from lactococci to *Clostridium acetobutylicum* (Yu and Pearce, 1986). Before efficient transformation by electroporation was established (refer Section 1.5.4.6), naturally transformable organisms such as *S. sanguis* were used as intermediate donors of plasmid constructs to conjugatively mobilized across 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 (Macrina *et al.*, 1983; Dao and Ferretti, 1985).

The discovery of a conjugal genetic transfer system in lactococci has also provided evidence for the plasmid linkage of diverse characteristics including lactose metabolism, proteolytic activity (Gasson and Davies, 1979, 1980b; Kempler and McKay, 1979a; McKay and Baldwin, 1984), restriction/modification systems (Chopin *et al.*, 1984), the production of antagonistic compounds *viz* diplococcin and other bacteriocins (Davey and Pearce, 1982; McKay and Baldwin, 1982; Scherwitz *et al.*, 1983; Davey, 1984; Gasson, 1984; Neve *et al.*, 1984) and resistance to nisin and bacteriophage (McKay and Baldwin, 1984). For commercial applications, conjugation has been used not only to select a nisin producing strain with dramatically increased yields (Tsai and Sandine, 1987) but also for the construction of bacteriophage-resistant dairy starters (Sanders *et al.*, 1986).

1.5.4.3 Transposition

Transposable elements or transposons (Tn) are genetic elements defined as discrete segments of DNA that are capable of moving or transposing from one replicon to another. In general there are a number of classes of transposons but the conjugative class is unique to members of the genus *Streptococcus* (Fitzgerald and Gasson, 1988). Tn916, from *Enterococcus faecalis* DS16 and Tn919 from *S. sanguis* FC1 are the best studied transposons that have been put into lactococci (Franke and Clewell, 1981; Tomich *et al.*, 1980; Clewell *et al.*, 1974; Hartley *et al.*, 1984; Fitzgerald and Clewell, 1985; Fitzgerald *et al.*, 1987; 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^{-8} per donor (Franke and Clewell, 1981; Gawron-Bourke and Clewell, 1982; Fitzgerald and Clewell, 1985). Both elements have been transferred to a number of lactococcal species by the filter mating technique and have also been cloned into *E. coli* (Gawron-Bourke and Clewell, 1984; Fitzgerald and Clewell, 1985).

Tn 916 promotes conjugal transfer among strains of *Enterococcus faecalis*, *S. mutans*, *S. agalactiae* and *L. lactis* ssp. *lactis* (Clewell and Gawron-Burke., 1986) and will insert at different sites on the bacterial chromosome. Tn916 and its related transposons have been used to locate the genes of hemolysin production on the pAD1 plasmid of *Enterococcus faecalis* (Clewell *et al.*, 1982) and those of malolactate, maltose, mannose and arginine metabolism traits in *L. lactis* ssp. *lactis* IL 1441 (Renault and Heslot, 1987).

Tn919 has been observed to transfer conjugatively among strains of *Enterococcus faecalis*, *L. lactis* ssp. *lactis*, *L. lactis* ssp. *lactis* var. *diacetyllactis*, *L. lactis* ssp. *cremoris*, *Lactobacillus plantarum* and *Leuconostoc cremoris* and was reported to insert into chromosomal Tn919 of *L. lactis* ssp. *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. However, the introduction of pMG600 (a deletion plasmid derived from pLP712) into a *L. lactis* ssp. *lactis* CH919 donor (*L. lactis* ssp. *lactis* strain MG1363 harbouring Tn919 on its chromosome), to produce *L. lactis* ssp. *lactis* CH001, resulted in a significant improvement in the transfer frequency of Tn919 to *L. lactis* ssp. *lactis* CK50. This new frequency was approximately 1.25×10^{-4} per recipient (Hill *et al.*, 1987).

The high frequency transfer of Tn919 into *L. lactis* ssp. *lactis* var. *diacetyllactis* 18-16S has also been observed, with inserts in transconjugants occurring at different sites on the bacterial chromosome as well as on plasmid DNA. *L. lactis* ssp. *lactis* CH001 was however unable to transfer the transposon into *L. lactis* ssp. *cremoris*, lactobacilli or *Leuconostoc cremoris* strains during 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 offers considerable potential for exploitation especially for the exploration of chromosomally controlled genes. (Gasson and Davies, 1984).

1.5.4.4 Protoplast Transformation and Transfection

The cell wall of lactococci has been considered a barrier to DNA uptake and thus it hinders the development of successful transformation systems. To circumvent this barrier, the possibility of protoplast transformation was investigated (Sanders and Nicholson, 1987). Protoplast production and regeneration involves cell wall digestion with lysozyme (Gasson, 1980; Okamoto *et al.*, 1985), N-acetyl-muramidase, mutanolysin (Hart, 1982; Kondo and McKay, 1982; Cocconcelli *et al.*, 1986; Reed, 1987) or a combination of amylase and lysozyme (Okamoto *et al.*, 1983a). The first report of the production and regeneration of lactococcal protoplasts was from Gasson (1980).

The first successful lactococcal protoplast transformation was achieved by Kondo and McKay (1982) using polyethylene glycol (PEG) treated *L. lactis* ssp. *lactis* ML3 protoplasts to introduce a transductionally shortened lactose plasmid. Protoplast treatment using PEG is used commonly in the transformation of streptomycetes and bacillus (Bibb *et al.*, 1978; Chang and Cohen, 1979).

A similar procedure has been used to transfect bacteriophage DNA into *L. lactis* ssp. *lactis* var. *diacetyllactis* F7/2 at a frequency of 5×10^4 cfu/ μ g DNA (Geis, 1982). Subsequent to these reports many protocols have been developed and optimized to increase protoplast transformation frequency from 10^4 to 10^6 cfu/ μ g DNA and are applicable to a limited number of *L. lactis* ssp. *lactis* strains (Kondo and McKay, 1984; Gasson and Anderson, 1985; von Wright *et al.*, 1985; Simon *et al.*, 1986; Woskow and Kondo, 1987) as well as *S. thermophilus* (Mercenier *et al.*, 1988) and *Enterococcus faecalis* (Smith, 1985; Wirth *et al.*, 1986). Very low frequency protoplast transformation of *L. lactis* ssp. *cremoris* has also been reported (Simon *et al.*, 1985; Woskow, 1987).

Protoplast transformation and transfection procedures have not been regarded as successful and there have been very few reports using these procedures to transform genetic material directly into lactococci. An obvious obstacle is the regeneration of the cell wall of the bacterial protoplast after transformation. Successful protoplast transformation depends critically on the ability to achieve a controlled cell wall digestion

such that the osmotically fragile protoplasts can be later regenerated to their normal state. Moreover, there are many factors influencing protoplast formation and recipient stability such as the growth state of the cells, the amount of lysozyme used, PEG treatment, regeneration medium and the osmotic stabilizer required. Furthermore, the procedures have often been found difficult to reproduce. Finally, the limited number of transformable strains available is also a factor of concern. In spite of these difficulties successful transformations of plasmid constructs from intermediate hosts such as *E. coli* or *B. subtilis* to lactococci have been documented (De Vos, 1987). Whether protoplast transformation will be widely suitable for the movement of genes among lactococci, however, remains an open question.

1.5.4.5 Protoplast fusion

Protoplast fusion is a process of genetic transfer whereby the osmotically stabilized bacterial protoplasts are combined together (i.e. fusion) and then regenerate into a single recombinant progeny cell, the so-called "fusant". The fusion process is induced either by a chemical substance, polyethylene glycol (PEG), a widely used fusion inducing agent, also called the fusagent (Gasson, 1980; Simon *et al.*, 1985; van der Vossen *et al.*, 1988) or by an electrical field (Reed, 1987). Protoplast fusion has been found widely applicable to strains of *L. lactis* ssp. *lactis* or *L. lactis* ssp. *cremoris* (Okamoto *et al.*, 1985; Simon *et al.*, 1985) other lactococci, lactobacilli (Coconcelli *et al.*, 1986) and *B. subtilis* (Baigori *et al.*, 1988; van der Vossen *et al.*, 1988).

The transfer of genetic information among lactococci by protoplast fusion and the subsequent regeneration of fusants into normal whole cells was first reported by Gasson (1980). Transfer of both plasmid and chromosomal DNA between genetically marked derivatives of *L. lactis* ssp. *lactis* strain 712 was also achieved (Gasson, 1980). Compared with plasmid transfer, transfer of chromosomal markers was shown to be of relatively low frequency (Okamoto *et al.*, 1985).

The observation that fusants may lose their plasmids during regeneration to whole cells (Novick *et al.*, 1980) has proved to be a valuable technique for eliminating plasmids

from the cells. This phenomenon in lactococci has been referred to as "protoplast curing" (Davies and Gasson, 1983), and protoplast formation and regeneration has become one of the plasmid curing methods. A comprehensive series of cured derivatives of *L. lactis* ssp. *lactis* strain NCDO 712 has been obtained for use in analysis of plasmid functions (Gasson, 1983a). By the same technique two plasmid-free strains of *L. lactis* ssp. *cremoris* have also been isolated (Davey *et al.*, 1986).

Protoplast fusion provides a useful technique for inducing genetic recombination since it generates large numbers of random recombinants (Okamoto *et al.*, 1985). In addition, it is a very useful technique for the development of new starter strains (Gasson and Davies, 1984) because it is non-specific at the strain, species or genus rank and it also involves the total genome rather than the unidirectional transfer of pieces of DNA. This total contact of genetic material during the fusion process and the retention of the complete genetic information will encourage future investigation of the fusant's characteristics.

1.5.4.6 Electroporation

Electroporation is an extremely efficient transformation method that uses an electrical current for the generation of cell membrane distortions. This results in a temporary change in the membrane permeability which allows the treated cells to take up DNA (Fiedler and Wirth, 1988). The process involves subjecting viable cells to a rapidly changing high-voltage electric field, thereby producing transient pores in their outer membranes. Fusion of the cells is facilitated when pores develop in adjacent cells. Diffusion and exchange of intracellular and extracellular components can take place during the lifespan of the pores and occurs in a brief period of time (in terms of "milliseconds") during a high voltage pulse (Fromm *et al.*, 1985; Zerbib *et al.*, 1985). This mechanism is referred to as "electrotransfusion" (Zimmermann and Vienken, 1982) and the generation of small localized pores in biological membranes by electric field induction is termed "electroporation". Exponential growth phase cells or their protoplasts are used. Cells have to be washed extensively with low ionic-strength solution and resuspended in a buffer of very low conductivity. Such cells are referred

to as electroporatable or electrocompetent cells. Although first developed for eukaryotic cells (Zimmermann, 1983), the technique has proved to be a universally applicable genetic transfer technique with successful applications involving animal (especially mammalian), plant, fungal, protozoan and bacterial cells (Chassy and Flickinger, 1987; Harlander, 1987; Powell *et al.*, 1988). Very high transformation efficiencies have been recorded using this technique; for example in *E. coli*, 10^9 to 10^{10} transformants/ μg DNA, have been achieved under optimal conditions (Calvin and Hanawalt, 1988; Dower *et al.*, 1988). The transformation rates obtained for the Gram-positive bacteria are lower at about 10^4 to 10^5 transformants/ μg DNA (Fiedler and Wirth, 1988) but are high enough to allow gene transfer from shotgun cloning experiments (refer section 1.6).

A number of its successful applications to bacterial systems have been reported, however, for lactococci, electroporation has been used with both protoplasts (i.e., lysozyme treated cells) and intact cells (Fiedler and Wirth, 1988). Powell *et al.* (1988) found 10 strains of lactococci with which electroporation could be used successfully to achieve genetic transformation including transfection via electroporation. However, the first report of successful electroporation transformation in lactococci using intact cells was published a year earlier (Harlander, 1987). Plasmids pSA3 and pGB301 were transferred into *L. lactis* ssp. *lactis* LM0230 with the efficiency from 10^2 up to 10^4 transformants/ μg DNA. In practice, there is a wide variation in electroporation efficiency existing between different lactococcal strains.

None-the-less, a useful range of lactic acid bacteria has been transformed successfully by electroporation and included amongst which are *L. lactis* ssp. *lactis* (Luchansky *et al.*, 1988; Powell *et al.*, 1988; Xu *et al.*, 1990), *L. lactis* ssp. *cremoris* (van der Lelies *et al.*, 1988), *S. thermophilus* (Somkuti and Steinberg, 1988), *Lactobacillus acidophilus*, *Lactobacillus casei*, *Enterococcus faecalis* and *Leuconostoc dextranicum* (Chassy and Flickinger, 1987; Luchansky *et al.*, 1988), *Leuconostoc paramesenteroides* (David *et al.*, 1989).

According to Harlander (1987) there are a number of important advantages offered with this electrical-pulse-induced transformation system. First, the high voltage pulse used generates an electroporation at both cell walls and membranes of host cells and therefore it is not necessary to prepare protoplasts. As a consequence osmotic stabilizing and toxic fusing agents, such as polyethylene glycol, are not required. Second, the recovery of the host cells takes place within 48 h rather than 5 to 10 days as required for protoplast regeneration. Finally, one can use only small amounts of DNA (of the order of nanogram quantities) and/or of average quality, such as from miniprep or ligation mixtures (refer section 2.7.6).

Because of its simplicity, universal applicability to all cell types and high transformation efficiency, electroporation has now become the most widely used genetic transfer technique (Miller *et al.*, 1988). It is now used routinely in lactococcal genetic research instead of protoplast transformation. Harlander (1987) has also proposed that the successful development of this technique and its application to lactococcal cultures has important implications for strain improvement and serves as the foundation for its application to other industrially important micro-organisms.

1.6 Lactococcal gene cloning

Although most of the earlier lactococcal genetic studies emphasized functional properties of their plasmids (Davies and Gasson, 1981; McKay, 1983), considerable progress in the development of gene cloning technology has been made in more recent years resulting in the establishment of gene cloning systems that are applicable to industrially important microorganisms. Since the first description of a lactococcal vector (Kok *et al.*, 1983), gene cloning has become an alternative strategy to more traditional genetic transfer techniques.

Application of gene cloning technology to lactococci can be directed either to the improvement of their existing properties or to the construction of novel strains capable of efficiently producing new dairy or non-dairy products. The first option provides a means whereby existing classical strain improvement methods may be complemented

to enable either the establishment of favourable traits or the abolition of undesired properties and as well, the stabilization of desired characteristics. The second option is possibly an alternative use for lactococci where they may be regarded as producers of new products such as heterologous proteins (i.e., non-dairy products). In this latter application it would be appropriate to compare the efficacy of lactococci with that of the established, genetically well-studied production organisms such as *E. coli*, *B. subtilis* or yeast (De Vos, 1986c). Efficient gene replication and gene expression are prerequisites for the application of gene cloning technology in lactococci host-vector systems.

1.6.1 Host-Vector systems

Since the first report on a successful plasmid transformation protocol (Kondo and McKay, 1982) several host-vector systems have been developed which allow various cloning and expression studies in lactococci (De Vos, 1987). In an ideal host-vector system, efficient transfer construct stability, optimal expression and non-interference of other host systems of the cloned DNA is necessary. Therefore, host functions and the properties of vector interactions have to be defined to achieve compatibility (i.e., an ability to replicate in the host cells and the ability to express the cloned DNA).

1.6.1.1 Lactococci host strains

A suitable host is a basic requirement for those gene cloning strategies which will allow transfer, stability and efficient expression of the cloned DNA. In general, host strains originating from related genera and species are found to be the most appropriate to meet these requirements. As mentioned earlier all strains of lactococci investigated harbour a characteristic large number of different plasmids (Davies and Gasson, 1981; McKay, 1983; Gasson and Davies, 1984). In gene cloning and other DNA-transfer experiments where plasmids are used, the presence of resident plasmids in a host strain can lead to difficulties in interpreting transfer results. In addition, if the transferred plasmid belongs to the same incompatibility group as one of the resident plasmids, the final result may be the curing of any one of them with perhaps the loss of the desired plasmid (De Vos, 1986c). For these reasons most plasmid transfer experiments have been performed using

plasmid-free host strains which have been constructed by mean of repeated plasmid curing experiments. In this regard the most widely used host strains are derivatives of the *L. lactis* ssp. *lactis* NCDO712 and its derivatives ML3 and C2 (Davies *et al.*, 1981; Gasson and Anderson, 1985). A number of plasmid-free derivatives of all three main species of lactococci are now available and are listed in Table 5.

Among the plasmid-free lactococcal strains, the plasmid-free derivatives from *L. lactis* ssp. *lactis* generally serve as better host strains than those from plasmid-free derivatives of *L. lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* var. *diacetylactis* (De Vos, 1987). The transformation of pGB301 (Kondo and McKay, 1984) into *L. lactis* ssp. *lactis* LM0230, a plasmid cured derivative of *L. lactis* ssp. *lactis* strain C2 (Efstathiou and McKay, 1977), produced a strain that has become a suitable host in recombinant DNA experiments and direct molecular cloning of plasmid DNA fragments among lactococci (dos Santos and Chopin, 1987). *L. lactis* ssp. *lactis* strain MG1363, a plasmid-free derivative of strain *L. lactis* ssp. *lactis* strain NCDO 712 has also proved particularly useful for transformation experiments (Gasson, 1983a; Kok *et al.*, 1984; von Wright *et al.*, 1985; Gasson and Anderson, 1985). In addition, successful transformations have been reported for the plasmid-free *L. lactis* ssp. *lactis* strain IL1403 (Chopin *et al.*, 1984) and strain H1-4125 (Davey and Pearce, 1982).

Plasmid-free strains can be used as recipients in genetic studies but are not suitable as commercial starter strains since they have lost their essential metabolic plasmids. However, their use in gene cloning studies offers two additional and important advantages. First, incompatibility problems are avoided which may arise when the plasmid vectors used are related to a resident plasmid. Second, in some cases restriction-modification systems which are widely distributed in lactococci, are plasmid-encoded (Klaenhammer, 1987) and may cause degradation of transformed DNA in the recipient. Homologous recombination with newly introduced DNA containing DNA sequences common to host plasmid DNA sequence is also avoided. Elimination of these systems thus greatly reduces the chance of loss of the incoming DNA in the cloning experiment (Chopin *et al.*, 1984).

Table 5 : Lactic streptococci strains which have been used (*) or have potential to be used as host in gene cloning experiments^b.

Parent strain	Derivative	Relevant phenotype	References
<i>S. lactis</i> ssp. <i>lactis</i>			
C2	LM0230 ^a	PF Lac ⁻	Efstathiou and McKay, 1977
714	MG1363 ^a	PF Lac ⁻	Gasson, 1983a
IL594	IL1403 ^a	PF Lac ⁻	Chopin <i>et al.</i> , 1984
H1	H1-4125	PF Lac ⁻	Davey and Pearce, 1982
ML3	MMS36	Rec ⁻ Lac ⁻	Anderson and McKay, 1983
<i>S. lactis</i> ssp. <i>diacetylactis</i>			
BU2	BU2-60	PF Lac ⁻	Neve <i>et al.</i> , 1984
176 ^b	ND	PF Lac ⁻	Gasson <i>et al.</i> , 1987
<i>S. lactis</i> ssp. <i>cremoris</i>			
4802	4843	PF Lac ⁻	Davey <i>et al.</i> , 1986
4803	4847	PF Lac ⁻	Davey <i>et al.</i> , 1986
1200 ^b	ND	PF Lac ⁺	Gasson and Davies, 1984
4365	4510	PF Lac ⁻	Limsowtin <i>et al.</i> , 1986
4358	4508	PF Lac ⁻	Limsowtin <i>et al.</i> , 1986
PF = plasmid-free; ND = not described; ^a = NCDO strain; Lac ⁻ = lactose-deficient; Rec ⁻ = recombinant-deficient.			
^b According to De Vos, 1987			

1.6.1.2 Lactococcal vectors

Major progress in both construction and application of lactococcal vectors has been made since the first description of a lactic streptococcal vector (Kok *et al.*, 1983). Vectors can now be divided into four classes according to their origin and construction.

The first class of vectors was constructed from the plasmids pIP501 (30 kb) and pAMB1 (26 kb), both members of a family noted for macrolide lincosamides (MLS) resistance. Both are low copy number, broad host-range conjugal plasmids which have been disseminated into various Gram-positive species (Hershfield, 1979). They do not contain Gram-negative replicons and therefore cannot replicate in *E. coli*. Deletion derivatives of these two plasmids resulted initially in the construct of pGB301 (Behnke *et al.*, 1981) and pHV1301 (Chopin *et al.*, 1986), respectively. These plasmids replicate in *S. sanguis* and *B. subtilis*. Both derivatives retained the ability to replicate and express the erythromycin resistance determinant, (Em^r), in lactococci (De Vos, 1987).

The second class of vectors is comprised of the *Escherichia coli*-*Streptococcus* shuttle plasmids. These were constructed by combining 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), pVA838 (Macrina *et al.*, 1982); pVA891 (Macrina *et al.*, 1983) and the pMU series (Achen *et al.*, 1986). These vectors can replicate in both *E. coli* and lactococci but their transformation efficiencies are relatively low in lactococci as compared with efficiencies in *E. coli*. Gene cloning studies with these vectors usually involve cloning in *E. coli* or *Streptococcus sanguis* followed by the introduction of the recombinant DNA into lactococci.

Members of the third class of vectors are those derived from small cryptic lactococcal plasmids as exemplified by pFX1 which was the first to be developed. These plasmids do not contain easily selectable markers such as antibiotic resistance genes. Therefore, two approaches have been used to convert them into useful plasmid vectors. The first approach was to introduce an antibiotic resistance selectable marker derived from Gram-positive, high copy number plasmids (Kok *et al.*, 1984). This resulted in the

construction of pGK12 and its derivative, pGKV2 (Kok, 1985; van der Vossen *et al.*, 1985) and which are based on the *L. lactis* ssp. *cremoris* pWV01 (Otto *et al.*, 1982) and pCK1 and its derivatives (Anderson and Gasson, 1985) which are based on the *L. lactis* ssp. *lactis* pSH71 (Gasson, 1983a). The second approach used was to randomly select restriction fragments from various lactococci plasmids that had the ability to sustain replication of a selected DNA fragment containing two antibiotic resistance genes. The most versatile vector resulting from this approach appeared to be pNZ12 which contained a 1.7-kb fragment from pSH71 (De Vos, 1986a). Since it has been shown that pWV01 was able to replicate in *B. subtilis* (Vosman and Venema, 1983), all initial vector constructions were performed in this Gram-positive model host, which has the advantage of being an efficient plasmid transformation system (Chang and Cohen, 1979). Subsequent studies showed that the similar sized pWV01 and pSH71 plasmids have comparable physical maps and have sequences in common (De Vos, 1986a). Therefore, it might be speculated that they belong to a family of plasmids which are present in both *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* (De Vos, 1987).

However, in contrast to the plasmids pCK1 and pNZ12 (Anderson and Gasson, 1985; De Vos, 1986c), all pWV01 derivatives have a low copy-number in *L. lactis* ssp. *lactis*, indicating that subtle differences may exist between the two replicons. A unique property, which discriminates this class of vectors from the first class of vectors (based on pIP501 and pAMB1) is the ability of pWV01 and pSH71 derivatives to replicate not only in Gram-positive bacteria but also in some strains of *E. coli* as first reported for the plasmid pGK12 (Kok *et al.*, 1984). The broad host-range of these vectors is of particular value for two reasons. First, the introduction of a Gram-negative bacterium's replicon becomes unnecessary. This makes *E. coli* an intermediate host for transformation in which several alternative cloning strategies can be used. Second, it allows the investigation of the lactococcal replication requirements in *E. coli* and *B. subtilis* (De Vos, 1987). To date, replication of pNZ12 has been observed in *E. coli* and many Gram-positive bacteria including *L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *L. lactis* ssp. *lactis* var. *diacetylactis*, *B. subtilis*, *Staphylococcus aureus* and also *Lactobacillus casei* (De Vos, 1986b; 1987).

Members of the fourth class of vectors were not designed for use as general cloning vectors, but for other kinds of genetic studies in lactococci. These are promoter-probe vectors i.e. promoter-detecting *E. coli*-*L. lactis* shuttle vector for identifying DNA sequences which regulate gene expression, e.g. pGKV110, pGKV210 (van der Vossen *et al.*, 1985), pNZ220 (De Vos, 1987), 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 Glutche *et al.*, 1989); the terminator-detecting vector, pGKV259 (van der Vossen *et al.*, 1987) and the chromosome integration vector pHV60 (Leenhouts *et al.*, 1989).

Most of these vectors are based on the promoter-less chloramphenicol-acetyl-transferase (CAT) gene derived from *B. pumilus* (Williams *et al.*, 1981). This CAT gene, preceded by a polylinker containing multiple cloning sites, was introduced into those lactococcal cloning vectors based on pWV01 and pSH71 and resulted in pGKV110 and pNZ220, respectively.

Moreover, with the broad host-range of the employed replicons these vectors have been used to isolate, identify and evaluate the expression signals in both homologous hosts, e.g. *L. lactis* ssp. *lactis* and heterologous hosts, e.g. *E. coli* and *B. subtilis*.

A second generation of cloning vectors as exemplified by the first of its kind, pFX1, has been developed by inserting DNA fragments containing multiple cloning sites. For example, pNZ17 and pNZ18 are based on the plasmids pNZ121 and pNZ12 (Simon *et al.*, 1985) and another pIL204, was derived from pHV1301 (Simon and Chopin, 1988).

These vectors offer more cloning flexibility than previously available. The useful lactococcal plasmid vector pFX3 (4.3kb) was also constructed by inserting multiple cloning sites and a selectable marker from the *E. coli lacZ* gene, viz the chloramphenicol resistance determinant, Cm^r, as well as T3 and T7 promoters (Xu *et al.*, 1990).

1.6.2 Lactococcal gene cloning and studies

Many lactococcal genes of both plasmid and chromosomal origin have been cloned and a number sequenced. The information is summarized in Table 6 which shows data for: cell-wall associated proteinases (Kok *et al.*, 1985; Gasson *et al.*, 1987; von Wright *et al.*, 1987; Kok *et al.*, 1988; Kiwaki *et al.*, 1989; Haandrikman *et al.*, 1989; Vos *et al.*, 1989; Xu *et al.*, 1990); genes involved in lactose metabolism, *lac* genes (Harlander *et al.*, 1984; Kondo and McKay, 1984); phospho- β -galactosidase, *pbg* (Maeda and Gasson, 1986; De Vos, 1986a; Inamine *et al.*, 1986; Boizet *et al.*, 1988); genes coding for D-tagatose 1,6-bis-phosphate aldolase, (Limsowtin *et al.*, 1986); *lacG* (De Vos and Gasson, 1989); the repressor gene of the Lac-PTS system, *lacR* (van Rooijen and De Vos, 1990); the genes coding for the lactose specific enzymes and phospho- β -galactosidase, *lacFEGX* (De Vos *et al.*, 1990); the entire Lac-PTS operon, *lacABCD FEGX* (van Rooijen *et al.*, 1991); bacteriophage resistance, *hsp* (Hill *et al.*, 1989; Hill *et al.*, 1990); phage repressor, *bpi* (Lakshmidēvi *et al.*, 1990); phage lysin (Shearman *et al.*, 1989); citrate permease gene, *citP* (David *et al.*, 1990); a small protein antibiotic nisin gene, *spaN* (Buchman *et al.*, 1988); the nisin gene (Kaletta and Entian, 1989); a bacteriocin production gene, Bac (van Belkum *et al.*, 1989); a nisin resistance determinant, Nis^r (Froseth *et al.*, 1988; Froseth and McKay, 1991), etc.,

The translation initiation signal sequences, identified by promoter-probe vectors, have provided an understanding of gene transcription in lactococci. These promoter sequences have the following features: the presence of typical canonical -35 and -10 sequences which are also observed in other eubacteria promoter consensus sequences (Hawley and McClure, 1983; Harley and Reynold, 1987); -16-18 bases spacings between the hexanucleotides of the promoter; a ribosomal binding site sequences which exhibits complementarity with the 3' end (3'-UCUUUCCUCCA-5') of lactococcal 16S ribosomal RNA (Ludwig *et al.*, 1985); a high incidence of TG preceding the -10 sequence and AT-rich sequences upstream of the -35 sequence (AT-box) within the range of 64% to 78%. These promoter sequences are followed by a ribosomal binding site, a translational initiation codon (ATG) and an open reading frame (ORF).

Table 6 : Summary of some of the lactococcal genes cloned in both lactococcal hosts and heterologous hosts.

Gene	Origin (plasmid)	Host for cloning	Vector	References
lac*	<i>S. lactis</i> C2 (pLM2001)	LM0230	pGB301	Kondo and McKay, 1984
pbg	<i>S. lactis</i> 712 (pLP712)	<i>S. sanguis</i>	pDB101	Harlender <i>et al.</i> , 1984
		<i>E. coli</i>	pAT153	Maeda and Gasson, 1986
		MG1363	pNZ12	De Vos, 1986a
		<i>E. coli</i>	pNZ12	De Vos, 1986a
pbg	<i>S. cremoris</i> H2 (pJI70)	<i>B. subtilis</i>	pNZ12	De Vos, 1986a
		<i>E. coli</i>	pUC18/19	Inamine <i>et al.</i> , 1986
lacG	<i>L. lactis</i> ssp. <i>lactis</i> NCDO712 (pMG820)	<i>E. coli</i> MG1363	pNZ12	De Vos and Gasson, 1989
pbg T6A	<i>S. lactis</i> Z268 <i>S. lactis</i> 4122 (pDI1)	<i>E. coli</i>	pUC18	Boizet <i>et al.</i> , 1988
		<i>E. coli</i>	pACYC184	Limsowtin <i>et al.</i> , 1986
lacABCD	<i>lactis</i> MG1820 (pMG820)	<i>E. coli</i> MG1363	pUC18/pET8c pNZ12/pIL305	van Rooijen <i>et al.</i> , 1991
prt	<i>S. cremoris</i> Wg2 (pWV05)	MG1363	pGKV2	Kok <i>et al.</i> , 1985
		<i>B. subtilis</i>	pGKV2	Kok <i>et al.</i> , 1985

Abbreviations: lac*=lactose PTS gene cluster encoding enzyme II, factor III and 6-phospho- β -D-galactosidase; pbg,lacG=phospho- β -galactosidase; lacFEGX=lactose specific enzymes of the Lac-PTS; lacABCD=tagatose 6-phosphate gene cluster; lacR=repressor gene of Lac-PTS; prt=proteinase; UV^R=resistance to UV irradiation; Tc^r=resistance to tetracycline; T6A=tagatose-1,6-bisphosphate aldolase; lacZ= β -D-galactosidase; PC=prochymosin; citP=citrate permease; thyA=thymidilate synthase; lcnA=lactococcin A; ldh=lactate dehydrogenase; Nis^r=nisin resistance; Bac=Bacteriocin producing gene; spaN=Small Protein Antibiotic Nisin gene; lysin=bacteriophage lysin gene.

Table 6 (continued) : Summary of some of the lactococcal genes cloned in both lactococcal hosts and heterologous hosts.

Gene	Origin (plasmid)	Host for cloning	Vector	References
<i>prt</i>	<i>S. cremoris</i> SK11 (pSK11)	MG1363	pNZ121	De Vos, 1986b
		<i>B. subtilis</i>	pNZ121	De Vos, 1986b
		<i>E. coli</i>	φEMBL3	De Vos, 1986b
<i>prt</i>	<i>S. lactis</i> NCDO 712 (pPL712)	MG1363	pCK1	Gasson <i>et al</i> 1987
		<i>B. subtilis</i>	pCK1	Gasson <i>et al.</i> , 1987
<i>prt</i>	<i>S. lactis</i> ssp. <i>lactis</i> SSL135	MG1614	pVS2	von Wright <i>et al.</i> , 1987
<i>prt</i>	<i>S. cremoris</i> Wg2 (pGKV500)	<i>E. coli</i>	M13mp10/11	Kok <i>et al.</i> , 1988
<i>prt</i>	<i>L. lactis</i> ssp. <i>cremoris</i> H2 (pDI21)	<i>E. coli</i> pop-13	λNM1149	Xu <i>et al.</i> , 1990
		4125		
Nis ^r	<i>S. lactis</i> ssp. <i>diacetylactis</i> DRC3 (pNP40)	<i>E. coli</i>	pSA3	Froseth <i>et al.</i> , 1988
<i>spaN</i>	<i>S. lactis</i> ATCC 11454	<i>E. coli</i>	λJ1	Buchman <i>et al.</i> , 1988
Bac	<i>L. lactis</i> ssp. <i>cremoris</i> 9B4 (p9B4-6)	<i>E. coli</i>	pWV01	van Belkum <i>et al.</i> , 1989

Abbreviations: *lac**=lactose PTS gene cluster encoding enzyme II, factor III and 6-phospho-β-D-galactosidase; *pbg*,*lacG*=phospho-β-galactosidase; *lacFEGX*=lactose specific enzymes of the Lac-PTS; *lacABCD*=tagatose 6-phosphate gene cluster; *lacR*=repressor gene of Lac-PTS; *prt*=proteinase; UV^R=resistance to UV irradiation; Tc^r=resistance to tetracycline; T6A=tagatose-1,6-bisphosphate aldolase; *lacZ*=β-D-galactosidase; PC=prochymosin; *citP*=citrate permease; *thyA*=thymidilate synthase; *lcnA*=lactococcin A; *ldh*=lactate dehydrogenase; Nis^r=nisin resistance; Bac=Bacteriocin producing gene; *spaN*=Small Protein Antibiotic Nisin gene; lysin=bacteriophage lysin gene.

Table 6 (continued): Summary of some of the lactococcal genes cloned in both lactococcal hosts and heterologous hosts.

Gene	Origin (plasmid)	Host for cloning	Vector	References
repA	<i>S. lactis</i> NCDO 712 (pSH71)	<i>E. coli</i>	pUC9	De Vos, 1986a
UV ^R	<i>S. lactis</i> IL594 (pIL7)	IL1403	pHV1301	Chopin <i>et al.</i> , 1986
Tc ^R	<i>S. lactis</i> IL1458 (Tn916)	IL1403	pIL204	dos Santos and Chopin, 1987
lacZ	<i>E. coli</i>	MG1363	pNZ17	De Vos and Simons, 1988
PC	bovine	MG1363	pNZ12	Simons and De Vos, 1987
citP	<i>L. lactis</i> ssp. <i>lactis</i> 176 (pCT176)	<i>E. coli</i>	pBR328	David <i>et al.</i> , 1990
thyA	<i>L. lactis</i> ssp. <i>lactis</i>	<i>E. coli</i>	pLAFR1	Ross <i>et al.</i> , 1990
lcnA	<i>L. lactis</i> ssp. <i>cremoris</i> LMG2130 IL1403 (55kb plasmid)	<i>E. coli</i> IL1403	pUC18 pIL253::pUC18	Holo <i>et al.</i> , 1991
ldh	<i>L. lactis</i> ssp. <i>cremoris</i> US3	<i>E. coli</i>	λGEM-11	Llanos <i>et al.</i> , 1992
lacR	<i>L. lactis</i> ssp. <i>lactis</i> MG1820 (pMG820)	<i>E. coli</i> MG1363	pUC18 pNZ18	van Rooijen and De Vos, 1990
lacFEGX	<i>L. lactis</i> ssp. <i>lactis</i> MC1820 (pMG820)	<i>E. coli</i> MC1061 MG1363	pUC18/19 pNZ12/pIL305	De Vos <i>et al.</i> , 1990
lysin	<i>L. lactis</i> ssp. <i>lactis</i> NCDO1200	MG1363 <i>E. coli</i>	λgt10	Shearman <i>et al.</i> , 1989

Abbreviations: lac*=lactose PTS gene cluster encoding enzyme II, factor III and 6-phospho-β-D-galactosidase; pbg,lacG=phospho-β-galactosidase; lacFEGX=lactose specific enzymes of the Lac-PTS; lacABCD=tagatose 6-phosphate gene cluster; lacR=repressor gene of Lac-PTS; prt=proteinase; UV^R=resistance to UV irradiation; Tc^r=resistance to tetracycline; T6A=tagatose-1,6-bisphosphate aldolase; lacZ=β-D-galactosidase; PC=prochymosin; citP=citrate permease; thyA=thymidilate synthase; lcnA=lactococcin A; ldh=lactate dehydrogenase; Nis^r=nisin resistance; Bac=Bacteriocin producing gene; spaN=Small Protein Antibiotic Nisin gene; lysin=bacteriophage lysin gene

1.7 Carbohydrate metabolism in Lactic acid bacteria

Conversion of carbohydrates to lactate by lactic acid bacteria is considered to be the most important fermentation process employed in food processing industries. The term "lactic acid bacteria" is used as a collective name for those bacteria which produce lactate from glucose (Garvie, 1984). There are two kinds of mechanisms found: firstly, homofermentative conversions by which lactate is the sole product and secondly, heterofermentative systems where other products, mainly acetate and CO₂ are formed with lactate. Lactococci are usually homofermentative if presented with hexose phosphates with the gluco-configuration. However, species and strains differ among each other in the mechanisms used for the fermentation of other carbohydrates such as lactose. This leads to quite different sets of end-products. [see the publications of Kandler, 1983; Hall and Franks, 1985 for a more detailed descriptions of the metabolic pathways functioning in these bacteria].

1.7.1 Lactose

Lactose is a common carbohydrate substrate and is known as either milk or mammalian sugar because of its abundance [5-5.5% (w/v)] in secretions from the mammary gland. Lactose is a disaccharide consisting of a D-glucose and a D-galactose linked by a β -1,4 glycosidic bond. It is also found in many by-products of dairy processes since milk is the principal raw material used. Enzymatic hydrolysis of lactose with β -galactosidase yields the two hexose constituents, D-glucose and D-galactose which are themselves useful in confectionary and pharmaceutical industry applications (Prentice and Neaves, 1986). The bioconversion of lactose in milk to lactic acid by lactic starter cultures is a vital dairy fermentation by which the lowered pH precipitates, the milk's protein, the curd, which is used in other processes.

1.7.2 Lactose utilization pathways in microorganisms

Microorganisms can utilize lactose by either or both of two metabolic pathways. The first of these is found commonly in Gram-negative and some Gram-positive bacteria and

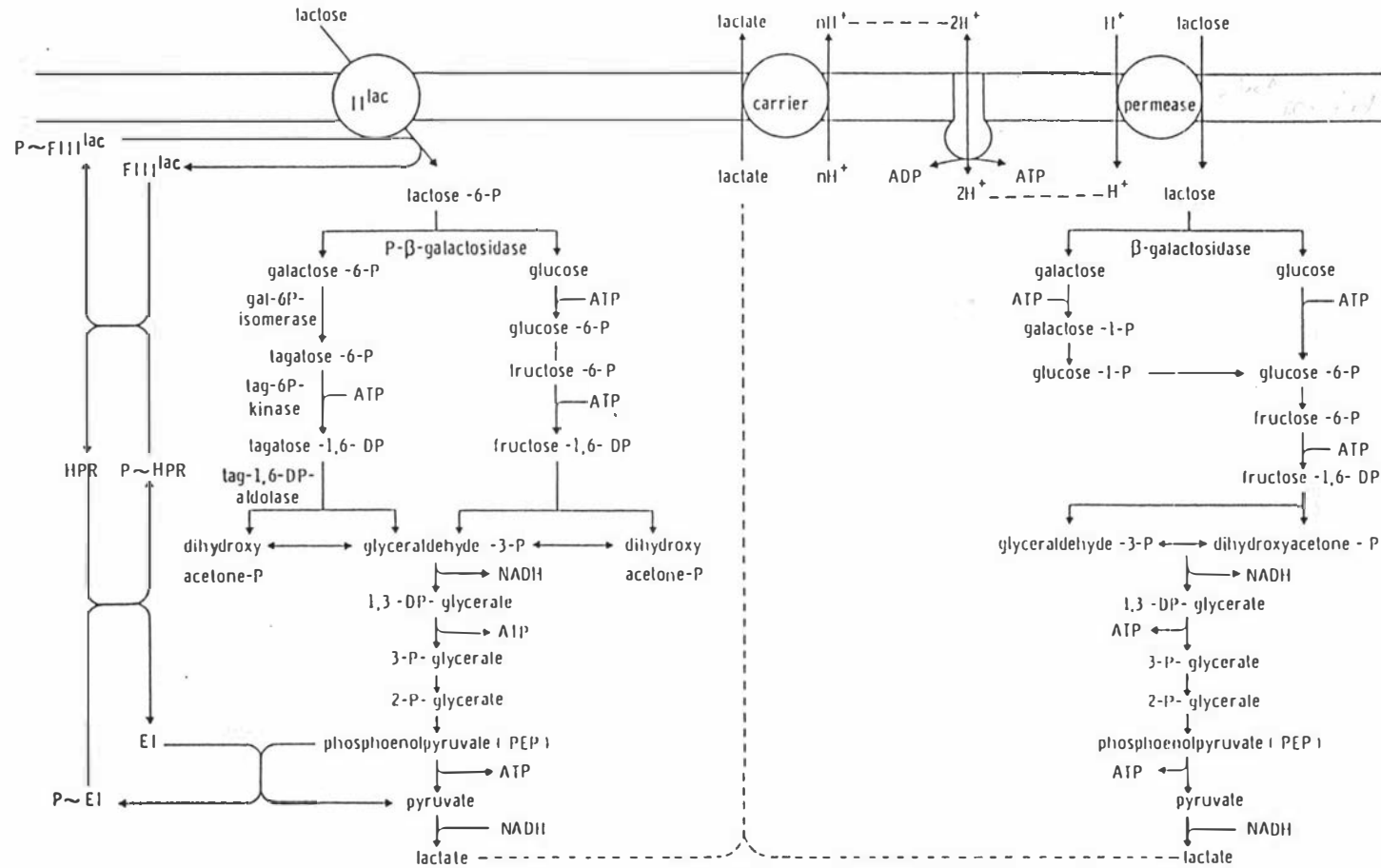
involves lactose permease and β -galactosidase. The second pathway is known as the phosphoenol-pyruvate-dependent phosphotransferase (PEP:PTS) lactose system and is widely distributed among Gram-positive bacteria (McKay *et al.*, 1970; Dills *et al.*, 1980; Saier, 1985).

1.7.3 Lactose metabolism in lactococci

Lactose metabolism is one of the major metabolic traits of lactococci and the conversion of lactose of milk into lactic acid is vital for the dairy fermentation. Under normal growth conditions lactococci are homolactic fermentors and obtain their energy by converting around 95% of incoming carbohydrate into lactic acid (Thomas, 1976). They contain no cytochromes so that energy production depends entirely on substrate level phosphorylation (Hall and Franks, 1985). A rapid homolactic fermentation of milk lactose is the principal requirement of lactococci used as lactic starters (Crow and Thomas, 1984).

1.7.4 Lactose transport system

The mesophilic (*L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*) and thermophilic (*S. thermophilus*) lactococci are the common dairy lactics used in industrial dairy fermentations. They contain two different lactose transport systems characterised by two distinct lactose hydrolysing enzymes, phospho- β -galactosidase (P- β -Gal, EC 3.2.1.85) and β -galactosidase (β -Gal, EC 3.2.1.23), respectively (Law and Sharpe, 1978; McKay, 1982; Konings and Otto, 1983; De Vos and Simons, 1988). The biochemical pathways involved (Figure 1) in lactose transport and degradation have been elucidated in these bacteria. The phospho- β -galactosidase catalyses hydrolysis of lactose after it has been translocated by the so-called "lactose phosphoenolpyruvate (PEP) dependent phosphotransferase system (PTS), whereas the β -galactosidase catalyses hydrolysis after the lactose has been transported via the lactose permease system (PS).



1.7.5 Biochemistry of lactose utilization using the PEP:PTS system

Lactose utilization by the PEP:PTS system has been shown to operate only in Gram-positive bacteria such as *Staphylococcus aureus* (Hengstenberg *et al.*, 1969), *L. lactis* ssp. *cremoris* (McKay *et al.*, 1970; Okamoto and Morichi, 1979), *S. faecalis* (Okamoto and Morichi, 1979), *L. lactis* ssp. *lactis* (Farrow, 1980), *S. mutans*; *S. salivarius* (Hamilton and Lo, 1978), *S. thermophilus* (Herman and McKay, 1986), *Lactobacillus casei* (Premi *et al.*, 1972), and *Lactobacillus acidophilus* (Fisher *et al.*, 1985). In this system, lactose is transported into the cells as lactose phosphate (Postma and Lengeler, 1985). It was first described in *Staphylococcus aureus* (Hengstenberg *et al.*, 1970; Simoni *et al.*, 1973). Translocation of lactose through the cell membrane requires energy from the high energy phosphate bond in phosphoenol pyruvate (PEP). To catalyze the transfer of the phosphoryl group from PEP to lactose, four proteins are required (Simoni *et al.*, 1968). Enzyme I and HPR (histidine-containing phosphocarrier protein) are soluble cytoplasmic components which are synthesised constitutively and are necessary for phosphorylation of many sugar substrates of the PTS system (Hays *et al.*, 1973; McKay *et al.*, 1969). A catalytic function of a lactose-specific membrane-associated protein, enzyme II^{lactose} is responsible for lactose recognition and lactose binding (Schafer *et al.*, 1981). The lactose-specific soluble cytoplasmic protein, enzyme III^{lactose} serves as an immediate phosphate donor to lactose in the presence of enzyme II^{lactose} (Nakazawa *et al.*, 1971; Deutscher *et al.*, 1982). Enzyme II^{lactose}, enzyme III^{lactose}, and phospho- β -galactosidase are synthesized after induction of the system. The lactose phosphate formed is then hydrolyzed by P- β -gal (Johnson and McDonald, 1974), resulting in glucose and D-galactose 6-phosphate (Hengstenberg *et al.*, 1970; Thompson, 1979). Glucose is subsequently metabolized via the glycolytic pathway (Embden-Myerhof-Parnas pathway) to lactic acid (Bissett and Anderson, 1973). D-galactose 6-phosphate is converted to D-tagatose 6-phosphate by the enzyme D-galactose 6-phosphate isomerase, then further phosphorylated by the enzyme D-tagatose 6-phosphate kinase to D-tagatose 1,6-bisphosphate which is then split to two triose phosphate molecules by the enzyme D-tagatose 1,6-bisphosphate aldolase. Thus, D-galactose 6-phosphate is metabolized via the tagatose bisphosphate pathway (Bissett and Anderson, 1974; Dills *et al.*, 1980; Saier, 1985).

The regulation and expression of the genes involved in lactose utilization in these bacteria is complex. Glucose, at least, partially represses expression of the lactose utilizing genes, even though the bacteria are reported to lack cAMP. Involvement of cAMP in catabolite repression is unlikely as it is not found in physiologically significant amounts in Gram-positive bacteria (Blumenthal, 1972; Botsford, 1981).

In *Staphylococcus aureus*, the chromosomally located phospho- β -galactosidase and determinants for PEP:PTS enzyme II^{lactose} and enzyme III^{lactose} are linked (Hengstenberg *et al.*, 1970). Constitutive mutants have shown coordinated increased activities of the three proteins (Morse *et al.*, 1968). The expression appears to be subjected to catabolic repression (McClatchy and Rosenblum, 1963). The preferred inducer for the system is galactose-6-phosphate not IPTG (isopropyl- β -D-thiogalactoside) a potent inducer of the *E. coli lac* operon (Morse *et al.*, 1968; Hengstenberg *et al.*, 1969).

The PEP:PTS system in lactococci appears to be analogous to that in *Staphylococcus aureus*. Enzymes involved in the pathway are found to be plasmid-encoded which can be transferred by conjugation and transduction (Anderson and McKay., 1977; Chassy *et al.*, 1978; Gasson and Davies, 1980a; Lee *et al.*, 1982; Crow *et al.*, 1983; McKay, 1983; Inamine *et al.*, 1986). In *L. lactis* ssp. *lactis* expression of the genes is induced by lactose or galactose and repressed by glucose (McKay *et al.*, 1970; Bissett and Anderson, 1974). In *Lactobacillus casei*, a β -galactoside is required for induction of the *lac* PEP:PTS and *lac* genes. Neither galactose nor galactose-6-phosphate is able to induce expression (Lee *et al.*, 1982).

1.7.6 Biochemistry of lactose utilization using the lactose permease system

The lactose permease system is a membrane located transport mechanism in which the lactose permease is linked to a proton motive force (pmf) generated by an ATP dependent membrane bound ATPase (Maloney, 1982; Maloney and Hansen, 1982). Therefore, lactose is brought into the cells as free sugar without phosphorylation and hydrolyzed into D-glucose and D-galactose by the enzyme β -galactosidase. Lactose may be transported in symport with a proton as has been found in *L. lactis* ssp. *lactis*

(Kashket and Wilson, 1972). Efflux of lactate is accompanied by the extrusion of protons and may generate a proton-motive-force as reported for *L. lactis* ssp. *cremoris* (Konings and Otto, 1983). As depicted in Figure 1, the resulting D-galactose is converted to glucose 6-phosphate by the Leloir pathway and together with the resulting glucose moiety of lactose, is metabolized to lactate via the Embden-Myerhof-Parnas pathway (Kandler, 1983, De Vos and Simons, 1988).

1.7.7 Lactose hydrolysing enzymes in lactococci

Lactose can be metabolized by two different enzymes, β -galactosidase and phospho- β -galactosidase. The distribution of lactose hydrolysing enzymes in lactococci has been surveyed in the past (Citti *et al.*, 1965; McKay *et al.*, 1970; Okamoto and Morichi, 1979; Somkuti and Steinberg, 1979; Farrow, 1980; Smart *et al.*, 1993) and it has been found that most of the wildtype mesophilic lactococci and the thermophilic strains possess high β -gal activity while mesophilic dairy lactic starters possess high P- β -gal activity. Lactose permease and β -galactosidase are repressed in streptococci and only function in strains lacking the Lac-PTS system. However, the rates of lactose fermentation and growth are significantly lower in such strains (Thomas, 1976; Thompson and Thomas, 1977). This general observation correlates with the suggestion that the presence of a lactose PTS and a P- β -gal is a prerequisite for the rapid homolactic fermentation of lactose by the dairy lactic starters (Lawrence and Thomas, 1979). Wildtype strains of lactococci are normally slow lactose fermentors. Lactose hydrolysis is mediated either by a β -gal as in the case of *L. lactis* ssp. *lactis* strain ATCC 7962 (Citti *et al.*, 1965), or by P- β -gal. However, in *L. lactis* ssp. *lactis* strain ATCC 7962 a low P- β -gal activity is rate limiting as a consequence of accumulated lactose-6-phosphate which might be deleterious to these bacteria (Crow and Thomas, 1984). It has been suggested that galactose-6-phosphate which is formed during degradation of either lactose or galactose is an inducer of the P- β -gal gene (McKay *et al.*, 1970; Cords and McKay, 1974) by analogy with the situation in *Staphylococcus aureus* (Morse *et al.*, 1968). Glucose appears to be a repressor of the synthesis of P- β -gal in many strains of lactococci (Maeda and Gasson, 1986). Both lactose and galactose induce the β -gal activity.

1.7.8 Molecular analysis of lactose hydrolysing genes of the Lac PEP:PTS system of Lactic Acid Bacteria.

The plasmid encoded P- β -gal genes have been cloned and expressed in both homologous (i.e. lactococcal hosts) and heterologous hosts (i.e. *E. coli* hosts) as listed in Table 7. Several other genes have also been cloned from the PEP:PTS systems of various Gram-positive bacteria. Lee *et al.*, (1982) and Porter and Chassy (1988) cloned the P- β -gal gene for *Lactobacillus casei* strain 64H which was expressed in *E. coli* and coded for a protein of M_r 43,000. Breidt and Stewart (1986) cloned the P- β -gal gene of *Staphylococcus aureus* which was found to possess a derepressed constitutive P- β -galactosidase due to the insertion of Tn915. Inamine *et al.* (1986) cloned the P- β -gal gene of *L. lactis* ssp. *cremoris* strain H2 and identified that the lac-PTS region was organised as an operon with a putative phospho- β -galactosidase regulatory region (lac-PTS genes) followed by the P- β -gal gene. The P- β -gal genes of *L. lactis* ssp. *lactis* strain NCDO 712 (Maeda and Gasson, 1986), strain Z268 (Boizet *et al.*, 1988) and of *L. lactis* ssp. *cremoris* strain H2 (Inamine *et al.*, 1986) have been cloned and expressed in *E. coli*. De Vos (1987) cloned the P- β -gal gene of strain NCDO 712 into the plasmid-free strain MG1363 derived from its original host. Using a homologous host-vector system they found that the gene was maintained stably in both high and low copy number vectors. It was observed that the only difference between the P- β -gal activities specified by the *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* Lac⁺ plasmids was the non-inducibility of the *L. lactis* ssp. *cremoris* P- β -gal activity (Inamine *et al.*, 1986). This is in contrast with the inducible phenotype of the *L. lactis* ssp. *lactis* strain NCDO 712 P- β -gal activity (Maeda and Gasson, 1986). The P- β -gal activity specified by the cloned genes in *E. coli* and *L. lactis* ssp. *lactis* was not inducible by lactose (Maeda and Gasson, 1986) and seemed to depend on the orientation of the inserted DNA (Inamine *et al.*, 1986). The level of expression (i.e. its specific activity) appeared to be much lower than that of the original Lac⁺ plasmid in *L. lactis* ssp. *lactis* (Maeda and Gasson, 1986; Inamine *et al.*, 1986). Based on the sequence it was suggested that the P- β -gal gene forms an operon-like structure with an intergenic promoter sequence immediately preceding the P- β -gal gene. The entire Lac-PTS operon (*lacR*, *lacFEGX* and *lacABCD*)

Table 7 List of cloned phospho- β -galactosidase genes in lactic acid bacteria

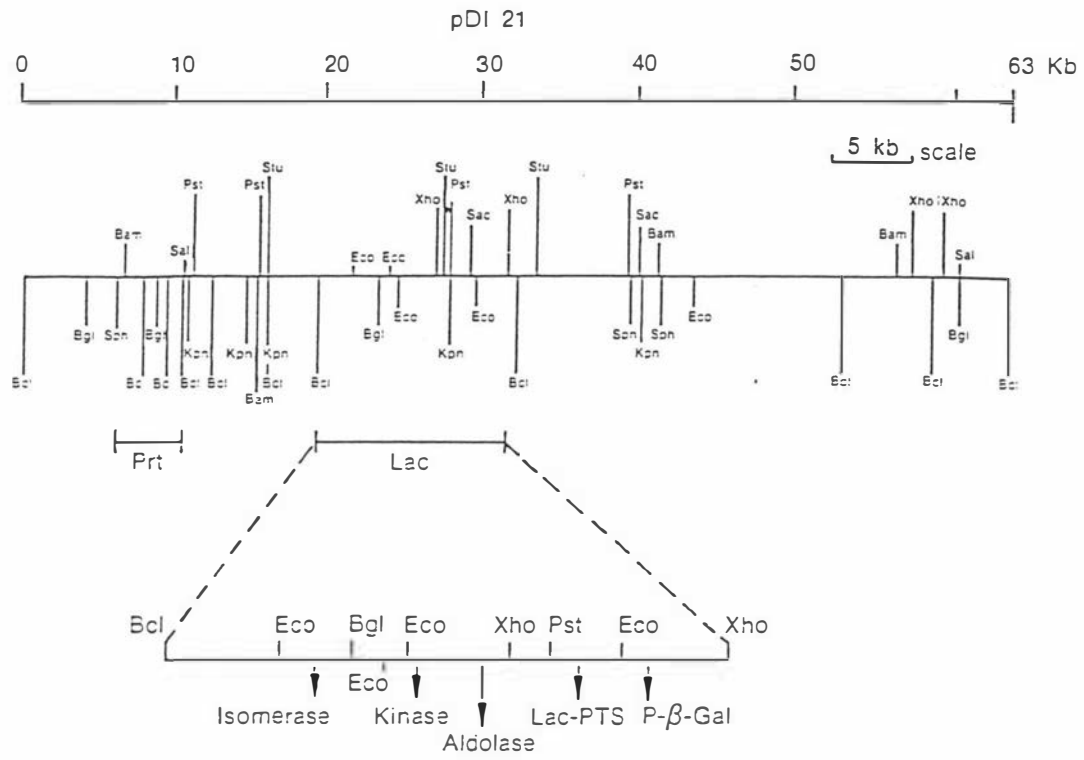
Bacterial strain	Source of DNA	Cloned fragment	Vector	Bacterial host	Reference
<i>Lactococcus lactis</i> ssp. <i>lactis</i> LM0232	Plasmid pLM2001	23 kb <i>Kpn</i> I	pDB101	<i>S. sanguis</i> Challis	Hardender <i>et al.</i> , 1984
<i>Lactococcus lactis</i> ssp. <i>lactis</i> NCDO712	Plasmid pMG820	10.4 kb <i>Pst</i> I	pAT153	<i>E. coli</i> HB101	Maeda and Gasson, 1986
<i>Lactococcus lactis</i> ssp. <i>lactis</i> NCDO712	Plasmid pMG820	4.4 kb <i>Xho</i> I	pNZ12	<i>E. coli</i> MC1061	De Vos and Gasson, 1989
<i>Lactococcus lactis</i> ssp. <i>lactis</i> Z268(L13)	Plasmid pUCL13	4.4 kb <i>Xho</i> I	pAT153	<i>E. coli</i> C600	Boizet <i>et al.</i> , 1988
<i>Lactococcus lactis</i> ssp. <i>cremoris</i> H2	Plasmid pJI70	3.5 kb <i>Ava</i> I	pUC19	<i>E. coli</i> TB1	Inamine <i>et al.</i> , 1986
<i>Lactobacillus casei</i> 64H	Plasmid pLZ64	7.9 kb <i>Pst</i> I	pBR322	<i>E. coli</i> X1849	Lee <i>et al.</i> , 1982

of *L. lactis* ssp. *lactis* MG1820 has been cloned and sequenced (van Rooijen and De Vos, 1990; De Vos *et al.*, 1990; van Rooijen *et al.*, 1991). Transcription studies showed that *lacABCD* are the first four genes of the entire lactose-inducible-Lac-PTS operon of *L. lactis* ssp. *lactis* MG1820 consisting of *lacABCDFEGX* in order. The *lacR* gene encodes the repressor of the Lac-PTS system, *lacFEGX* encodes for Enzyme III^{lac} (Factor III), Enzyme II^{lac}, phospho- β -galactosidase, and protein X of unknown function, respectively. *LacABCD* encodes a tagatose 6-phosphate pathway gene cluster; *lacAB* encodes the multimeric galactose-6-phosphate isomerase (15 and 19-kDa subunits), *lacC* encodes the tagatose-6-phosphate kinase (33-kDa) and *lacD* encodes the 36-kDa of tagatose-1,6-diphosphate aldolase (van Rooijen *et al.*, 1991; De Vos *et al.*, 1990). Van Rooijen and colleagues suggested that the *lacABCD* genes evolved from a common ancestor because there is a basic homology existing between the deduced amino acid sequences of *L. lactis* ssp. *lactis* LacD and *Staphylococcus aureus* LacD (van Rooijen *et al.*, 1991), i.e. between the Gram-positive coccal bacteria. De Vos *et al.* (1990) suggested that the individual *lacF*, *lacE*, and *lacG* genes, and possibly other components of the *lac*-operon, have been acquired independently and/or have been reshuffled after acquisition, possibly in order to allow optimal gene expression and regulation.

1.7.9 Molecular analysis of Lactose permease/ β -galactosidase system

Comparisons have been made of the lactose permease system of lactococci with that of the well characterized *E. coli* system. The lactose permease system in *E. coli* is part of the *lac* operon (Jacob *et al.*, 1960; Jacob and Monod, 1961). There are four genes, *lacZYA* and *lacI* responsible for lactose utilization and they are located at 9 min on the *E. coli* map (Bachmann, 1990). Three of these structural genes (*lacZ*, *lacY*, and *lacA*) constitute a polycistronic operon transcribed in the direction of *lacZ* to *lacA*. The *lacZ* codes for the β -galactosidase enzyme, *lacY* directs the synthesis of the hydrophobic lactose permease required for active transport of lactose into the cell (Rickenberg *et al.*, 1956), and *lacA* codes for the β -galactoside transacetylase, a detoxification enzyme regarded as nonessential for cell growth (Lederberg, 1950; Miller and Reznikoff, 1980). The *lacI* gene codes for the repressor, which is active in inhibiting *lac* transcription and is produced constitutively at a low rate from a separate promoter (Fox *et al.*, 1966). A

number of β -galactosidase genes have been cloned and some have been sequenced as listed in Table 8. The *lacZ* gene of *E. coli* has been sequenced (Kalnins *et al.*, 1983). Dickson and Markin (1978) cloned the β -galactosidase gene from *Kluyveromyces lactis* in *E. coli* and found that its structure and regulation was different to that of the native *E. coli* gene. The *lac* gene of *Klebsiella pneumoniae* (*K. pneumoniae lacZ*) has been cloned and sequenced by Buvinger and Riley (1985). Herman and McKay (1986) cloned the β -galactosidase gene, designated as *β -gal*, from *S. thermophilus* strain ATCC19258 in *E. coli*. This β -galactosidase gene was significantly different at the DNA level to the native β -galactosidase gene of *E. coli*. Schroeder *et al.* (1991) also cloned and sequenced the β -galactosidase gene, designated as *lacZ*, from *S. thermophilus* strain A054. The two cloned chromosomal fragments of these two strains of *S. thermophilus* (ATCC19258 and A054) showed physical similarities (i.e. restriction map and the deletion analyses). The lactose catabolic genes including β -galactosidase structural gene, the lactose permease, thiogalactoside transacetylase and the lactose repressor protein were also isolated from a genomic library of *S. bovis* constructed in bacteriophage λ 47.1 and subcloned into pBR322 and expressed in *E. coli* (Gilbert and Hall, 1987). Hirata *et al.* (1984) cloned two β -galactosidase genes from *B. stearothermophilus*. This bacterium possessed three β -galactosidase enzymes (β -galI, β -galII, β -galIII) the subunits of which were coded for by two β -galactosidase genes, *bgaA* for β -galII, β -galIII and *bgaB* for β -galI. These two genes are located very close to each other on the chromosome and differ from one another as well as from those of *E. coli*. Schmidt *et al.* (1989) cloned and sequenced the β -galactosidase gene, *lbu* from chromosomal DNA of *Lactobacillus bulgaricus* strain B131 into the vector pKK233-3. The deduced amino acid sequence encoded by the *lbu* exhibited an average homology to that of *E. coli*. Hancock *et al.* (1991) cloned and sequenced the β -galactosidase gene from *Clostridium acetobutylicum* strain NCIB 2951 (*cbgA*) in *E. coli*. It was found that the deduced amino acid sequence of *cbgA* showed homology to the deduced amino acid sequences derived from *E. coli*, *lacZ* (54%); *E. coli*, *ebgA* (53%); *Klebsiella pneumoniae*, *lacZ* (52%); and *Lactobacillus bulgaricus*, *lbu* (65%) but no significant similarity to the *bgaB* of *B. stearothermophilus* (Hancock *et al.*, 1991).



Some of the physical properties of the sequenced β -galactosidases are listed in Table 9. Poolman *et al.* (1989) cloned *lacS* gene encoding the lactose permease system (i.e. the pmf-linked lactose transport system) from the chromosomal library of *S. thermophilus* into *E. coli*. The *lacS* gene was transcribed from its own promoter and encoded a protein of M_r 69,454 containing 634 amino acids. It was found that there was no similarity between the *lacS* protein of *S. thermophilus* and the lactose permease (*lacY*) from *E. coli*. Leong-Morgenthaler *et al.* (1991) cloned the β -galactosidase gene and the lactose permease gene of *Lactobacillus bulgaricus* strain ATCC 11842 into *E. coli*. The lactose permease gene of *Lactobacillus bulgaricus* codes for the protein containing 627 amino acid residues. The lactose permease gene of *Lactobacillus bulgaricus* and the lactose permease gene of *S. thermophilus*, *lacS*, both encoded by the lactose operon are highly homologous but differ from the lactose operons of other organisms which also metabolize lactose by the same transport system. Their lactose permease genes are located upstream from the β -galactosidase genes while the others are located downstream from the β -galactosidase genes. However, they both have homology to the melibiose transport protein and still contain the important residues of the lactose permease of *E. coli*. In addition, the deduced amino acid sequences derived from the *lacZ* of *S. thermophilus* and of *Lactobacillus bulgaricus* exhibited 48% homology (Schroeder *et al.*, 1991). Samek *et al.* (1991) cloned the β -galactosidase gene from chromosomal DNA of *Lactobacillus lactis* strain 447 into *E. coli*. The β -galactosidase gene of *Leuconostoc lactis* strain NZ6009 was also isolated from its plasmid DNA, pNZ63 and cloned in *E. coli* by using pACYC184 (David *et al.*, 1992). From the nucleotide sequence analysis it was shown that this cloned fragment contained two partially overlapping genes, *lacL* (1,878 bp) and *lacM* (963 bp) that encode two differently sized proteins, LacL and LacM. David *et al.* (1992) studied the DNA sequences of the *lacL* and *lacM* genes of *Leuconostoc lactis* and the sequence of the plasmid-encoded β -galactosidase gene of *Lactobacillus casei* strain ATCC 393. They found that a close similarity existed between these genes and also between the deduced protein levels. They concluded that the *lac* genes from *Leuconostoc lactis* and *Lactobacillus casei* have evolved from a common ancestor and may have diverged only very recently.

Table 9 Comparison of some physical properties of β -galactosidases from the sequenced β -galactosidase genes.¹

Source of β -galactosidase	Number of amino acid	Molecular weight (Da)	Average residual mol.wt	Reference
<i>E.coli lacZ</i>	1,023	116,353	113.7	Kalnins <i>et al.</i> , 1983
<i>E.coli ebGA</i>	963	109,652	113.8	Stokes <i>et al.</i> , 1985
<i>Klebsiella pneumoniae lacZ</i>	1,033	117,385	113.6	Buvinger and Riley, 1985
<i>Bacillus stearothermophilus bgaB</i>	672	78,052	116.2	Hirata <i>et al.</i> , 1986
<i>Lactobacillus bulgaricus Lbu</i>	1,006	113,915	113.2	Schmidt <i>et al.</i> , 1989
<i>Streptococcus thermophilus lacZ</i>	1,026	116,860	113.9	Schroeder <i>et al.</i> , 1991
<i>Leuconostoc lactis lacL</i>	626	72,113	115.2	David <i>et al.</i> , 1992
<i>lacM</i>	321	35,389	100.9	David <i>et al.</i> , 1992
<i>Clostridium acetobutylicum cbgA</i>	897	105,000	117.1	Hancock <i>et al.</i> , 1991

¹ according to Schmidt *et al.* (1989)

1.8 Background and aims of the study

An intense interest has been directed in recent years towards developing a more detailed understanding of the lactose hydrolysing systems of lactococci since the fermentation of this disaccharide is of obvious and vital importance to the dairy industry. Most of the dairy lactococci do not have the enzyme β -galactosidase available for the hydrolysis of lactose. It has been suggested that these lactococcal starter strains in which lactose utilization occurs by the Lac-PEP:PTS system may have arisen through evolutionary selection mechanisms by which rapid lactose fermentation to lactate confers clear advantages to them, particularly with respect to allowing their adaptation to milk. It has also been suggested that starter strains are of recent origin (Hirsch, 1952) and that their evolution has progressed through acquisition by wildtype strains of the lactose plasmid (Lawrence *et al.*, 1976; McKay, 1982; Porter and Chassy, 1988).

Useful starter-type strains are those exhibiting rapid lactose fermentation which is possible because of high phospho- β -galactosidase activities and low or non-detectable β -galactosidase activities. *L. lactis* ssp. *cremoris* strain H2 utilize lactose via the Lac-PEP:PTS system. Its lactose metabolism (Lac^+) as well as its proteinase positive phenotype (Prt^+) is found to be associated with a 63 kb plasmid, pDI21. This plasmid is a conjugative plasmid which had been previously transferred by conjugation into *L. lactis* ssp. *lactis* strain 4125, a plasmid-free recipient (Davey, *et al.*, 1984). Plasmid pDI21 is identical to plasmid pJI70 since each is derived from the same conjugation type of experiment carried out by two groups of researchers, pJI70 by Inamine *et al.* (1986) and pDI21 by Yu *et al.* (1989). Inamine *et al.* (1986) cloned genes for the lactose phosphotransferase system and phospho- β -galactosidase into *S. sanguis* and *E. coli*. 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 according to what Inamine *et al.* (1986) mapped pJI70. The restriction map of pDI21 and the localization of the gene for its Lac and Prt region is shown in Figure 2.

Table 8 List of cloned β -galactosidase genes in lactic acid bacteria and some other bacteria

Bacterial strain	Source of DNA	Cloned fragment	Vector	Bacterial host	Reference
<i>Streptococcus thermophilus</i>					
ATCC19258	Chromosome	7.0 kb <i>Pst</i> I	pBR322	<i>E.coli</i> JM108	Herman and McKay, 1986
A054	chromosome	7.0 kb <i>Pst</i> I	pBR322	<i>E.coli</i> JM107	Schroeder <i>et al.</i> , 1991
<i>Lactobacillus bulgaricus</i>					
B 131	Chromosome	7.0 kb <i>Hind</i> III	pKK223-3	<i>E.coli</i> JM105	Schmidt <i>et al.</i> , 1989
ATCC11842	Genomic	7.0 kb <i>Sal</i> I	pBR322	<i>E.coli</i> JM105-8	Leong-Morgenthaler <i>et al.</i> , 1991
<i>Lactobacillus lactis</i>					
477 MC4100	Chromosome 1991	<i>Sau</i> 3A I	pTZ18R	<i>E.coli</i>	Samek <i>et al.</i> ,
<i>Leuconostoc lactis</i>					
NZ6009	Plasmid pNZ63(23kb)	16 kb <i>Bam</i> HI	pACYC184	<i>E.coli</i>	David <i>et al.</i> , 1992
<i>Clostridium acetobutylicum</i>					
NCIB2951	Genomic	5.2 kb <i>Eco</i> RI	pSUP202	<i>E.coli</i> PB2959	Hancock <i>et al.</i> , 1991
<i>Streptococcus bovis</i> H3					
	Genomic	7.8 kb <i>Hind</i> III	pBR322	<i>E.coli</i> MC1061	Gilbert and Hall, 1987
<i>Streptomyces lividans</i> 1326					
	Chromosome	15.5kb <i>Sph</i> I	pJ16	<i>E.coli</i>	Eckhardt <i>et al.</i> , 1987
<i>Klebsiella pneumoniae</i>					
	Chromosome	4.8 kb <i>Hind</i> III	pBR322	<i>E.coli</i> JM103	MacDonald and Riley, 1983
<i>Bacillus stearothermophilus</i>					
IAM 11001 (ATCC8005)	Chromosome	2.9 kb <i>Eco</i> RI	pUB110	<i>Bacillus subtilis</i> MI111	Hirata <i>et al.</i> , 1984

In contrast to such starter type strains, wildtype strains usually ferment lactose slowly and produce a variety of end products. They are often found associated with non-dairy sources (e.g. plant material, insect gut) in which fast fermentation of lactose would not be a selective advantage (Farrow, 1980). The lactococci and dairy streptococci, a few other mesophilic lactococcal wildtype strains and the thermophilic streptococci (*Streptococcus thermophilus*) all possess a relatively high β -galactosidase activity. The best characterized strain of lactococci possessing β -galactosidase activity is *L. lactis* ssp. *lactis* strain ATCC 7962 and was first recognized for its unusual characteristic by Citti and his coworkers in 1965. The other lactococcal bacteria that also contain a relatively high β -galactosidase is *L. lactis* ssp. *lactis* strain NCDO 2091 which was isolated from soft rot radish (Farrow and Garvie, 1979). *L. lactis* ssp. *lactis* strain ATCC 7962 contains both lactose transport systems but rather possesses a relatively high β -galactosidase activity and a relatively low phospho- β -galactosidase activity (McKay *et al.*, 1970; Molskness *et al.*, 1973; Farrow and Garvie, 1979; Farrow, 1980; Crow and Thomas, 1984). As illustrated in Figure 3 this strain appears to take up lactose into the cell as the free sugar (McKay, 1982) and contains the enzymes of both the tagatose-6-phosphate and the Leloir pathways (Bissett and Anderson, 1974). The specific activities of its β -galactosidase and phospho- β -galactosidase as determined by Farrow and Garvie (1979), was 160×10^3 and 10×10^3 moles of o-nitrophenol released per mg protein per minute, respectively as shown in Table 10. Even though it was originally isolated from a milk sample (Rahn *et al.*, 1938), it has a slow growth rate in milk (Crow and Thomas, 1984). Its doubling time on lactose in complex broth had been determined by Crow and Thomas (1984) and found to be approximately 100 min in contrast to 32 to 44 min for normal lactic starters. Also, approximately 15% of the lactose fermented appears as lactic acid (Thomas, 1976). Being such a slow lactose fermenter it has never been used as commercial lactic starter. The classification of strain ATCC 7962 is in question for a number of its unusual biochemical reasons. It contains both lactose transport systems and possess a relatively high in β -galactosidase activity while the other lactococci do not and it exhibits different antigenic properties to the other group N streptococci in that its cell wall extracts do not react with the group N antiserum. (McKay *et al.*, 1969).

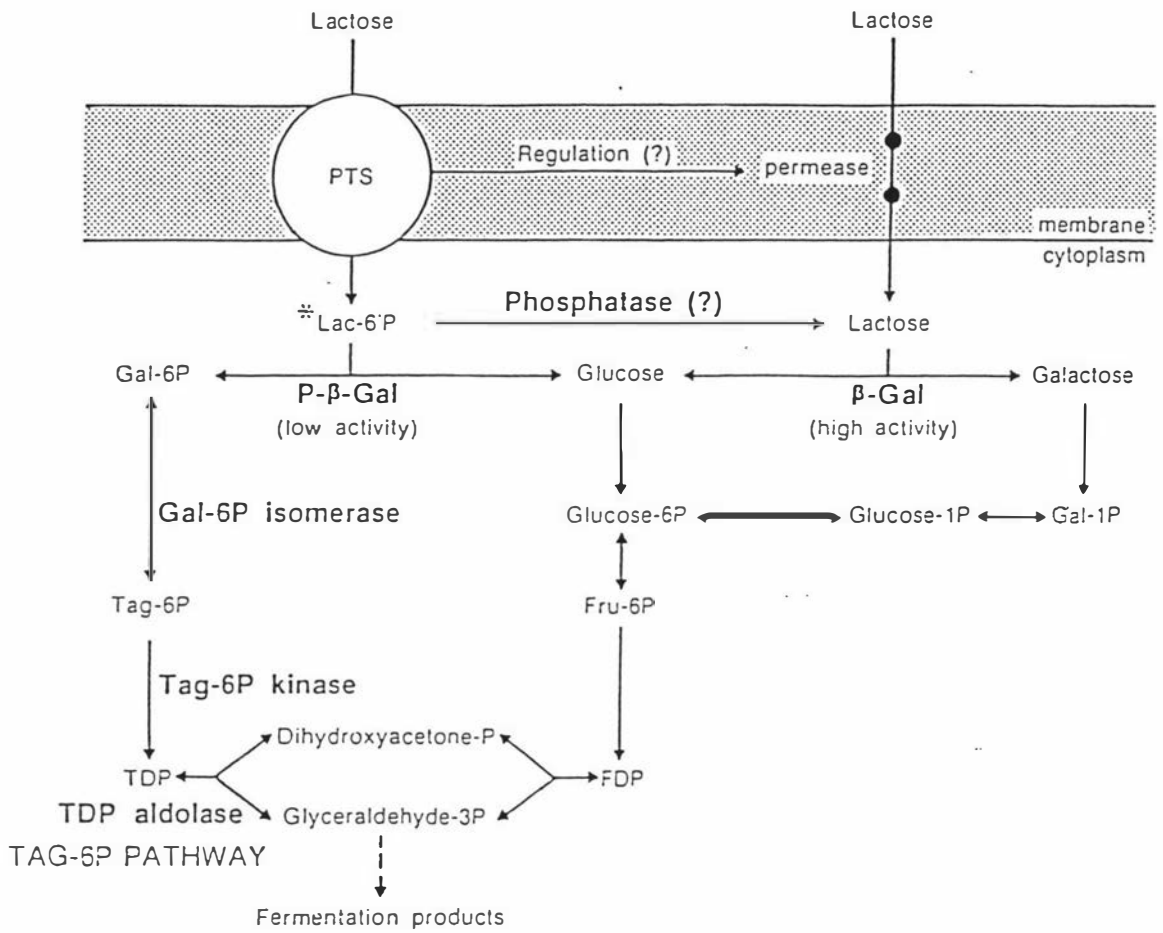


Table 10 The specific activities of β -galactosidase and phospho- β -galactosidase in strains of *Lactococcus lactis* ssp. *lactis* (Farrow and Garvie, 1979).

Strain	Enzyme activity	
	β -gal activity $\times 10^3$	P- β -gal activity $\times 10^3$
NCDO 712	0	103
NCDO 890	0	129
NCDO 2091	43	27
ATCC 7962	160	10

Specific activity = moles o-nitrophenol released per milligram of protein per minute.

However it has been classified as *Lactococcus lactis* (Sandine *et al.*, 1962) because of its morphology and its ability to ferment lactose. Farrow and Garvie (1979) proposed that *L. lactis* ssp. *lactis* ATCC 7962 should be recognized as an atypical lactococcal strain because of its %G+C content is within the range for the lactococci and because of the degree of hybridization possible between its DNA and the DNA of typical lactococcal strains. The seemingly unique properties of this strain, i.e. its two lactose transport systems, its preference to transport lactose as a free sugar, its slow fermentation of lactose make *L. lactis* ssp. *lactis* strain ATCC 7962 an interesting lactococcal strain for further study concerning its relationships to other lactococcal strains and the control mechanisms that govern its two lactose transport systems. To date the lactococcal β -galactosidase gene has not been cloned and sequenced. Interesting experiments in DNA hybridization by Schroeder *et al.* (1991) have shown that there was no detectable homology between the lactose metabolizing genes (*lacZ* and *lacS*), of *S. thermophilus* and the DNA from *L. lactis* ssp. *lactis* ATCC 7962. Similarly, no hybridization has been found between the β -galactosidase gene from *Lactobacillus bulgaricus* and the DNA from strain ATCC 7962 (Smart *et al.*, 1993). β -galactosidase activity of strain ATCC 7962 has been confirmed recently by , Crow and Thomas (1984) and Pillidge and Pearce (1991) as shown in Table 11. However, based on plasmid curing experiment its β -galactosidase activity was found to be encoded by its plasmid DNA (Davey, unpublished data) an observation that contrasts markedly with the fact that most of the β -galactosidase genes isolated from other organisms were chromosomally located except that from *Leuconostoc lactis* strain NZ6009 and *Lactobacillus casei* strain ATCC 393 (David *et al.*, 1992).

Table 11 Specific activities of phospho- β -galactosidase and β -galactosidase of strains of *Lactococcus lactis* ssp. *lactis* ATCC 7962

Strain	Enzyme activity		Reference
	P- β -gal ^a	β -gal ^a	
ATCC 7962 Wildtype ^b	0.041	0.49	Crow and Thomas, 1984
NZDRI5191/pSA3 ^c	0.049	1.64	Pillidge and Pearce, 1991
NZDRI5201/pDI21 ^d	1.56	ND ^e	Pillidge and Pearce, 1991

^aSpecific activities are expressed as μ moles of o-nitrophenol released per minute per milligram of protein.

^b This strain contains all four natural occurring plasmid i.e. three other plasmids and pDI3.

^c This strain contains two plasmids i.e. pDI3 and pSA3.

^d This strain is a plasmid-free strain derivative of ATCC7962 wildtype strain and contains pDI21.

^e ND = Not detectable

1.8.1 The Research Programme:

The research programme embraced two distinct themes under the general topic of a molecular study of lactose metabolising genes from *Lactococcus lactis*. They are as follows:

The first part of the research programme was to sequence the genes of the entire Lac region of plasmid pDI21 which is responsible for the metabolism of lactose by the Lac-PEP:PTS system in *L. lactis* ssp. *cremoris* strain H2. The initial part of this programme was to sequence a DNA fragment encoding D-tagatose 1,6-bisphosphate aldolase. This part of the programme was abandoned as the clones provided contained *E. coli purM* and *purN* sequences.

The second part of the research programme was to undertake a genetic study of lactose metabolism in the unusual lactococcal strain, *Lactococcus lactis* ssp. *lactis* strain ATCC 7962.

The individual aims for the second part of the research programme were as follows:

To generate background genetic information on the lactococcal plasmid, pDI3 which is derived from the unusual lactococcal strain, *Lactococcus lactis* ssp. *lactis* ATCC 7962.

To localize, clone and characterize the plasmid-borne β -galactosidase gene from plasmid pDI3 of *Lactococcus lactis* ssp. *lactis* ATCC 7962.

To study the expression of this lactococcal β -galactosidase gene in an *E. coli* host strain.

To make a further contribution to the understanding of the genetic factors governing lactose metabolism in *Lactococcus lactis* ssp. *lactis* strain ATCC 7962.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are described in Table 12.

2.2 Preparation of Stock Solutions and Culture Media

The stock solutions used in the preparation of culture media and the culture media are described.

Chemicals used were obtained from: E. Merck P.O. Box 4119, D-6100 Darmstadt, Frankfurter Strasse 250, Germany; BDH Limited, Broom Road, Poole, BH12 4NN, England; May & Baker Ltd., Dagenham, England and Sigma Chemical Co., St Louis, MO, USA. Stock solutions of the chemicals were made up at appropriate concentrations (i.e. usually were at ten times concentration). If the chemical required to add in to the preparation of the media after the media had been sterilized, the chemicals stocks were sterilized at appropriated conditions and kept at sterile conditions.

Lactose Stock solution was prepared at the concentration of 10 mg/ml (w/v) by dissolving 0.1 g of DL-lactose in 10 ml of water and sterilization at 10 lb/in² for 20 min. The lactose stock solution was stored at 4°C.

Glucose Stock solution was prepared at the concentration of 20 mg/ml (w/v) by dissolving 0.2 g of DL-glucose in 10 ml of water and sterilization at 10 lb/in² for 20 min. The glucose stock solution was stored at 4°C.

X-gal stock solution was prepared to obtain the concentration of 20 mg/ml by dissolving 250 mg of X-gal [5-bromo-4-chloro-3-indoyl galacto-pyranoside, Sigma catalog no. D4252] in 12.5 ml of dimethylformamide, stored in a dark bottle and kept at -20°C.

Table 12 Bacterial strains and plasmids used in this study

Bacterial strains, Plasmid	Relevant Characteristics	Source or Reference
<i>Lactococcus lactis</i> <i>ssp. lactis</i>		
ATCC ¹ 7962	wildtype, β -gal ⁺	NZDRI ² Crow and Thomas, 1984
5191	plasmid-cured strain containing pDI3 β -gal ⁺	Davey, G.P. unpublished data (NZDRI)
5201	plasmid-free strain derivative of 7962 wildtype	Davey, G.P. unpublished data (NZDRI)
LM0230	Prt ⁻ Lac ⁻ , plasmid-free	Efstathiou and McKay, 1977
MG1363	Prt ⁻ Lac ⁻ , plasmid-free	Gasson, 1983a
4760	Prt ⁺ Lac ⁺ , containing pDI21	Yu <i>et al.</i> , 1989 (NZDRI)
<i>Escherichia coli</i>		
DH5 α	<i>endA1, recA1, hsdR17, (r_k⁻m_k⁺) F', Δ 80d, lacZ Δ M15, supE44</i>	
JM109	Δ (<i>lacZYA-argF</i>), <i>thi-1</i> <i>endA1, recA1, hsdR17, (r_k⁻m_k⁺) (F'<i>traD36, proAB, lacI^qZ</i> Δ M15) <i>relA1, supE44, thi-1</i></i>	Hanahan, 1983 Yanisch-Perron <i>et al.</i> , 1985
MC1022	<i>araD¹³⁹ Δ(ara leu)⁷⁹⁶⁷ lacZ Δ M15</i>	Casadaban and Cohen, 1980
PB2959	F- Δ <i>lac pheC</i>	Bergquist and Adelberg, 1972
XL-1 Blue	<i>endA1, recA1, hsdR17 (r_k⁻m_k⁺), relA1 supE44, thi-1</i>	
PN1153	LE392	Bullock <i>et al.</i> , 1987 MMCC ³
PN1226	HB101/pKH107	Hancock <i>et al.</i> , 1991
PN1227	HB101/pKH179	Hancock <i>et al.</i> , 1991
PN1338	MC1022/pSY115	This study
PN1346	JM109/pSY102	This study
PN1347	JM109/pSY103	This study
PN1348	JM109/pSY101	This study
PN1349	JM109/pSY105	This study
PN1350	JM109/pSY104	This study

Table 12 (Continued)

Bacterial strains, Plasmid	Relevant Characteristics	Source or Reference
PN1351	JM109/pSY202	This study
PN1352	JM109/pSY201	This study
PN1353	JM109/pSY203	This study
PN1354	JM109/pSY204	This study
PN1355	JM109/pSY302	This study
PN1356	JM109/pSY303	This study
PN1357	DH5 α /pSY303	This study
PN1378	XL-1 Blue/ pSY303: Δ pSY303 (ratio 1:9)	This study
PN1379	XL-1 Blue/pSY303: Δ pSY303 (ratio 9:1)	This study
PN1380	XL-1 Blue/pSY303	This study
PN1383	XL-1 Blue/pSY303	
PN1384	XL-1 Blue/pSY303: Δ pSY303 (ratio 1:1)	This study
PN1394	PB2959/pSY303	This study
Plasmids		
pBR322	Am ^r Tc ^r	Bolivar <i>et al.</i> , 1977
pBR328	Am ^r Cm ^r Tc ^r	Soboron <i>et al.</i> , 1980
pBH401	pGEM-3Z clone containing 4.4 kb <i>EcoRI</i> fragment from pDI21	Hodge, 1989
pBH501	pGEM-3Z clone containing 4.4 kb <i>EcoRI</i> fragment from pDI21	Hodge, 1989
pDI3	70 kb, β -gal ⁺	Davey, G.P. unpublished data
pDI21	63 kb, Prt ⁺ Lac ⁺	Yu <i>et al.</i> , 1989
pFX1	Cm ^r	Xu <i>et al.</i> , 1990
pKH179	pSUP202 clone containing 5.2 kb <i>EcoRI</i> fragment <i>CbgA</i> from <i>Clostridium acetobutylicum</i>	Hancock <i>et al.</i> , 1991
pSA3	Cm ^r Tc ^r Em ^r	Dao and Ferretti, 1985
pSY101	pBR328 clone containing 1.2 kb <i>EcoRI</i> fragment from pDI3	This study
pSY102	pBR328 clone containing 2.5 kb <i>EcoRI</i> fragment from pDI3	This study
pSY103	pBR328 clone containing 3.6 kb <i>EcoRI</i> fragment from pDI3	This study
pSY104	pBR328 clone containing 3.8 kb <i>EcoRI</i> fragment from pDI3	This study

Table 12 (Continued)

Bacterial strains, Plasmid	Relevant Characteristics	Source or Reference
pSY105	pBR328 clone containing 4.3a kb <i>EcoRI</i> fragment from pDI3	This study
pSY115	pUC18 clone containing 4.3a kb <i>EcoRI</i> fragment from pDI3	This study
pSY201	pBR322 clone containing 1.7 kb <i>PstI</i> fragment from pDI3	This study
pSY202	pBR322 clone containing 4.2b kb <i>PstI</i> fragment from pDI3	This study
pSY203	pBR322 clone containing 6.7 kb <i>PstI</i> fragment from pDI3	This study
pSY204	pBR322 clone containing 8.3 kb <i>PstI</i> fragment from pDI3	This study
pSY301	pSA3 clone containing 2.5 kb <i>SaI</i> fragment from pDI3	This study
pSY302	pBR322 clone containing 5.5 kb <i>SaI</i> fragment from pDI3	This study
pSY303	pBR322 clone containing 13.6 kb <i>SaI</i> fragment from pDI3	This study
pUC18	Am ^r	Yanisch-Perron <i>et al.</i> , 1985

¹ ATCC American Type Culture Collection, Maryland, USA

² NZDRI New Zealand Dairy Research Institute, Palmerston North, New Zealand

³ MMCC Massey University Microbiology Culture Collection, Palmerston North,
New Zealand

IPTG stock solution was prepared to obtain the concentration of 24 mg/ml by dissolving 0.24 g of IPTG [isopropyl thiogalactopyranoside, Sigma catalog no. I5502] in water. The IPTG stock solution was stored at -20°C.

Antibiotics were obtained from Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178, USA. Antibiotic stock solutions were dissolved at the stock concentration [mg/ml Ampicillin (Ap), 100; Tetracycline (Tc), 25; Chloramphenicol (Cm), 20; Erythromycin (Em), 20] in methanol except Ap which was dissolved in sterile MilliQ water. All antibiotic stock solutions were stored at -20°C. Stock solutions of Cm, Em and Tc were all stored in a dark bottle at -20°C. Appropriate antibiotics were added to freshly autoclaved media after they had cooled to approximately 50°C to give the required final concentrations ($\mu\text{g/ml}$ Ap, 100; Tc, 12.5; Cm, 25) for *E. coli* and ($\mu\text{g/ml}$ Cm, 5; Em, 5) for lactococci.

Dehydrated media were obtained from Difco Laboratories Inc., Detroit, Michigan 48232, USA.; Gibco Laboratories, Madison, Wisconsin 53713, USA., and Oxoid Ltd., Basingstoke, Hants., England. All media were prepared in the laboratory, resuspended in MilliQ water and sterilized at 15 lb/in² for 15 min, otherwise specified. Solid medium was made up to contain Bacto-agar at a final concentration of 1.5% (w/v). Soft medium was made up to contained Bacto-agar at a final concentration of 0.7% (w/v).

AI (Miller, 1972). Composition (g/l): K₂HPO₄, 10.5; KH₂PO₄, 4.5; (NH₄)₂SO₄, 1; Sodium citrate.2H₂O, 0.5. Prior to use AI was supplemented with thiamine, MgSO₄ and glucose to a final concentration of 20 $\mu\text{g/ml}$, 1 mM and 0.4% (w/v), respectively.

Luria Broth (LB) (Miller, 1972). Composition (g/l): Bacto-tryptone, 10; Bacto-yeast extract, 5; NaCl, 5. LB was adjusted to pH 7.5 before autoclaving.

LBAX contained ampicillin, X-gal and Bacto-agar at a final concentration of 100 $\mu\text{g/ml}$, 40 $\mu\text{g/ml}$ and 1.5% (w/v), respectively in LB.

LBAXI contained ampicillin, X-gal, IPTG and Bacto-agar at a final concentration of 100 µg/ml, 40 µg/ml, 200 µg/ml and 1.5% (w/v), respectively in LB.

LBLAX contained lactose, ampicillin, X-gal and Bacto-agar at a final concentration of 0.1% (w/v), 100 µg/ml, 40 µg/ml and 1.5% (w/v), respectively in LB.

M17 broth (Terzaghi and Sandine, 1975). Composition (g/l): DL-lactose, 5; Bacto-polypeptone, 5; Bacto-beef extract, 5; Bacto-soy peptone, 5; Bacto-yeast extract, 2.5; ascorbic acid, 0.5; sodium-β-glycerophosphate, 19; 1 M MgCl₂, 1 ml. Before autoclaving its pH was adjusted to 7.2.

M17G contained M17 broth and DL-glucose at a final concentration of 0.5% (w/v).

M17X contained M17 broth plus X-gal and Bacto-agar at a final concentration of 40 µg/ml and 1.5% (w/v), respectively.

M17SG contained M17 broth and a final concentration of 0.5 M sucrose.

MM56 base medium (Berquist and Adelberg, 1972). Composition (g/l): KH₂PO₄, 5.36; Na₂HPO₄, 8.69; (NH₄)₂SO₄, 2; 10% MgSO₄.7H₂O, 2 ml; 1% Ca(NO₃)₂, 1 ml; 0.027% FeSO₄.7H₂O, 1 ml. Its pH was adjusted to 7.2. With the above composition the medium was diluted with an equal volume of deionized water for use as a basal medium.

M56 contained MM56 base medium supplemented with glucose, phenylalanine and thiamine at a final concentration of 0.4% (w/v), 1% (w/v) and 20 µg/ml, respectively.

M56LAX contained M56 plus a final concentration of lactose, X-gal, ampicillin and Bacto-agar of 0.1% (w/v), 40 µg/ml, 100 µg/ml and 1.5% (w/v), respectively.

SOB (Maniatis *et al.*, 1982). Composition (g/l): Bacto-tryptone, 20; Bacto-yeast extract, 5g; 1 M NaCl, 10 ml; 1 M KCl, 2.5 ml.

SOC contained a final concentration of 0.02 M of MgSO_4 and 0.02 M of DL-glucose.

2.3 Buffers and Solutions

Buffers and solutions used were prepared according to Maniatis *et al.* (1982). MilliQ water was used in all preparations.

2.3.1 TAE Buffer contained 40 mM Tris-HCl, 20 mM glacial acetic acid, 2 mM Na_2EDTA at pH 8.2.

2.3.2 TBE Buffer pH 8.3 containing 89 mM Tris-HCl, 2.5 mM Na_2EDTA , 89 mM boric acid and pH 8.3. In practice a ten times concentrated stock solution was prepared by dissolving 107.7 g Tris, 9.3 g Na_2EDTA and 55 g boric acid in 1 litre of MilliQ water, pH 8.3.

2.3.3 TE Buffer (10:1) pH 8.0 containing 10 mM Tris-HCl, 1 mM Na_2EDTA at pH 8.0 was prepared to the required concentration from 1 M Tris-HCl (pH 8.0) and 250 mM Na_2EDTA (pH 8.0) stock solutions.

2.3.4 TES Buffer (10:1:100) pH 8.0 containing 10 mM Tris-HCl, 1 mM Na_2EDTA , 100 mM NaCl at pH 8.0 was prepared to the required concentration from 1 M Tris-HCl (pH 8.0) and 250 mM Na_2EDTA (pH 8.0) and 5 M NaCl stock solutions.

2.3.5 Tris-Equilibrated Phenol was prepared by placing a 500 g bottle of Crystalline analytical grade phenol (Merk) in a water bath at 50°C until melted and 8-hydroxy-quinoline (an antioxidant, a partial inhibitor of RNase and a weak chelator of metal ions) was added to give a final concentration of 0.1% (w/v). An equal volume of 1.0 M Tris-HCl was added to the liquified phenol at room temperature and stirred for 15 min. The phenolic phase was retained using a separation funnel and washed 2-3 times with 1.0 M Tris-HCl (pH 8.0), essentially until the pH of the phenolic phase was > 7.8 (DNA will partition into the organic phase if the pH is acidic). After the equilibration was completed the phenolic phase was retained and washed 2-3 times with 100mM Tris-HCl

(pH 8.0) containing 0.02% (v/v) β -mercapto ethanol. This equilibrated phenol solution was stored under 100 mM Tris-HCl (pH 8.0) in a dark bottle and kept at 4°C.

2.3.6 10x Universal buffer contained 60 mM Tris-HCl pH 7.6; 100 mM $MgCl_2$ and 100 mM β -mercaptoethanol

2.3.7 SDS Loading Buffer contained 1% (w/v) sodium dodecyl sulphate (SDS), 0.02% (w/v) bromophenol blue, 20% (w/v) sucrose and 5 mM Na_2EDTA (pH 8.0).

2.3.8 STET Buffer contained 8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM Na_2EDTA (pH 8.0) and 50 mM Tris-HCl (pH 8.0).

2.3.9 Solutions used for isolation of lactococcal DNA (Anderson and McKay, 1983).

2.3.9.1 Solution A contained 6.7%(w/v) sucrose, 50mM Tris-HCl pH 8.0 and 1 mM Na_2EDTA , pH 8.0.

2.3.9.2 Solution B contained 10 mg/ml of lysozyme (Sigma, catalog no.L6876) in 25mM Tris-HCl pH8.0.

2.3.9.3 Solution C contained 50 mM Tris-HCl (pH 8.0) and 0.25 M Na_2EDTA .

2.3.9.4 Solution D contained 20% (w/v) SDS in 50 mM Tris-HCl pH 8.0 and 20 mM Na_2EDTA pH 8.0.

2.3.9.5 Solution E contained freshly prepared 3.0 N NaOH.

2.3.9.6 Solution F contained 2.0 M Tris-HCl pH 7.0.

2.3.9.7 Solution G contained 5.0 M NaCl.

2.3.9.8 Phenol saturated with 3% NaCl was prepared by melting crystalline analytical grade phenol (500 g, Merck) in a water bath at 50°C and 8 hydroxy-quinoline was added to give a final concentration of 0.1% (w/v). One liter of 3% (w/v) NaCl solution was prepared and used to equilibrate the liquefied phenol solution. The phenol/NaCl mixture was mixed thoroughly until the phenolic phase was separated. The phenol saturated with 3% NaCl was stored in a dark bottle and kept at 4°C.

2.3.9.9 Solution H contained 10 mM Tris-HCl pH 7.5 and 1 mM Na₂EDTA pH7.5

2.3.10 20x SSC (Maniatis *et al.*, 1982) contained 3.0 M sodium chloride and 0.3 M sodium citrate in water (pH 7.2).

2.3.11 2x SSC was prepared by dilution of 20x SSC 10 fold with water.

2.3.12 50x Denhardt Solution (Maniatis *et al.*, 1982) contained 1% (w/v) Ficoll (Sigma 70 catalog no. F2637), 1% (w/v) polyvinylpyrrolidone (Sigma PVP-10, catalog no.P2307) and 1% (w/v) BSA fraction V (Sigma catalog no.A4503) in water. The solution was filter sterilized and stored frozen at -20°C.

2.3.13 Hybridization Buffer (Southern, 1975): 50 ml of 1M Hepes {(N-[2-Hydroxy ethyl]piperazine-N'-[2-ethanesulfonic acid]); Sigma, catalog no. H1016}, pH 7.0; 150 ml of 20x SSC (section 2.3.10); 6.0 ml of phenol extracted herring sperm DNA (3 mg/ml, Sigma); 5 ml of 20% (w/v) SDS; 2 ml of *E. coli* transfer RNA (10 mg/ml, Sigma, catalog no. R0383); 100 ml of 50x Denhardt solution (section 2.3.12) and water to make up to 1 litre.

2.3.14 CTAB/NaCl solution (Murray and Thompson, 1980) containing 10% (w/v) of CTAB (hexadecyltrimethyl ammonium bromide) in 0.7 M NaCl was prepared by dissolving 4.1 g of NaCl in 80 ml water and to the solution was slowly added 10 g of CTAB (Sigma catalog no. H6269) while heating to 65°C and stirring until all of CTAB dissolved and made up to 100 ml volume with water.

2.4 Growth and Maintenance of Bacterial Strains

E. coli were incubated aerobically in LB at 37°C for overnight or 2-4 h. where early log-phase cells were required (Miller, 1972).

Lactococci were grown overnight at 22°C in M17 or M17G (section 2.2) or for 2-4 h at 30°C where early log-phase cells were required (Terzaghi and Sandine, 1975).

E. coli working cultures were maintained in LB or on LB agar plates (section 2.2) at 4°C while lactococcal working cultures were maintained in M17 or M17G at 4°C.

Growth of bacteria in broth cultures was determined, where required, by measurement of cell suspension absorbance at 600 nm (A_{600}) and or/by dilution plating.

Stock cultures of both *E. coli* and lactococcal strains were stored at -20°C in vials with glycerol at a final concentration of 15% (w/v). This was done by adding 200 µl of sterile glycerol to either 800 µl of an overnight culture of *E. coli* in LB and an early log phase lactococcal culture (i.e. 2-4 h) in either M17 or M17G, as appropriate.

For long term maintenance, all cultures were stored in 50% (v/v) glycerol at -70°C.

2.5 Isolation of Lactococcal DNA

2.5.1 Small scale preparations of plasmid DNA from lactococci

Lactococcal plasmid DNA was prepared from exponential growth phase culture grown for 3-4 h in M17 broth at 30°C (sections 2.2 and 2.4) using the Anderson and McKay (1983) protocol as summarized in Table 13.

2.5.2 Large scale plasmid DNA preparation from lactococci

A 2-4 l of a 4 h culture of lactococcal strain grown at 30°C in M17 broth (section 2.2) was used for large scale preparations of lactococcal plasmid DNA. A 5% (v/v) inoculum was used to initiate these cultures. Cells were pelleted by centrifugation at 12,000 x g for 15 min at 4°C. Then the Anderson and McKay (1983) protocol as described in Table 13 was followed. The method was identical to the small scale procedure but larger proportions of reagents were used accordingly. The DNA was further purified by ultracentrifugation in a CsCl-buoyant density gradient (see also 2.7.1).

Table 13 Lactococcal plasmid preparation protocol (Anderson and McKay, 1983).

Step	Details	
	Screening (1.5-10 ml) ¹	Preparative (600 ml) ¹
	Microfuge ²	Refrigerated Centrifuge (Sorvall) ²
Resuspend pellets cells in solution A (2.3.9.1)	379 µl	30 ml
warm to 37°C		
Add solution B (2.3.9.2)	96.5 µl	7.5 ml
Incubate at 37°C for 10 min.		
Add solution C (2.3.9.3), mix gently	48.2 µl	3.75 ml
Add solution D (2.3.9.4)	27.6 µl	2.25 ml
Mix immediately		
incubate for 5-10 min at 37°C to complete lysis		
Vortex at highest speed for 30 sec	Eppendorf	15 ml per tube (25 by 150 mm)
Add solution E (2.3.9.5)	49.6 µl	2.4 ml
Mix gently by intermittent inversion or swirling for 10 min	Inversion	Swirl in 250-ml centrifuge bottle
Add solution F (2.3.9.6)	49.6 µl	3.9 ml
Continue gentle mixing for a further 3-5 min		
Add solution G (2.3.9.7)	71.7 µl	5.7 ml
Add phenol saturated with 3% NaCl (2.3.9.8)	700 µl	55.8 ml
Mix thoroughly		
Centrifuge	5 min	5000 rpm in GSA rotor, 10 min
Remove upper phase and extract with Chloroform:isoamyl alcohol (24:1)	700 µl	55.8 ml
Remove upper phase, precipitate with 1 volume of isopropanol		
incubate at -20°C	>30 min	>60 min
Centrifuge	20 min	8,000 rpm in GSA rotor, 20 min
Remove excess isopropanol and resuspend in TE buffer	20 µl	1.2 ml

¹ The volume of the culture used in each protocol is indicated in parentheses.

² Centrifuge used in each protocol

2.5.3 Isolation of total genomic DNA from lactococci

Total lactococcal genomic DNA was isolated from 1.5 ml of late log phase cells grown at 30°C in M17 broth (section 2.2). The protocol was carried out according to the Anderson and McKay (1983) until the cells lysis stage then followed the protocol of Wilson *et al.* (1990). After cell lysis, proteinase K (EC.3.4.21.14, Sigma catalog no.P2308) was added to give a final concentration of 100 µg/ml and incubated 1 h at 37°C. To the solution 100 µl of 5 M NaCl was added and mixed thoroughly. Protein and polysaccharide were precipitated from the lysate by adding 80 µl of CTAB/NaCl solution (section 2.3.14) to the solution, mixed and incubated at 65°C for 10 min. The solution was extracted with an equal volume of chloroform/isoamyl alcohol (section 2.7.2). The upper phase was precipitated at -20°C overnight with two volumes of ice-cold absolute ethanol (section 2.7.3). The DNA pellet was washed in 70% (v/v) ethanol and dried *in vacuo*. The DNA was resuspended in TE buffer and kept at 4°C.

2.6. Isolation of *E. coli* DNA

2.6.1 Small Scale Isolation of *E. coli* plasmid DNA by Rapid boiling method

The rapid boiling method of Holmes and Quigley (1981) was routinely used in various experiments for the rapid isolation and detection of plasmid DNA from *E. coli*.

A 1.5 ml overnight culture of *E. coli* was prepared from a single colony which had been cultivated on an agar medium containing the particular antibiotic. The *E. coli* cells were harvested by centrifugation in a microfuge at full speed for 1-2 min and collected into an Eppendorf tube. The cell pellet was resuspended in 350 µl of STET buffer (section 2.3.8) by sucking up and down with micropipette. Then 25 µl of lysozyme solution (10 mg/ml in 25 mM Tris-HCl pH 8.0) was added and mixed thoroughly by vortexing. The mixture was then placed in a boiling water bath for 40-45 sec and immediately centrifuged in a microfuge at full speed for 10 min. The cell debris at the bottom of the tube was removed from the supernatant with a sterile toothpick. The DNA in the supernatant was precipitated by the addition of an equal volume of ice-cold isopropanol

to the supernatant (approx. 250-300 μ l) and left at -20°C for 20-30 min (section 2.7.3). Phenol/ chloroform (1:1) extraction (section 2.7.2) was used at a 1:1 ratio of supernatant to solvent where greater purification of the DNA was required. The RNA was removed from the supernatant by treating with RNaseA (section 2.7.4) prior to the phenol /chloroform extraction. The DNA was collected by pelleting via centrifugation in a microfuge at full speed for 20-30 min. The DNA pellet was now washed twice with 70% (v/v) ethanol, dried *in vacuo* for 10-15 min and resuspended in the desired amount (usually 50-100 μ l) of either sterile water or TE buffer.

2.6.2 Small scale isolation of *E. coli* plasmid DNA by Rapid alkaline extraction method

The rapid alkaline extraction procedure for *E. coli* plasmid preparation was carried out according to the method of Birnboim and Doly (1979).

A 5.0 ml volume of an *E. coli* culture was centrifuged at $4340 \times g$ for 10 min and the bacterial pellet was washed once with TES buffer (section 2.3.4) and then resuspended in 100 μ l of *E. coli* lysis solution [50 mM glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM Na_2EDTA (pH 8.0)]. Lysozyme was added to give a final concentration of 2 mg/ml and the mixture incubated for 10 min at room temperature. Freshly prepared 200 μ l of an alkaline solution [0.1% (w/v) of SDS in 0.2 N NaOH] was added and mixed gently for 5 min until the supernatant was clear. 150 μ l of ice-cold 5M potassium acetate (prepared by mixing 29.44 g potassium acetate and 11.5 ml glacial acetic acid per 100 ml, pH 4.8), mixed thoroughly and left on ice for 20 min. The mixture was then centrifuged ($3020 \times g$ for 10 min at 4°C). The supernatant was transferred to a clean tube in which the DNA was treated with 20 $\mu\text{g}/\text{ml}$ final concentration of RNase A at 37°C for 20 min (section 2.7.4). To the solution an equal volume of phenol/ chloroform was added and centrifuged (section 2.7.2). The DNA in the upper phase was precipitated with 2 volume of absolute ethanol at -20°C for 1-2 h. The DNA was recovered by centrifugation at $13300 \times g$, 20 min, 4°C and the pellet was washed once with 70% (v/v) ethanol, dried *in vacuo* and resuspended in 20-100 μ l TE buffer.

2.6.3 Large scale isolation of *E. coli* plasmid DNA

A modification of the clear lysate procedure of Clewell and Helinski (1969) was used for improved yields of supercoiled DNA.

An overnight culture of *E. coli* in LB (2.5 ml) was inoculated into 100 ml of LB and incubated aerobically by shaking at 200 rpm at 37°C. The *E. coli* culture was incubated for a further 12-16 h and harvested by centrifugation (4340 x g, 10 min at 4°C). The pellet was resuspended in 10 ml of lysis solution [25% (w/v) sucrose solution in 0.05 M-Tris pH 8.0], then 2.5 ml of lysozyme (freshly prepared to give a final concentration of 2.0 mg/ml) was added and followed by 5.0 ml Na₂EDTA. The mixture was incubated on ice and after 10 min, 15 ml of Triton lysis mixture [0.25 M Na₂EDTA (25ml), 1 M Tris pH 8.0 (50 ml), 20% (v/v) Triton X-100 in 0.01 M Tris-HCl pH 8.0 (10 ml), and sterilized H₂O (60ml)] was added to the mixture with gentle inversion until lysis was complete. Care was taken to avoid the gelatinous chromosomal DNA pellet. The supernatant was purified by treating once with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) and followed by extracting once with an equal volume of chloroform/isoamylalcohol (24:1). Two volumes of absolute ethanol were added to the purified supernatant and the DNA precipitated either overnight or 1 h at -20°C. The DNA pellet was collected by centrifugation at 13300 x g for 20 min at 4°C. The DNA pellet was washed twice with 70% (v/v) ethanol, dried *in vacuo* and resuspended in TE buffer. The DNA solution was further purified by ultracentrifugation in a CsCl-buoyant density gradient (see also 2.7.1).

2.6.4. Isolation of genomic DNA from *E. coli*.

The method used was that described by Wilson *et al.* (1990).

E. coli cells were grown to late exponential phase in LB and 1.5 ml of the culture was pelleted by centrifugation in a microfuge at full speed for 1-2 min. The cell pellet was resuspended in 567 µl of TE buffer (2.3.3), then 30 µl of 10% (w/v) of SDS in water

was added followed by the addition of 3 μ l of Proteinase K (20 mg/ml). The solution was then mixed thoroughly and incubated at 37°C for 1 h. The protein from the lysate were precipitated by an addition of 100 μ l of 5M NaCl and mixed thoroughly. Then 80 μ l of CTAB solution (10% CTAB in 0.7M NaCl, section 2.3.14) was added, mixed and incubated at 65°C for 10 min. The mixture were then extracted with an equal volume of phenol/ chloroform solution (2.7.2) and centrifuged at full speed in a microfuge. The upper phase of the supernatant was extracted further with an equal volume of chloroform and collected. The DNA in the collected supernatant was precipitated with 0.6 volume of ice-cold isopropanol at -20°C for 1-2 h. After centrifugation the DNA pellet was washed with 70% (v/v) ethanol, dried *in vacuo* and resuspended in 100 μ l of TE buffer.

2.7 Purification of DNA

2.7.1 Ultracentrifugation in a CsCl-buoyant density gradient

The method used was that of Maniatis *et al.* (1982). Purification of DNA by ultracentrifugation in a CsCl-buoyant density gradient was normally performed using the large scale DNA preparation for both lactococcal and *E. coli* plasmid DNA. All the performances were carried out by using a Beckman L8-80 centrifuge. A VTi80 rotor was used and the centrifugation was carried out at 70,000 rpm for 5 h at 25°C. Prior to the gradient set up step, the DNA pellet was resuspended in 2 ml of TE buffer. The CsCl (Sigma catalog no. C4036) was added to the DNA solution either in solid form to give an approximate concentration of 1 g/ml or as a CsCl solution. For both operations the final refractive index (n , using a refractometer, Atago Illuminator) of the DNA-CsCl solution should be in the range 1.386-1.392. Ethidium bromide (Sigma catalog no. E2515, 10 mg/ml in TE buffer) was added to the DNA-CsCl solution at a final concentration of 300 μ g/ml. After the addition of ethidium bromide, all subsequent steps were carried out in the dark to avoid light damaged to the DNA. The mixture was centrifuged (4340 x g , 10 min, at 4°C) to pellet the ribonucleoprotein. The refractive index of the supernatant was finally checked and readjusted to $n = 1.386-1.392$. The DNA-CsCl-ethidium bromide solution was transferred to a Beckman ultracentrifuge tube.

After ultracentrifugation, the covalently closed circular plasmid (CCC) DNA was detected by long wave length UV light as the lowest band in the centrifuge tube. It was collected by removing into a syringe. Most of the chromosomal DNA was visualized as an upper band while the RNA was at the bottom of the tube. The DNA solution was further purified by extracting the ethidium bromide and removing the CsCl via dialysis. The purity and concentration of the DNA was determined as described in 2.8. The DNA solution was stored at 4°C.

Ethidium bromide was extracted from the DNA solution after ultracentrifugation in a CsCl-buoyant density gradient using CsCl-TE-saturated isopropanol. The extraction procedure was based on the partitioning of ethidium bromide into the organic solvent (pinkish upper phase) while the DNA solution remained in the aqueous (bottom) phase. The extraction was carried out in the dark or subdued light. Sequential extraction was required to remove ethidium bromide at which point the upper phase was clear.

The cesium chloride was removed by dialysing against a large volume of sterile TE buffer pH 8.0 (section 2.3.2) at 4°C with at least two changes of TE buffer during the 24 h period. Following the ethidium bromide extraction the DNA solution from the bottom phase was filled into the prepared dialysis tubing with a closure at the other end. Dialysis tubing was prepared according to the method described by Maniatis *et al.* (1982). The dialysis tubing was cut into 15 cm lengths and boiled in a large volume of 2% (w/v) NaHCO₃ and 1 mM Na₂EDTA for 10 min. The tubing was rinsed thoroughly in distilled water and boiled for another 10 min in distilled water. After cooling, the tubing was stored submerged in sterile deionized water at 4°C. Prior to use, the tubing was washed both inside and outside with sterile distilled water. The dialysis tubing closures were sterilized and stored in sterile water until required.

2.7.2 Extraction with phenol/chloroform solution

The following procedures were used to purify both small and large scale DNA preparations as well as for subsequent DNA manipulations. A mixture of chloroform:isoamyl alcohol (24:1 v/v) was used to remove proteins from the DNA

preparations (Maniatis *et al.*, 1982). DNA was purified by adding to the preparation an equal volume of phenol. After mixing thoroughly, the preparation was centrifuged at top speed for 3-5 min in a microfuge. The upper (aqueous) phase was collected and to this was added an equal volume of phenol/chloroform solution (i.e. phenol:chloroform:isoamyl alcohol 25:24:1, v/v). After centrifuging, the upper (aqueous) phase was collected and to it was added an equal volume of chloroform. The upper (aqueous) phase was recollected after centrifugation. The purification cycle was repeated as many times as desired. Finally, the DNA in the upper aqueous phase was precipitated using 2 volume of absolute ethanol.

2.7.3 Precipitation of DNA

DNA precipitation was accomplished using 2 to 2.5 volumes of absolute ethanol (99% to 99.7% v/v) containing either 2.5 M ammonium acetate pH 7.5 or 0.3 M sodium acetate pH 5.2. The mixture was mixed by inversion. Precipitation was allowed to proceed either at -20°C for at least 1 h or preferably overnight or -70°C for 20-30 min. The DNA was collected by centrifuging at top speed in a microfuge for at least 20 min. The DNA pellet was then washed at least once with 70% (v/v) ethanol, dried *in vacuo* and resuspended in either sterile water or TE buffer. Occasionally 0.6 volume of isopropanol was also used in place of ethanol, however, ethanol was used preferentially as it is more volatile and solutes (e.g. sodium chloride) are less easily coprecipitated, thereby minimizing coprecipitation of components that may prevent redissolution of the DNA.

2.7.4 RNaseA treatment

Any contaminating RNA contained in the DNA preparation was removed by treating the DNA preparation with DNase-free RNaseA at a final concentration of 2 mg/ml at 37°C for 30 min to 1 h. DNase-free RNaseA was prepared according to the method described by Maniatis *et al.* (1982). RNaseA (Sigma catalog no.R9009) was dissolved to give 10 mg/ml in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl. This solution was heated to

100°C for 15 min and allowed to cool slowly to room temperature. Aliquots were dispensed and stored at -20°C until required.

2.7.5 Column chromatographic method

Gel filtration was used as an alternative method for purifying DNA samples (e.g. for the separation of [α -³²P]dCTP labelled DNA fragment from unincorporated dNTPs. The method used was based on that of Maniatis *et al.* (1982).

Sephadex G-50 was prepared by suspending 30 g of Sephadex G-50 powder in 250 ml of TES buffer (2.3.4). This was heated at 65°C for 1-2 h and allowed to cool to room temperature. The supernatant was decanted and replaced with an equal volume of TE buffer (2.3.3). The Sephadex G-50 slurry was equilibrated in TES buffer and stored at 4°C in a wide-mouth bottle.

A Sephadex G-50 column was prepared in a 1 ml sterile disposable, Terumo, Tuberculin syringe (without needle) with siliconized glass wool at the bottom of the syringe and above this was packed with 0.9 ml of Sephadex G-50 suspension. The end of the syringe was then inserted into a decapped Eppendorf centrifuge tube and the syringe and tube were spun at speed 3 in a BTL bench top centrifuge (approximately 1500 x g) for 3 min at room temperature in a swinging bucket rotor. More Sephadex G-50 was added until the packed column volume was 0.9 ml. Then 0.1 ml of TES buffer was added to the column and recentrifuged at exactly the same speed and time as before and the void volume of the column was checked and repeated twice. The freshly packed column was inserted into the perforated cap of a 1.5 ml Eppendorf tube. The radioactive labelled DNA sample (total volume of 100 μ l) was loaded onto the prepared column and spun at the same speed and time used before then the purified DNA collected.

2.7.6 Micro-dialysis through millipore membrane

Micro-dialysis through a millipore membrane Type VS (pore size of 0.025 μ M) was used to purify small amounts of DNA samples (10-50 μ l), especial prior to

electroporation. The DNA sample was dropped onto the membrane and dialyzed against 10% (w/v) sterile glycerol solution at 4°C for 15-30 min.

2.8 Determination of DNA Purity and DNA Quantitation

Purified DNA samples were spectrophotometrically quantified by measurement of the UV absorbance at wavelength 260 nm and 280 nm against TE buffer (Maniatis *et al.*, 1982). The DNA solutions were diluted 50^x in TE. The standard value for UV absorption of double stranded DNA is that 1.0 absorbance unit at 260 nm (A_{260}) is equivalent to a concentration of 50 µg/ml.

Therefore the concentration of DNA in a sample [µl/ml DNA] was = 50 x dilution factor x A_{260}

Determination of DNA concentration for a small scale DNA preparation was accomplished by comparing its fluorescence intensity with ethidium bromide from serial dilutions of a known concentration of standard DNA (for example uncut λ DNA or any known linearized DNA fragment e.g. linearized pUC18) on agarose gel.

DNA purity was also determined from UV absorption at 230 nm, 260 nm, 280 nm and 300 nm. The following spectra ratios were regarded as satisfactory:

$$A_{260}/A_{280} \text{ nm } 1.8-2.0; A_{260}/A_{230} \text{ nm } 1.8-2.3$$

(i.e. pure preparations of DNA have an A_{260}/A_{280} ratio of 1.8 and contamination of protein or phenol decreases this ratio while RNA contamination increases this ratio)

DNA purity was also able to be assessed by checking whether the DNA was readily digested with a restriction enzyme. Normally, the pure DNA could be digested completely and gave satisfactory results.

2.9 Horizontal Agarose Gel Electrophoresis of DNA

Horizontal gel agarose gel electrophoresis was used for DNA separation and detection and the " wide mini slab gel chamber" (electrophoretic chamber from Bio-Rad Lab., Richmond, CA, USA) was employed except when rapid detection was required. In this case the mini gel chamber was used instead. The agarose (Sigma, Type I) was used at various concentrations (0.5, 0.7 and 1% w/v) for routine detection but when the DNA fragment was to be recovered from the gel a highly purified grade of agarose was used; SeaPlaque for recovery of the DNA fragment (section 2.11) was used. Agarose gel solutions were prepared by adding an appropriate quantity of agarose to the required volume of gel solution in electrophoresis buffer, TBE (section 2.3.2) and melted in a microwave. The gel solution was cooled to 50°C in a water bath, then poured into an electrophoretic chamber and allowed to set.

Prior to gel electrophoresis 2 µl of SDS loading buffer (section 2.3.7) was added to 10 ml of DNA sample. In some cases the DNA sample was heated to 60-65°C to destroy any annealing of cohesive ends before addition of the loading buffer. The bromophenol blue was used as a tracking dye to monitor the progress of the electrophoresis. The glycerol provided the sample with a high density so that it could be easily introduced into gel slots which was covered with the TBE buffer.

Electrophoresis was carried out at 1.5V/cm for 16 h for a better separation although 10 V/cm for 2.5 h provided conditions for rapid checking. The gel slots were connected with the cathode of the power supply since DNA mobility moved toward the anode. After electrophoresis gels were stained with 0.5 µg/ml ethidium bromide in water or TBE for 10-30 min, destained in water for 10-15 min, visualized using a UV transilluminator and photographed through a red filter with the polaroid camera, using polaroid film number 667.

2.10 Determination of Molecular Weights of DNA

A *Hind*III λ DNA digest was universally used as a standard to determine the molecular weight of DNA fragments. The relative mobilities of DNA fragments were measured and molecular weights determined graphically from a plot of relative mobility versus \log_{10} molecular weight (Sanger *et al.*, 1982). The Gel Frag Sizer programme written by Gilbert, D.G. (1989), dog star software, Biology Department, Indiana University, Bloomington, Indiana, USA was also used. BRL's 1kb ladder purchased from BRL Inc., MD, USA. was also used as another standard.

2.11 Recovery of DNA fragments from agarose Gel

The method used was a modification of that described by Weislander (1979). The DNA fragment was separated on a 0.7% (w/v) agarose gel (SeaPlaque, a low temperature melting at 45°C, GTG grade obtained from FMC Co. Rockland, ME, USA).

The SeaPlaque agarose gel solution was prepared by adding 1.4 g of SeaPlaque agarose into 200 ml of TAE buffer (section 2.3.1) and melted in a microwave. The gel solution was allowed to cool at 50°C in a water bath then poured and allowed to set in an electrophoresis chamber. The TAE buffer was poured into a chamber sufficient to cover gel slots.

The DNA sample was added into the gel slot and electrophoresed at an appropriate lower voltage to prevent from overheating during the electrophoresis which would result in melting the gel. Alternatively the electrophoresis was carried out in a cold room (in most cases it was carried out at 40 volts for 3-4 h on the bench). After gel electrophoresis the localized DNA band(s) of interest was excised from the gel using a sterile scalpel with the minimum amount of excess agarose. The cut band was then cut into small pieces and transferred into a 1.5 ml Eppendorf tube. The gel pieces were melted at 65°C for 10 min in a water bath and the melted gel was then mixed by inversion. A covering volume of Tris-equilibrated phenol (section 2.3.5) was added to the melted gel. The gel mixture was mixed by inversion and was frozen overnight at -

20°C. The Eppendorf tube was centrifuged in a microcentrifuge at top speed for 10 min. The aqueous phase was collected and extracted once with phenol/chloroform and then with chloroform (section 2.7.2). The preparation was centrifuged and the supernatant carefully collected to avoid contamination with the gel debris. Ethanol was used to precipitate the DNA from the supernatant in the presence of 0.3 M Na acetate (section 2.7.3).

2.12 Enzymatic Manipulation of DNA

The enzymes used were purchased from available commercial supplies (Boehringer Mannheim GmbH Biochemicals, Mannheim, W. Germany; Bethesda Research Laboratories Inc., MD, USA, New England Biolabs and Promega Co. WI, USA) and were as follows: restriction endonuclease enzymes, Calf intestinal phosphatase, T4 DNA ligase, Pancreatic DNase I, Klenow-large fragment of *E. coli* DNA polymerase I.

2.12.1 DNA digestion with restriction endonuclease

Restriction endonuclease cleavage was accomplished by incubating the enzyme(s) with DNA under appropriate conditions (buffer and incubation temperature) as specified in the manufacturer's recommendations.

A single digestion was done by digesting the DNA with one restriction enzyme for at least 1 h under recommended assay conditions. After the digestion was completed, the reaction was stopped by either incubation at 65°C for 10 min or by addition of 1/5 volume of SDS loading buffer (section 2.3.7) and the digestion was checked by gel electrophoresis (section 2.9). If the digestion was incomplete more enzyme was added and the mixture incubated for further 1 to 2 h.

A multiple digestion was carried out by digesting the DNA with more than one enzyme. Two or more enzymes were added at the same time to the digestion tube if it was appropriate to have the same reaction conditions for all enzymes. When different

reaction conditions were required (usually differences in NaCl concentration in the assay buffer) then the enzyme reacting at lowest NaCl concentration was added first and the digestion completed. Then the salt concentration of the reaction mixture was adjusted by adding sufficient 5 M NaCl to suit the second enzyme which was then added. An alternative was to use 10x Universal buffer (section 2.3.6) and to add NaCl to the required concentration in each digestion reaction.

2.12.2 Dephosphorylation of vector DNA

Calf intestinal phosphatase (CAP) was used to remove 5' phosphates from the vector DNA fragment before the ligation experiment. The method used was based on that described in Maniatis *et al.* (1982). Approximately 1.0 µg vector DNA was digested to completion by the appropriate restriction enzyme (section 2.12.1). One µl of CAP was added and the mixture was incubated for 30 min at 37°C. An additional 1.0 µl of CAP was added and the mixture was incubated for a further 30 min at 37°C. The mixture was heated at 65°C for 10 min, then extracted with phenol/chloroform (section 2.7.2) and precipitated with 2 volume of ethanol (section 2.7.3). The precipitated DNA was resuspended in sterile water.

2.12.3 DNA ligation

The insert and vector DNA were added into the ligation mixture (usually 30-50 µl) in the ratio 1:2 for the dephosphorylated vector (usually 0.2- 1.0 µg of vector DNA). T4 DNA ligase was used at a final concentration of 1 Weiss Unit/µl and freshly prepared ATP (ATP was prepared according to Maniatis *et al.* (1982) was added into the ligation buffer (10x concentration: 0.66 M Tris-HCl, pH 7.5, 100 mM MgCl₂, 100 mM DTT) to give a final concentration of 1 mM. The ligation mixture was incubated at 4°C overnight. An aliquot (2-3 µl) was checked by gel electrophoresis and used directly for transformation. If electroporation was desired for transformation experiment then the ligation mixture was further purified by micro-dialysis (as previously described in section 2.7.6).

2.12.4. Labelling DNA by using random primer

Two methods were used for the preparation of [α - 32 P]dCTP labelled probe DNA. Deoxycytidine 5' [α - 32 P] triphosphate (3000 Ci/mmol) was purchased from Amersham International plc, UK. dNTPs were supplied from Sigma Co., St.Louis, MO, USA.

The method used was described by Taylor *et al.* (1976). Each dNTPs was dissolved in 50 mM Tris-HCl pH 7.6 to give a final concentration of 20 mM. DNA (0.25-1.0 μ g) to be labelled was digested with *Hae*III restriction enzyme in a 25 μ l reaction mixture for 1 hr at 37°C. Random primers (100 μ g) obtained from Professor Barry Scott were added and the mixture was boiled for 5 min then chilled rapidly on ice. The following reagents were added in order and as follows: water, 2.5 μ l; 10x *Hae*III buffer, 1.5 μ l; dATP, 1.0 μ l; dGTP, 1.0 μ l; dTTP, 1.0 μ l; [α - 32 P]dCTP, 3 μ l; *E. coli* DNA polymerase I, 1.0 μ l. The mixture was incubated at 37°C for 1 h, and the reaction stopped by adding 2.0 μ l of 0.25 M Na₂EDTA. The mixture was either loaded onto mini spin Sephadex G-50 column (2.7.5) and the DNA peak collected. The labelled DNA was used as a probe for further hybridization experiments or otherwise stored at -20°C until needed.

DNA labelling with [α - 32 P]dCTP was also performed using the Ready-To-Go™ DNA Labelling Kit 27-9251-01 supplied from Pharmacia, P-L Biochemical, Inc. The method used was carried out according to the manufacturer's recommendation.

2.13 DNA Transfer

The DNA sample from agarose gel was transferred onto a membrane for hybridization experiment. Nitrocellulose membrane (Schleicher and Schull BA 85) and Hybond-N Nylon membrane (Amersham International plc, UK) were both used.

The DNA transfer technique used was that described by Southern, (1975). Gels were stained and photographed then rinsed with water and drained well. DNA was subjected to partial depurination by shaking the gels in 0.25 M HCl for 20 min and were then

washed twice with water, drained well then denatured by shaking for 20 min in 0.5 M NaOH, 0.5 M NaCl, washed twice with water, drained well and neutralized in 0.5 M Tris containing 2.0 M NaCl at pH 7.2 for at least 30 min to 1 h. The gel was then used in the Southern Blot procedure.

A glass tray was used to assemble the layers of Whatmann 3MM paper without air bubbles. The tray was wet with 20x SSC (section 2.3.10). Four pieces of Whatman 3 MM paper were cut to fit the bottom of the tray and saturated with 20x SSC. The assembly was then carefully wrapped with a piece of polyethylene sheet to avoid air bubbles and identified with appropriate label. The polyethylene sheet was cut smaller than the marked template to give a slightly smaller well. The gel from the neutralizing step was placed on top of the well and a nitrocellulose filter or a nylon membrane was cut to the same size as the gel. The membrane was wet with water and placed on the gel without entrapment of any air bubbles. Three pieces of 3MM filter paper were cut slightly larger than the gel. One of these three pieces was saturated with 20x SSC and put on top of the nitrocellulose or nylon membrane and on top of it was placed the other two dry pieces on top. A thick layer of paper towel was placed on top and a plexiglass plate was positioned on top of the paper towel layer. Finally, a weight placed on top provided a pressure on the gel. The following day the membrane filter was removed from the tray, washed briefly in 2x SSC (section 2.3.11), blotted dry and baked *in vacuo* at 80°C for 2 h. The membrane was ready for the hybridization procedure (see 2.14). The gel was restained and photographed to check for any residual DNA.

2.14 DNA-DNA Hybridization to Southern Blots

The following procedure was used for the hybridization of [α -³²P]dCTP labelled DNA probe to Southern blots. The filter membrane with baked DNA was transferred to a hybridization tube containing 25 ml of hybridization buffer (section 2.3.14). Prehybridization was conducted at 65°C for at least 2 h. Most of the prehybridization buffer was then drained off and 10 ml of hybridization buffer (section 2.3.13) was added together with the denatured [α -³²P]-labelled (10^7 cpm) DNA probe. The radiolabelled DNA probe (2.12.4) was denatured by boiling for 10 min and chilling quickly on ice

prior to hybridization. Hybridization proceeded at 65°C overnight. The filter was washed twice for 15 min with 2x SSC containing 0.1% SDS at room temperature and subjected to further washings for higher stringency with 0.1x SSC containing 0.1% SDS twice at room temperature for 15 min each wash. The washed filter was then blotted dry, covered in "Gladwrap" polythene plastic film and exposed to an X-ray film (Cronex, Du pont Ltd, Australia) in the presence of a Cronex intensifying screen at -70°C for 1-7 d. This exposure time varied with the expected radioactivity signal left on the filter after washing. If the filter had to be re-used, the probe DNA was removed totally by washing, firstly for 20 min in 20 mM NaOH then for 15 min in 0.5 M Tris, pH 7.4 containing 2.0 M NaCl and finally 15 min in 2x SSC. After air drying it was stored for further use.

2.15 Hybridization of Synthetic Oligonucleotide Probe to DNA Bound to Nylon Membrane.

2.15.1 Labelling 5 ' End by the Forward Reaction

The method used for 5 'end labelling was based on the method described by Chaconas and Van de Sande, (1980). The oligonucleotide used was synthesized using an automated DNA synthesizer and dephosphorylated at the 5'end to be ready for the enzyme T4 polynucleotide kinase (Biochemistry Department, Massey University, Palmerston North, New Zealand). The [γ -³²P]ATP with specific activity 3000 Ci/mmol was purchased from Amersham International, plc, UK. T4 polynucleotide kinase was supplied from Promega, Co, WI, USA.

The lyophilized form of the oligonucleotide was dissolved in deionized water to give a final concentration of 0.5 mg/ml and stored at -70°C until needed.

The labelling reaction was carried out in a total volume of 50 μ l with the following reagents: 4 μ l of oligonucleotide linker (2 μ g), 5 μ l of 10x linker-kinase buffer (0.5M Tris-HCl, pH 7.6, 0.1 M MgCl₂, 50 mM DTT, 1 mM spermidine and 1 mM EDTA), 3 μ l of T4 polynucleotide kinase (10 units were required), 15 μ l of [γ -³²P]ATP, 5 μ l of 10

μM ATP, 18 μl deionized water. The reaction mixture was incubated at 37°C for 30-40 min and stopped by adding 1 μl 0.5 M Na_2EDTA .

The [$\gamma\text{-}^{32}\text{P}$]-labelled oligonucleotide was purified from the unincorporated [$\gamma\text{-}^{32}\text{p}$]ATP via chromatography over DE-52 cellulose ion exchange column (Wallace and Miyada, 1987). The column was prepared as follows: 15g of DE-52 cellulose (Whatman) was resuspended in 400 ml of 0.5 M NaOH and brought to 500 ml with deionized water. The resin was collected on a Whatman No.41 or No.54 filter and sucked dry on a Buchner funnel. To the filter cake of DE-52 was added 200ml of water containing 1 M NaCl. The cake was then washed with 1 M HCl, filtered dry and then washed with 100 ml of 0.5 M NaOH, filtered again and washed with deionized water. The DE-52 cake was resuspended in 200 ml of 1x TBE buffer and kept at 4°C in screw capped container.

After the labelling reaction was completed, the reaction mixture was diluted to 1 ml with TE buffer (section 2.3.3) and loaded on to the DE-52 column. The DE-52 was packed into 1 ml disposable syringe of bed volume $\approx 0.2\text{ml}$ and equilibrated with TBE buffer (section 2.3.2), then washed with TBE buffer. The pre-elution wash with 2 volumes of TBE buffer containing 0.2 M NaCl was followed with a 3 volume wash with TBE buffer. The preparation was applied and rinsed with 2 volumes of TBE, then 2 volumes of TBE containing 0.2 M NaCl. Elution of the [$\gamma\text{-}^{32}\text{P}$]-labelled oligonucleotide was achieved using 0.5 ml of TBE containing 1.0 M NaCl. The collected aliquots were checked for radioactivity with a scintillation counter. The eluted oligonucleotide was either used directly in hybridization experiments or stored at -20°C for no longer than one week.

2.15.2 DNA transfer to hybridize with [$\gamma\text{-}^{32}\text{P}$]-labelled oligonucleotide probe

The method used to transfer DNA from the gel to a nylon membrane was described by Wallace and Miyada (1987). Southern blots were all transferred to nylon membranes and prepared for hybridization experiments in the same way as previously described (see 2.13) except that the depurination step was omitted. The DNA was first denatured by

treating with 0.5 M NaOH containing 1.5 M NaCl for 30-45 min at room temperature. The preparation was then neutralized in 1 M Tris-HCl containing 2.0 M NaCl, pH 7.0 for 30-45 min at room temperature. The technique used to transfer DNA from the gels was the same as that described above for Southern blot except that 10x SSC was used instead of 20x SSC.

2.15.3 Hybridization of DNA immobilized on nylon membrane with [γ - 32 P]ATP labelled oligonucleotide probe.

The hybridization method used was the modification of the method described by Miyada and Wallace (1987). Prehybridization buffer containing final concentration of 5x SSC, 20 mM sodium phosphate, pH 7.0, 10x Denhardt solution (see 50x Denhardt in section 2.3.12), 100 μ g/ml of denatured salmon sperm DNA and 7% (v/v) SDS was added to the hybridization tube and the mixture incubated for at least 2 h at 30°C. The pre hybridization buffer was drained off then the hybridization buffer (i.e. prehybridization buffer with 10% (w/v) dextran sulfate) was added and followed with the addition of 2×10^6 cpm/ml of [γ - 32 P]ATP labelled oligonucleotide probe. The hybridization was allowed to proceed for at least 16 h. at 30°C. The filter membrane was washed twice at 30°C with 200 ml of washing solution (30 ml 20x SSC; 10 ml 200 mM sodium phosphate, pH7.0; 40 ml 50x Denhardt solution; 50 ml 20% (w/v) SDS; 70 ml H₂O) for 30 min each at 30°C. After another wash with 1x SSC, 1% SDS for 30 min at 30°C the membrane was blotted dried and wrapped with " Gladwrap" polythene plastic film. An X-ray film (Cronex, Du pont, Ltd, Australia) was exposed to the washed filter in an X-ray cassette with an intensifying screen at -70°C for 2-3 d. Visualization was possible of any hybridized DNA.

2.16 Transformation of plasmid DNA

Transformation was carried out both in *E. coli* and lactococci via electroporation. Also, in *E. coli* the CaCl₂ method was used for transformation experiments.

2.16.1 CaCl₂ transformation in *E. coli*

Preparation and transformation of *E. coli* cells was performed by the following method which was modified from that described in Maniatis *et al.* (1982). To prepare competent *E. coli* cells using the CaCl₂ method, a 1% (v/v) inoculum of *E. coli* was prepared from an overnight culture of *E. coli* JM109 or DH5 α grown in 100 ml of LB medium. The culture for transformation experiments was grown with vigorous shaking at 37°C for 2-3 h. Cells were harvested by centrifugation at 4340 x g for 10 min at 4°C and the cells kept chilled (on ice). Cells were washed with 100 ml of ice-cold 100mM MgCl₂, 10 mM Tris at pH8.0 and centrifuged at 1485 x g for 10 min at 4°C. The cell pellet were chilled on ice and gently resuspended in 100 ml of ice-cold solution of 100 mM MgCl₂, 10 mM Tris-HCl at pH 8.0, kept on ice for 20 min, then harvested by centrifugation at 1485 x g for 10 min at 4°C. The cells were gently resuspended in 50 ml of ice-cold solution of 50 mM CaCl₂ in 10 mM Tris-HCl at pH8.0, kept chilled on ice for 20 min then centrifuged at 1485 x g for 10 min at 4°C. Cells were gently resuspended in 2.5 ml of ice-cold solution of 50 mM CaCl₂ in Tris-HCl at pH8.0 containing 15% (w/v) glycerol and kept chilled on ice until used or dispensed into aliquot of 200 μ l to Eppendorf tubes and stored at -70°C for at most 6 month. All the steps involved were carried out using aseptic technique and at 0-4°C.

The transformation was carried out in an Eppendorf tube. Competent cells of *E. coli* (100 μ l) were added to 10 μ l of ligation mix, gently mixed and incubated on ice for 30 min to 1 h with occasional gentle mixing. The mixture was then heat shocked at 42°C for 2 min and immediately returned to ice for 5 min. Then 1ml of LB medium was added and incubated at 37°C without shaking for 30 min. A further incubation for 1-1.5 h was conducted on the rotating shaker at 37°C. The transformation mixture was then plated on selective agar and positive colonies were sought.

2.16.2 Electroporation in *E. coli*

The method used was according to the method described by Dower *et al.* (1988). To prepare electrocompetent *E. coli* cells, a 1 l shake culture was harvested from a 1-liter

at mid-log growth phase (A_{600} of 0.5 to 1) from LB medium at 37°C by centrifugation at 4340 x g, 10 min at 4°C. The cells were washed with 2 x 1 l of ice-cold sterile Milli Q water, centrifuged at 1485 x g 10 min at 4°C and resuspended in 2 to 4 ml of sterile 10% (w/v) glycerol. On dispensing into 100 µl aliquots, tubes were frozen on dry ice and stored at -70°C.

Electroporation in *E. coli* was performed using a Gene Pulser apparatus (Bio-Rad Lab., Richmond, CA, USA). The parameter of electroporation was set for the pulse generator at 25 µF capacitor, 1.25 kV/cm and 600 Ω in parallel with the chamber which contained 2 mm Gene Pulser cuvettes. The electrocompetent cells of *E. coli* were thawed at room temperature and placed on ice. From 1 to 10 µl of the ligation mix or DNA was gently dripped on one side of a chilled cuvette then 40 µl of thawed cells were added and the preparation was mixed by flicking the cuvette. The outside of the cuvette was dried and it was placed between electrodes in the safety chamber. The pulse was applied after which the cuvette was immediately removed from the electrodes and 960 µl of ice-cold SOC medium was added, mixed and transferred into an Eppendorf tube. The broth was then incubated with shaking at 37°C for 1 to 1.5 h. At the end of this expression period, the cells were plated on appropriate selective LB agar to screen for positive transformants.

2.16.3 Transformation of lactococci by electroporation

The procedure was a modified method of Powell *et al.* (1988). To prepare electrocompetent cells of lactococci, 2.5 ml overnight culture of lactococci were used to inoculate 100 ml of modified M17 medium (M17 broth + 0.5% glucose and 40mM DL-Threonine). After 2-4 h grown at 30°C (i.e. to A_{600} of 0.2 - 0.6) cells were collected by centrifugation in a chilled rotor at 1085 x g for 15 min, washed in 25 ml of ice-cold EPB2 buffer (0.5 M sucrose containing 1 mM HEPES, pH7.4) and recentrifuged as above. On resuspending the cells in 2.5 ml of ice-cold EPB2 buffer they were kept on ice until needed or stored at -70°C for no longer than 2 months.

The electroporation of lactococci was also carried out by using the same Gene Pulser apparatus and 2mm cuvette as described for *E. coli*. The electrocompetent cells were thawed and left on ice. The procedure used was identical to that described for the electroporation of *E. coli* (see 2.16.2) except that the mixture was pulsed at 1.25 kV/cm and 25 μ F with a Pulse Controller set at 200 Ω . The electroporated cells were immediately transferred into 800 μ l of ice-cold expression broth (M17GS, section 2.2) for 2-3 h and plated on selective M17G to enable the detection of positive transformants.

2.17 β -galactosidase enzyme assay

The agar plate plus a chromogenic substrate X-gal (5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside) which is cleaved by β -galactosidase to galactose plus the insoluble blue indoyl derivative was used for qualitative β -galactosidase assay. X-gal substrate solution was prepared to give a final concentration at 20 mg/ml in dimethyl formamide, stored in a dark bottle and kept at -20°C. X-gal solution was added into 100ml of agar media after sterilization and allowed to cool at 50°C. For lactococci, the medium agar used was M17, or M17G agar. For *E. coli*, the medium used was LB agar with an optional addition of either 0.1% (w/v) lactose or 60 μ l of IPTG stock solution (section 2.2). The 100 ml medium was poured into 4 agar plates. The positive transformants (Lac⁺) were detected as blue colonies on agar plates.

For quantitative determination of β -galactosidase activity from *E. coli* strain the method of Miller (1972) was used. The β -galactosidase activity in permeabilized *E. coli* cells was assayed using the chromogenic substrate o-nitro-phenyl- β -D-galactoside (ONPG). ONPG substrate was prepared by dissolving of 4 mg of ONPG into 1 ml of Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄·7H₂O and 50 mM β -mercaptoethanol) and then kept frozen at -20°C in a dark bottle. Half a milliliter of the exponential-phase cells, grown in minimal medium of either AI or M56 (section 2.2), were added to an equal volume of Z buffer. Then two drops of Triton-X solution [10% (w/v) in H₂O] and one drop of SDS [0.1% (w/v) in H₂O] was added. The mixture was vortexed for 10 sec and incubated for 5 min at 28°C. Two hundred μ l of ONPG was added and the assays were timed until a pale yellow colour developed. The reaction

was then stopped by addition of 450 μl of 1 M Na_2CO_3 . The cells were removed by centrifugation at top speed in a microfuge and the amount of o-nitro-phenyl (ONP) produced was determined by measuring the absorbance at 420 nm. The activity was measured as Units of β -galactosidase = $1000 \times A_{420}/\text{time} \times \text{vol} \times A_{600}$.

2.18 DNA Sequencing

The sequencing method performed in this study was based on the dideoxynucleotide chain termination method developed by Sanger *et al.* (1977). The DNA fragment subcloned into sequencing plasmid vector pGEM-3Z containing SP6 and T7 promoters primers were used for sequencing the DNA templates. The actual method using double-stranded closed circular plasmids as DNA templates was developed by Chen and Seeburg (1985). The series of DNA overlapping fragments used for sequencing strands were obtained by the *ExoIII* unidirectional deletion method. The 'Sequenase' version 2.0 sequencing kit (Tabor and Richardson, 1987) used was purchased from USB, Cleveland, OH, USA. [α - ^{35}S]ATP (specific activity 250 $\mu\text{Ci}/\text{mmole}$) was obtained from Amersham International, plc, UK. DNA was sequenced using a S2 model gel apparatus purchased from BRL inc., MD, USA.

2.18.1 The ExonucleaseIII unidirectional deletion procedure

The method used was provided in 'Erase-a-BaseTM System' technical manual (Promega Co., WI, USA). All chemicals, enzymes and buffers were supplied in an 'Erase-a-Base' kit. The double-digested DNA (suited for unidirectional digestion by *ExoIII*) was resuspended in 60 μl of 1x *ExoIII* buffer (10x: 660 mM Tris-HCl, pH8.0, 6.6 mM MgCl_2). Twenty five Eppendorf tubes containing 7.5 μl of S1 mix [(for 25 time laps sample): 172 μl deionized H_2O , 27 μl 7.4x S1 buffer (7.4x S1 buffer contained 0.3 M potassium acetate, pH 4.6, 2.5 M NaCl, 10 mM ZnSO_4 , 50% (w/v) glycerol), 60 units S1 nuclease] were left on ice. The DNA tube was pre-warmed to 37°C in a water bath. Exonuclease III 200-300 units was added and mixed as rapidly as possible. At 30 sec intervals 2.5 μl were removed into S1 tubes on ice. Following sampling all tubes were brought to and held at room temperature for 30 min when 1 μl of S1 stop buffer (0.3

M Tris base, 0.05 M EDTA) was added and the tubes were heated at 70°C for 10 min. The extent of unidirectional digestion was analysed by loading 2µl sample from each time interval on to 1% (w/v) agarose gel for checking by gel electrophoresis. All samples were transferred to 37°C and 1µl of Klenow mix [30µl 1x Klenow buffer (20 mM Tris-HCl, pH 8.0, 100 mM MgCl₂), 3-5 units Klenow DNA polymerase were added to each each tube and incubated for 3 min. Then 1 µl of dNTPs mix was added and the incubation continued for 5 min. The samples were removed to room temperature and 40µl of ligase mix [(freshly prepared for each experiment): 790 µl deionized H₂O, 100 µl 10x ligase buffer (500 mM Tris-HCl, pH 7.6, 100 mM MgCl₂, 10 mM ATP), 100µl 50% PEG, 10µl 100 mM DTT, 5 units T4 DNA ligase] were added to each, mixed well and incubated at 4°C overnight. The DNA sample from each time interval was used to transform competent *E. coli* cells using the CaCl₂ method.

An alternative procedure for generating unidirectional digestion by Exonuclease III involving filling in the 5' protruding end with α-phosphorothioate nucleotides (α-dNTPs) was also used according to the 'Erase-a-Base' system protocol supplied by Promega Co., WI, USA. DNA was digested to completion with the chosen restriction enzyme for the vector protecting site, then purified with phenol/chloroform and precipitated with ethanol. The DNA was then resuspended in 1x Klenow buffer. α-dNTPs mix was added to give a final concentration of 40 mM. Then 1 mM DTT and Klenow polymerase (50 units/ml) were added and the mixture was incubated at 37°C for 10-15 min. After heating at 70°C for 10 min the DNA was purified and subjected to a second restriction digestion. After the second digestion was completed, the DNA was purified and then digested with *ExoIII* digestion procedure as described above.

2.18.2 Sizing of the Exonuclease III deletion subclones

The size of DNA isolated from the Exonuclease III subclones at each time interval was determined by either single digestion with the restriction endonuclease which resulted in a linear DNA fragment or by double digestion with an appropriate pair of restriction endonucleases, which resulted in an isolated insert fragment from the plasmid vector.

The digested DNA samples were then checked on a 0.7% (w/v) gel electrophoresis at 20 volts for 16 h (see section 2.9).

2.18.3 Preparation of double-stranded DNA template

DNA templates were isolated from the Exonuclease III subclones using the method described in 2.6.2. When more purified DNA preparation was required the DNA was subjected to CsCl-buoyancy density gradient ultracentrifugation (as described in 2.7.1).

2.18.4 Alkaline denaturation of double-stranded DNA template

The method used was according to Hattori and Sakaki (1986). DNA was diluted to a volume of 18 μ l in a small Eppendorf tube to give a final concentration of 2-4 μ g and denatured with 2 μ l of 2 M NaOH at room temperature for 5 min. Then 8 μ l of 5 M ammonium acetate, pH 7.5 was added followed with 100 μ l of absolute ethanol. The preparation was kept at -70°C for 10-20 min. On recovering the DNA by centrifugation in the microfuge at full speed for 15-20 min it was washed with 75% (v/v) ethanol, dried *in vacuo* and resuspended in 7.0 μ l of H₂O, prior to the annealing step. Alternatively, it was stored at -20°C and used within 1 wk.

2.18.5 The annealing and labelling reaction

The procedures used in annealing and labelling reaction were according to the Sequenase Kit version 2.0 protocol provided by USB, Cleveland, OH, USA.

2.18.6 Gel electrophoresis in DNA sequencing

The S2 model gel apparatus (BRL Inc., MD, USA) was used.

The preparation of gel solution and gel assembly were carried out according to the protocol provided by the company.

2.18.6.1 Preparation and assembly of glass plates

Glass plates were cleaned thoroughly with non-abrasive detergent, rinsed well and wiped over with ethanol. The inside of the top plate was siliconized with dimethyldichloro silane (BDH), and the plates were wiped with ethanol and polished dry. Spacers were placed on the side of the back plate, then the top plate. After checking for a good alignment between both plates and the spacers, they were all wrapped with binding tape to ensure no gaps. The sides were then clamped.

2.18.6.2 Preparation of gel solutions and sequencing gel

An acrylamide stock [40% (w/v)] was prepared as follows: 380 g of acrylamide (ultra pure grade, BRL, USA) and 20 g of bisacrylamide (ultra pure grade) were dissolved in water and brought to a final volume of 1 l. The solution was then filtered through 3MM Whatman paper No.1 and stored in the dark at 4°C.

A standard 6% (w/v) polyacrylamide sequencing gel was used and prepared as follows: 50 g of urea (ultra pure grade, BRL, USA) was dissolved in 35 ml of deionized H₂O then 15 ml of 40% (w/v) acrylamide stock and 10 ml of 10x TBE sequencing buffer (89 mM Tris-HCl, 8.9 mM boric acid, 12 mM Na₂EDTA, pH8.3) were added, mixed well and the mixed gel solution was then de-gassed. Freshly prepared (10% w/v in aqueous) ammonium persulphate (1ml) and 10 µl of TEMED (N,N,N',N'-Tetramethylethylenediamine; stored dark at 4°C) were added into the mixed gel solution. The mixed gel solution was immediately poured between the sandwich glass plate, ensuring that no air bubbles were present and that there were no leaks from the gel solution. The back of the sharktooth comb was inserted up to the guide line of the comb. The gel sandwich was allowed to polymerize for at least 2-3 h.

2.18.6.3 DNA sequencing gel electrophoresis

After the gel sandwich was completely set, the surface was washed and the binding tape removed. The combs were flushed with water and removed. The wells of the gel were

flushed out with 1x TBE sequencing buffer, and re-inserted with the sharksteeth side until the teeth just penetrated the gel. The gel sandwich was then mounted firmly on to the S2 model sequencing apparatus according to the operation manual. Normal strength TBE sequencing buffer was added to the top and bottom reservoirs. The gel was pre-run at 1500 V, 40 mA, 60 W for at least 1 h. The wells were marked off and cleaned by flushing with TBE sequencing buffer from the top reservoir before the DNA sample was loaded. The labelled DNA template samples were heated at 80°C for 2-3 min and immediately returned to ice. Each sample from all 4 reactions was loaded (1.5-2.5 µl) into the cleaned wells so as to avoid air bubbles and in the order G, A, T, C. The gel was run for both a short (2 h) and long (5 h) period. After the completion of the run, the top plate was carefully removed and the gel still attached to the bottom plate was fixed for 20 min with 2 l of fixing solution: [10% (v/v) Methanol and 10% (v/v) glacial acetic in water]. The fixing solution was drained off. The gel was blotted dry, transferred to 3MM Whatman pape, covered with a piece of polyethylene sheet and dried in a gel dryer (Bio-Rad) at 80°C for 2-3 h. The dried gel was exposed to X-ray film (Cronex 4, Du Pont Ltd., Australia) for 2-4 d, at room temperature without an intensifying screen and then developed.

2.18.7 Assembly and analysis of sequence data

Assembly and analysis of sequence data was carried out with the assistance of three software computing programmes: The Clone Manager Program Version 2.3, Serial No. 32065 and the Align Program Version 1.01, Serial No. 41040 were produced by Scientific and Educational Software, USA. The GCG (Genetics Computer Group) software package was developed by the University of Wisconsin Genetics Computer Group and installed on a VAX 750 (Devereux *et al.*, 1984).

CHAPTER 3: SEQUENCING OF A 4.4 kb *EcoRI* FRAGMENT THAT ENCODES D-TAGATOSE 1,6-BISPHOSPHATE ALDOLASE.

3.1 Introduction

Dairy lactococcal strains metabolize lactose via the phosphoenolpyruvate (PEP)-dependent:phosphotransferase system (PTS) in which lactose is phosphorylated and metabolized further by β -D-phosphogalactosidase (P- β -gal), enzymes of the tagatose-6-phosphate pathway and then via the glycolytic pathway. Tagatose 1,6-bisphosphate aldolase (TBP-aldolase) is one of the three enzymes of the tagatose-6-phosphate pathway and hydrolyses D-tagatose 1,6-bisphosphate to the triose-3-phosphates, dihydroxy acetone phosphate and glyceraldehyde-3-phosphate. Previous work has reported that a 4.4 kb *EcoRI* fragment from pDI21 of *L. lactis* ssp. *cremoris* strain H2 encodes the tagatose 1,6-bisphosphate aldolase (Yu *et al.*, 1989).

3.2 Results

The starting material for this work was two clones of pGEM-3Z with a 4.4 kb *EcoRI* fragment from pDI21 in both orientations i.e. pBH401 and pBH501 (Table 12). The DNA sequencing method performed in this study is that described in section 2.18. The double stranded DNA templates used were derived from a series of unidirectional nested deletions. Plasmids pBH401 and pBH501 were isolated from the *E. coli* subclones as described in section 2.6.3 and purified using ultracentrifugation (section 2.7.1). The exonuclease III nested deletions were constructed by Hodge (1989) using restriction enzyme *HindIII* and the created 3'recessive ends were filled in with dNTPs. The 5' overhangs used for exonuclease III digestion were generated using the restriction enzyme *XbaI* as described in section 2.18.1. The nested deletions were then religated and transformed into *E. coli* JM109 and DH5 α (sections 2.12.3 and 2.16.1, respectively). Prior to sequencing the double stranded DNA templates were isolated using the methods described in section 2.6.2 and the size of the DNA templates were determined according to the method described in section 2.18.2. The DNA sequencing was carried out using a Sequenase kit (version 2.0) as mentioned in section 2.18 and the sequence

data generated was analysed using the Wisconsin GCG software package as mentioned in 2.18.7.

A restriction map of this fragment as previously determined by Hodge (1989) is shown in Figure 4. The programme Gel Enter was used to enter the reading sequence data from the sequenced DNA template. Using the programme Gel Assemble sequence data was assembled into contigs and one such contig so-called 401-36a. There were altogether five DNA templates derived from pBH401 and five DNA templates derived from pBH501 that were used to assemble the contig 401-36a as illustrated in Figure 5. The consensus sequence and the diagnostic restriction enzyme sites of the above contig 401-36a is also shown in Figure 6 with many ambiguities remaining. The %G+C content of the 401-36a contig was also determined using the computer data analysis and found to be approximately 52%. The consensus sequence of the contig 401-36a was screened against the GenEMBL DNA sequence data base to identify any sequences that were similar by using the FastA programme. A 95.7% similarity to the *E. coli purM* was demonstrated and an alignment using the programme GAP with this sequence is shown in Figure 7. Analysis of the other consensus sequence 401-46 derived from another contig (data not shown) demonstrated a 97.2% homology to *E. coli purN*, a region downstream of *E. coli purM*. A restriction enzyme map for the *pur* MN region of *E. coli* was drawn from the sequence data available for this region (accession number M13747.Ba) and is shown in Figure 8.

3.3 Discussion

With respect to the present sequencing study and analysis of the data generated, no homology was found between the analogous genes of either closely related bacteria or others including the lactococcal bacteria. Results obtained from the FastA search demonstrated that the sequence data templates derived from the 4.4 kb *EcoRI* are most likely to be *E. coli* DNA and not lactococcal DNA, as the 401-36a consensus sequence obtained showed 95.7% homology to the *E. coli purM* gene (Figure 7) for 1029 nucleotides. The 401-46 consensus sequence obtained also showed 97.2% homology to

Figure 4

Restriction enzyme map of the 4.4 kb *EcoRI* of pBH501 according to Hodge (1989).

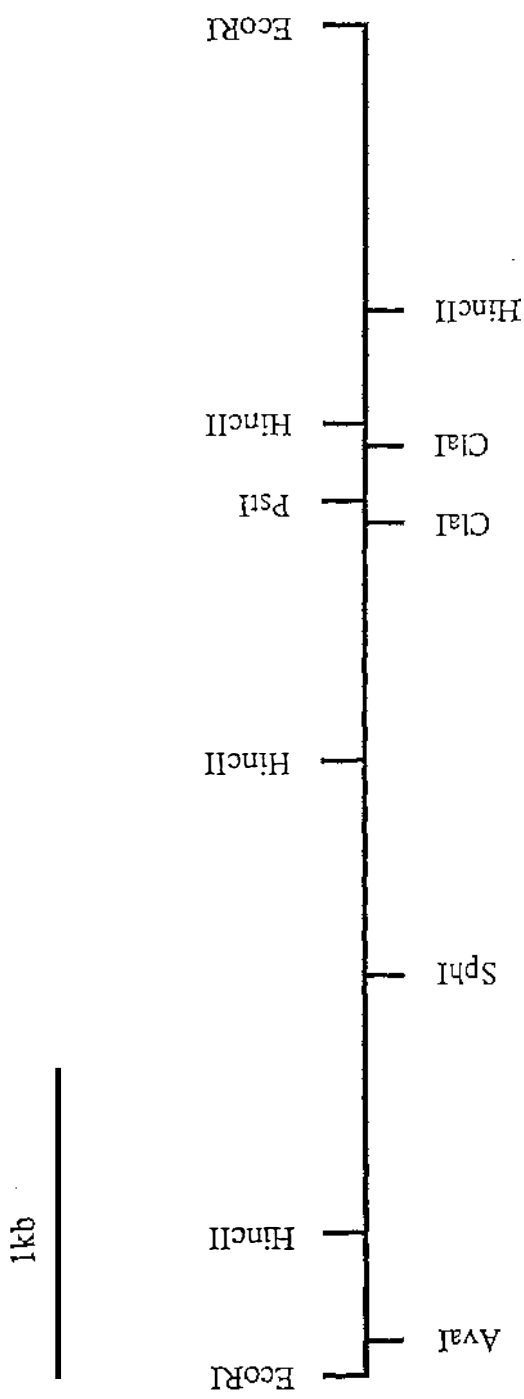
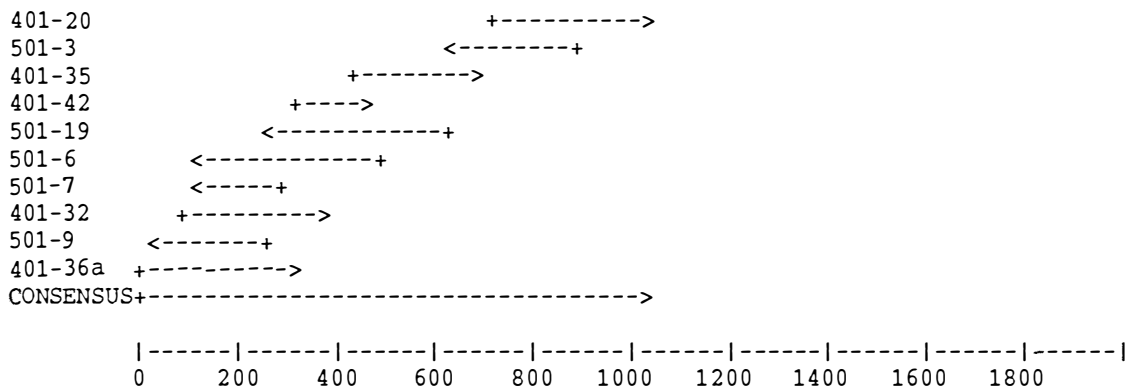


Figure 5

The 401-36a contig demonstrating the gelassembly of the DNA templates from both strands using the Gelassemble and Bigpicture programme of the GCG package, University of Wisconsin.

All fragments



Fragments sorted by strand

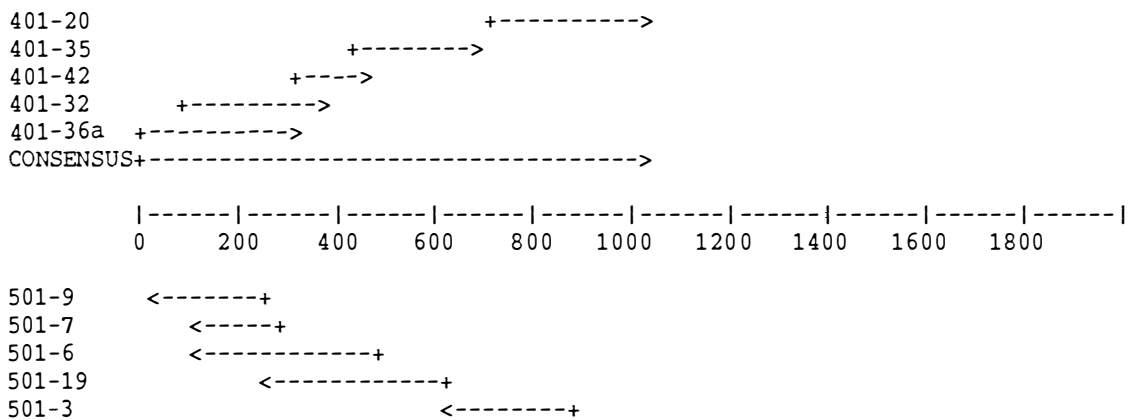


Figure 6

The consensus sequence of the 401-36a contig illustrating the internal *Sph*I and *Bsp*MI restriction sites.

1 CCGGTGTGA TATTGACGCG GGTAATGCTC TGGTTGGAAG AATCaAAGGC
Bsp MII
51 GTAGTGAAGA AAACGCGTCG TCCGGAAGTG ATGGGCGGTC TGGGCGGCTT
101 CGGtgCgCTG TgtgCaTtGC cGCaAAAATA TCGTGaacCC GTGCTgGtTT
151 CTgGCACTgA CGgcgTAgtGT ACCAAGCTgC gTCTgGCAAT gGACTtaAAA
201 CgTCACgACA CCATTGGTAT TGATCTGgTC gCCATGTGwC GTTAATgACC
251 tGGTGGaTGC AAGGTGCAGA GccGCTGTTT TtccTCgACT ATtACgCAAC
301 cgGAAAACTg GATGTTGATa ccgCTTcAGc GkkcAcTCAG CngGCATGTC
351 gGAAGGTTGT CTGCAATcAG GCTgTTcACT GGTgggTgGc ggAAACGGcA
401 gAAATGcccG gcGATGTATC ACGGTGAGGA TTAcGATGTC GCgGgTTct
451 gCGTTGGCGt cGGTAGAAAA ATcAGAAATc ATCGACGGCT CTAAAGTCAG
501 CGAcGGCGAT GTGCTGATTG CACTyGGTTC CAGCGGTCCA CACTCGAACG
551 GCTATTyGCT GGTGCGcAAA ATTCTTGAAG TCAGCGGTTs TGATCCGCAA
601 ACCACCGAAC TTGATGGTAA GCCATTAGCC GATCATCTkC kGsACCGACC
651 CGCATTTACG TGAAGTCAGT GCTGGAGTTG ATTGAAAAaG GTCGATGTGC
Sph I
701 ATGCCATTGC GnACCTGACC GGCGGCGGCT TCTGGnAAAA yATTCCGcgc
751 GTATTGCCAG ATAATACTCA GGCAGTGATT GATGAATCTT CCTGGCAGTG
801 GCCGGAAGTG TTCAACTGGC TGCAAACGGC AGGTAACGTT GAGCACCATG
851 AAATGTATCG CACCTTCAAC TGCGGCGTCG GGATGATTAT TGCCCTGCCT
Bsp MII
901 GCTCCGGAAG TGGACAAAGC CCTCGCCCTG CTCAATGCCA ACGGTGAAAA
951 CnGTGGAAAA TCGGTATCAT CAAAGCTCTG ATTCGACACG TGTATCGATA
1001 TGATATGTGT GCTTATTCGC ACGAGTATT

Figure 7

The alignment between the 401-36a contig and the *Eco purM* gene using the GAP programme of the GCG package, University of Wisconsin.

Symbol comparison table: Gencoredisk:[Gcgcore.Data.Rundata]Nwsgapdna.Cmp
CompCheck: 6876

Gap Weight:	5.000	Average Match:	1.000
Length Weight:	0.300	Average Mismatch:	0.000
Quality:	932.7	Length:	2907
Ratio:	0.906	Gaps:	10
Percent Similarity:	96.572	Percent Identity:	95.299

```

801 AGCTACAAAGATGCCGGTGTGATATTGACGGCGSTAATGCTCTGGTTGG 850
      |||
1 .....CCGGTGTGATATTGACGGCGSTAATGCTCTGGTTGG 37

851 AAGAATCAAAGCGTACTGAAGAAAACGGTCGTCCGGAAGTGATGGGCG 900
      |||
38 AAGAATCAAGCGTACTGAAGAAAACGGTCGTCCGGAAGTGATGGGCG 87

901 GTCTGGGCGGCTTCGGTGCCTGTGTGCATGCGCGCAAAAATATCGTGAA 950
      |||
88 GTCTGGGCGGCTTCGGTgCgCTGTGtgCaTtGCCcGCaAAAATATCGTGaa 137

951 CCCGTGCTGGTTTTCTGGCACTGACGGCGTACGTAACCAAGCTGCGTCTGGC 1000
      |||
138 cCCGTGCTgGtTtCTgGCACtGACGGcgTAgGTACCAAGCTgCgTCTgGC 187

1001 AATGGACTTAAAACGTCACGACACCCATTCGTATTGATCTGGTCCGCCATGT 1050
      |||
188 AATgGACTtaAAACgTCACgACACCCATTCGTATTGATCTGgTCgCCATGT 237

1051 G.CGTTAATGACCTGGTGG.TGCAAGGTGCAGAGCCGCTGTTTTTCCCTCG 1098
      | |||
238 GwCGTTAATgACCTGGTGGaTGCAAGGTGCAGAGccGCTGTTTTTccTCg 287

1099 ACTATTACGCAACCGGAAAACCTCGATGTTGATACCGCTTCAGCGGTGA.. 1146
      |||
288 ACTATtACgCAACcgGAAAACtGATGTTGATaccgCTTCAGcGkkaAct 337

1147 TCAGCGGCATTCGGGAAGGTTGTCTGCAATCAGGCTGTTCACTGGTGGGT 1196
      : |||
338 CAGCngGCATTCGcGAAGGTTGTCTGCAATcAGGCTgTTcACTGGTgggT 387

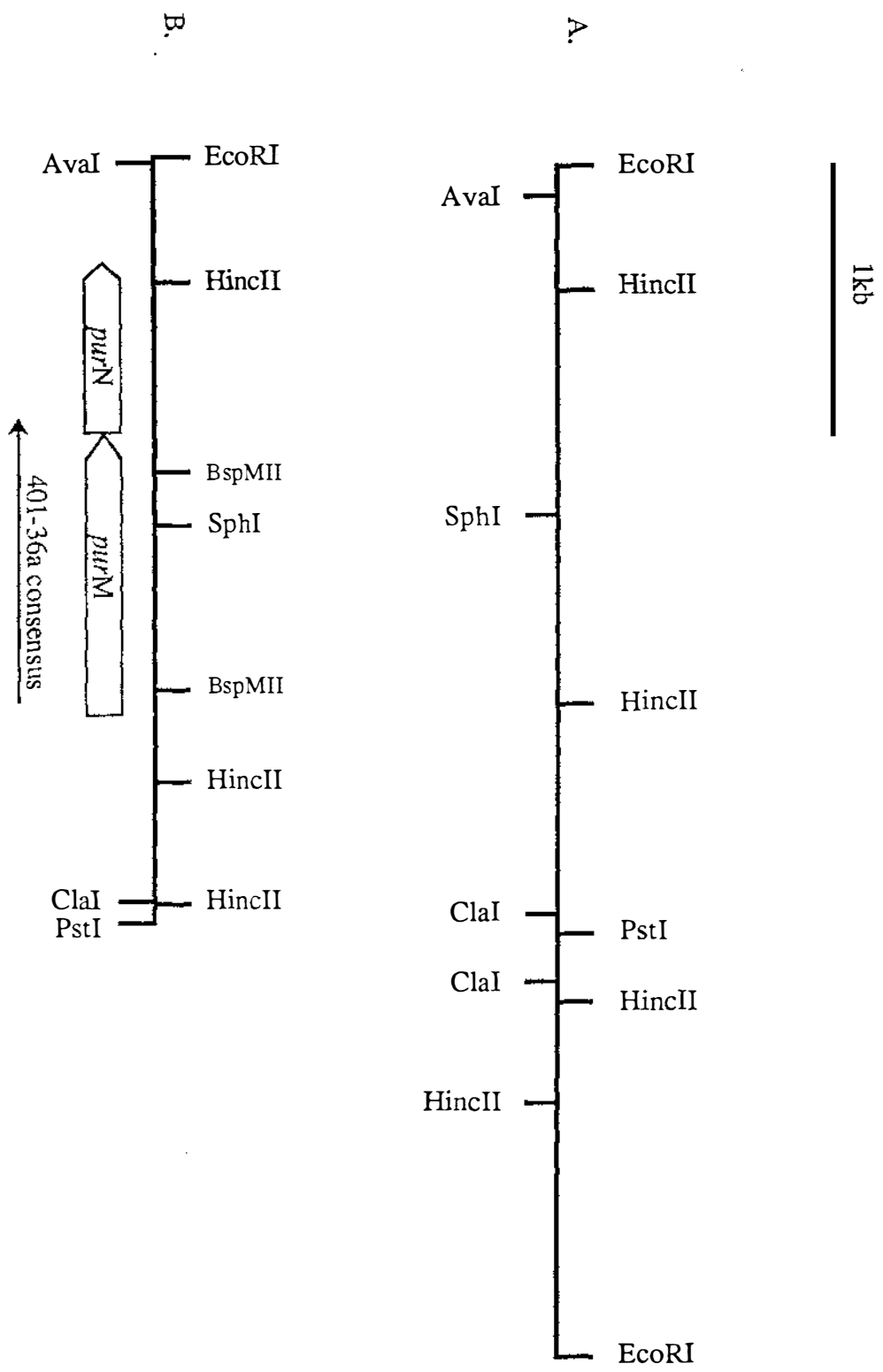
```


Figure 8 A

Restriction enzyme map of the 4.4 kb *EcoRI* fragment of pBH501

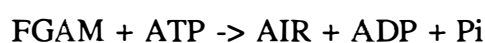
Figure 8 B

Restriction enzyme map of the 4.4 kb *EcoRI* fragment illustrating the location of the 401-36a contig and the *E.coli purM* gene and *purN* gene.



the adjacent gene, *E. coli purN* gene (Figure 8 B) within 713 nucleotides. The 401-36a consensus sequences contained the unique *SphI* site and *BspMII* site of the *EcopurM* gene as shown in Figures 6 and 8 B and the 401-46 consensus contained the unique *BsmI* site of the *EcopurN* gene (data not shown). The restriction sites of the two regions were correlated to the map of *E. coli purMN* gene (Smith and Duam III, 1986; 1987) as shown in Figure 8 B.

The *E. coli purM* and *purN* genes are of chromosomal origin in *E. coli* K-12 (Smith and Duam III, 1986). They encode for enzymes involved in purine nucleotide biosynthesis. The *E. coli purM* gene encodes the enzyme 5'-Phosphoribosyl-5-aminoimidazole synthetase (EC.6.3.3.1) which catalyses the ATP-dependent synthesis of 5'-phosphoribosyl-5-aminoimidazole (AIR) from 5'-phosphoribosyl-formylglycinamide (FGAM) as shown by following equation (Levenberg and Buchanan, 1957):-



This reaction catalyses the fifth step, i.e. the imidazole ring closure, in the *de novo* synthesis of the purine nucleotides. The 5'-phosphoribosyl-5-aminoimidazole synthetase encoded by *Eco purM* gene contains 344 amino acid residues and has a calculated M_r of 36,726.

The *E. coli purN* gene encodes the enzyme 5'-Phosphoribosylglycinamide transformylase (EC.2.1.2.2) which catalyses the N^{10} -formyl tetrahydrofolic acid (H_4 folate)-dependent synthesis of 5'-phosphoribosylformylglycinamide (FGAR) from 5'-phosphoribosyl glycinamide (GAR) as shown by the following equation (Levenberg and Buchanan, 1957):-



This reaction catalyses the third step and is the first of two transformylation reactions in the *de novo* synthesis of purine nucleotides. The GAR transformylase encoded by *Eco purN* gene has a calculated M_r of 23,241 and contains 212 amino acid residues.

The DNA sequence of the 4.4 kb *EcoRI* fragment from pBH401 and pBH501 used in this study shows strong homology to the *Eco purMN* operon region. The 2.9 kb *PstI-EcoRI* chromosomal insert derived from pLC1-41, was subcloned into pUC9 and transformed *E. coli* strain TX393 by Smith and Duam III (1986).

Because the original starting material proved to be *E. coli* DNA and not lactococcal DNA. It requires further comment in the form of a brief analysis of the origins of the provided starting material. As mentioned earlier the 4.4 kb *EcoRI* DNA fragment used in this study was initially reported to encode the tagatose 1,6-bisphosphate aldolase (Yu *et al.*, 1989). This 4.4 kb *EcoRI* fragment was originally isolated from an *EcoRI* gene library of pDI21. pDI21 is a plasmid resulting from the conjugation transfer between *L. lactis* ssp. *cremoris* strain H2 (a donor) and *L. lactis* ssp. *lactis* strain H1 (a plasmid-free recipient). The *EcoRI* gene fragment library of pDI21 was constructed by a complete *EcoRI* digestion of pDI21 followed by cloning into the bacteriophage vector λ NM1149. A clone designated λ NM1149-2 containing a 4.4 kb *EcoRI* fragment from pDI21 was reported to encode the tagatose 1,6-bisphosphate aldolase (Yu *et al.*, 1989). The λ NM1149-2 was adsorbed to *E. coli* host strain POP-13, amplified and the λ DNA isolated. The 4.4 kb *EcoRI* fragment was isolated from the λ NM1149-2 DNA and subcloned into *E. coli* plasmid vector, pGEM-3Z and transformed into *E. coli* strain MC1022 (Hodge, 1989). Two clones were found to harbour the 4.4 kb *EcoRI* insert with both orientations and were designated pBH4 and pBH5. These two 4.4 kb *EcoRI* DNA inserts were isolated from pBH4 and pBH5 and subsequently subcloned into a new stock of pGEM3Z and transformed into a new *E. coli* host strain, JM109. The clones were redesignated as pBH401 and pBH501, respectively (Hodge, 1989).

The results obtained from the FastA Serve Search become significant when comparisons are made with similar data from Gram-positive bacteria. The nucleotide sequences of the entire Lac-PTS operon of *L. lactis* ssp. *lactis* MG1820 (*lacR*, *lacFEGX*, and *lacABCD*) have now been determined (van Rooijen and De Vos, 1990; De Vos *et al.*, 1990; van Rooijen *et al.*, 1991, van Rooijen *et al.*, 1992). The transcriptional studies show that the *lacABCD* are the first four genes of the lactose-inducible-Lac-PTS operon of *L. lactis* ssp. *lactis* MG1820 consisting of *lacABCDFEGX* in that order. The *lacR*

gene encodes for the repressor of the Lac-PTS system, *lacFEGX* encodes for Enzyme III^{lac} (Factor III), Enzyme II^{lac}, phospho- β -galactosidase and a protein X of unknown function, respectively. The *lacABCD* encodes a tagatose 6-phosphate pathway gene cluster. *LacAB* encodes for the multimeric galactose-6-phosphate isomerase (15 and 19-kDa subunits), *lacC* encodes for the tagatose-6-phosphate kinase (33-kDa) and *lacD* encodes for the 36-kDa of tagatose 1,6-bisphosphate aldolase. Van Rooijen and colleagues suggested that in Gram-positive cocci the *lacABCD* have evolved from a common ancestor and there is a basic homology existing between the deduced amino acid sequences of LacD of *L. lactis* ssp. *lactis* and LacD of *Staphylococcus aureus* (i.e. between Gram-positive coccal bacteria). In addition, the nucleotide sequence and the deduced amino acid sequences of the *lac* genes of the Lac-PTS system of lactococcal bacteria are also highly homologous to those from *Staphylococcus aureus* and *Lactobacillus casei* (Porter and Chassy, 1988; De Vos and Gasson, 1989; De Vos *et al.*, 1990). De Vos *et al.* (1990) suggested that the individual *lacF*, *lacE*, and *lacG* genes and possibly other components of the lac-operon, have been acquired independently and/or have been reshuffled after acquisition, possibly in order to allow optimal gene expression and regulation.

The 4.4 kb *EcoRI* fragment expresses the synthesis of 41-, 23-, and 12- kDa proteins which correspond to the TBP-aldolase, Lac-PTS Enzyme II^{lac} and Factor III respectively, (Hodge, R.A. unpublished data). As mentioned earlier, the nucleotide and deduced amino acid sequences of the lactose genes of Lac-PTS system of lactococci are highly homologous to those from *Staphylococcus aureus* and *Lactobacillus casei*, (Porter and Chassy, 1988; De Vos and Gasson, 1989; De Vos *et al.*, 1990). In particular the deduced primary structures of lactose-specific components, Enzyme II^{lac} and a soluble protein, Factor III are homologous to those of *Staphylococcus aureus* (71% and 72% identity, respectively) and *Lactobacillus casei* (47% and 47% identity, respectively). The calculated molecular weights of Factor III and Enzyme II^{lac} of *L. lactis* ssp. *lactis* MG1820 are 11.4- kDa containing 105 amino acid residues and 61.5- kDa containing 586 amino acid residues, respectively (De Vos *et al.*, 1990). Factor III and Enzyme II of *Staphylococcus aureus* have molecular weights of 11.3- and 50- kDa, respectively. Although the size of Factor III expressed from this 4.4 kb *EcoRI* fragment was found

to be similar in size to the protein expressed by the highly homologous of the Lac-PTS genes, the size of Enzyme II^{lac} expressed from this 4.4 kb *EcoRI* fragment was found to be significant smaller (i.e. 23,000 dalton) compared to values of 50- and 61.5-kDa as reported for these genes of *Staphylococcus aureus* and *Lactobacillus casei*. Moreover, the *lacD* gene of *L. lactis* ssp. *lactis* MG1820 encoding tagatose 1,6-bisphosphate aldolase has a calculated molecular size of 36.4 -kDa and contains 326 amino acid residues (van Rooijen *et al.*, 1991). In contrast the *E. coli fba* gene encodes the class II fructose 1,6-bisphosphate aldolase with a calculated molecular size of 39.1- kDa and comprising 359 amino acids (Alefounder *et al.*, 1989).

Furthermore, the fragment sequenced expressed a 41- kDa protein which although slightly larger was speculatively thought to be the TBP-aldolase from *L. lactis* ssp. *cremoris*. However, the sizes of these proteins are in agreement with the results of the two enzymes encoded by the *Eco purMN* genes: i.e. 36.7- kDa of the enzyme encoded by *purM* gene, and 23.2-kDa of the enzyme encoded by *purN* gene (Smith and Duam III, 1986; 1987). In spite of the speculation concerning the protein expressed from *in vitro* transcription-translation studies there was no confirmation using a biochemical assay for the tagatose 1,6-bisphosphate aldolase activity on this particular 4.4 kb *EcoRI* fragment (Hodge, personal communication). The estimation of molecular weight of protein from *in vitro* expression studies provides inadequate data to draw conclusions about the success of the cloning. Such information must also be correlated with the results of molecular weight determinations using purified and isolated protein from the *in vivo* system together with biochemical testing (De Vos *et al.*, 1990; van Rooijen and De Vos, 1990; van Rooijen *et al.*, 1991).

According to the nucleic acid hybridization studies by Jarvis and Jarvis, (1981) and Garvie and Farrow (1982) and the phylogenetic studies using oligonucleotide cataloguing of 16S ribosomal RNA (Ludwig *et al.*, 1985) *L. lactis* ssp. *cremoris* is a close relative of *L. lactis* ssp. *lactis*. Thus the two organisms have a considerable proportion of their base sequences in common. Even though this 4.4 kb *EcoRI* fragment was supposedly derived from *L. lactis* ssp. *cremoris* and not from *L. lactis* ssp. *lactis* it should show an acceptable degree of homology to at least some of the DNA sequence data of the Lac-

PTS operon of *L. lactis* ssp. *lactis* strain MG1820 reported by van Rooijen *et al.* (1991). Moreover, the G+C content of the 4.4 kb *EcoRI* fragment also indicates that it is not derived from the lactococcal DNA. The G+C content range for the lactococcal bacteria is between 32%-39% (Stackebrandt and Teuber, 1988). The G+C content of the *lacD* encoding 1,6-bisphosphate aldolase gene of *L. lactis* ssp. *lactis* is 39% (van Rooijen *et al.*, 1991) whereas the G+C content of this particular 4.4 kb *EcoRI* DNA sequence ranges from 48% to 50% (i.e. the G+C content of the 401-36a consensus is 50%). Thus the G+C content of this particular DNA fragment is higher than the G+C content range for the lactococci or streptococci and is in the same range as the G+C content of *E. coli* DNA such as *E. coli fda* gene which encodes for Class II aldolase and which exhibits a 50% (Alefounder *et al.*, 1989).

3.4 Conclusion

The sequencing data obtained from this study sought to complete the earlier work on the molecular analysis of the genes encoding the enzymes involved in the Lac-PEP:PTS metabolic pathway of dairy lactococcal bacteria. Unfortunately, the sequence information obtained has shown quite unexpectedly that these sequences are not those of lactococcal DNA but rather *E. coli* chromosomal DNA from the *Eco purMN* genes region. These genes encode enzymes involved in purine nucleotide biosynthesis of *E. coli*. These unexpected results may have been a consequence of either the difficulties encountered by Hodge (1989) during the subcloning of the 4.4 kb *EcoRI* fragment from λ NM1149-2 into *E. coli* MC1022 or from the original work on *EcoRI* genomic library of pDI21 into bacteriophage vector λ NM1149. It is therefore concluded that the 4.4 kb *EcoRI* fragment which supposedly was derived from pDI21 of *L. lactis* ssp. *cremoris* strain H2 was in fact derived from *E. coli* chromosomal DNA.

CHAPTER 4: MOLECULAR CLONING AND CHARACTERIZATION OF THE β -GALACTOSIDASE GENE FROM *Lactococcus lactis* ssp. *lactis* STRAIN ATCC 7962

4.1 Introduction

In contrast to most lactococcal strains which contain only phospho- β -galactosidase *Lactococcus lactis* ssp. *lactis* strain ATCC 7962 contains both β -galactosidase and phospho- β -galactosidase. Plasmid curing experiments suggest that the β -galactosidase gene of strain 7962 is plasmid borne (Davey, G.P. personal communication). Strain 7962 contains four plasmids of 70, 45, 30, and 25 kb in size (i.e. 45, 30, 18.5, and 17.5 Md). Davey, G.P. (personal communication) deduced that the 70 kb plasmid, pDI3 encodes the β -galactosidase as strain NZDRI 5191, cured of all plasmids except pDI3, retained β -galactosidase activity whereas strain NZDRI 5201 which lacks all plasmids, also lacks β -galactosidase activity.

4.2 Confirmation that pDI3 encodes β -galactosidase

To confirm that β -galactosidase activity in *L. lactis* strain ATCC 7962 is associated with the presence of the plasmid, pDI3. Strain NZDRI 5191 (cured of all plasmids except pDI3), NZDRI 5201 (cured of all plasmids) and ATCC 7962 (wildtype) were plated on M17 medium containing 40 μ g/ml of X-gal and incubated at 30°C overnight (sections 2.2 and 2.17). Strain ATCC 7962 (Figure 9 A) and strain NZDRI 5191 (Figure 9 B) formed blue colonies on this medium demonstrating the presence of β -galactosidase activity whereas strain NZDRI 5201 (Figure 9 C) formed white colonies indicating that the β -galactosidase activity was either inactive or not being produced. The plasmid profiles of these strains were examined following 4 h of growth in M17 broth (section 2.2) as described in section 2.4. The plasmids from these bacterial strains were isolated according to the method described in section 2.5.1. The wildtype strain (ATCC 7962) contains four plasmids (Figure 10, lane 3), NZDRI 5191 (Figure 10, lane 1) contains only one plasmid of 70 kb in size, whereas NZDRI 5201 is completely free of plasmids (Figure 10, lane 2).

Figure 9

β -galactosidase activity of *Lactococcus lactis* ssp. *lactis* strain ATCC 7962 wildtype and plasmid cured derivatives.

Strains were grown on M17X (section 2.2) at 30°C overnight.

- A *Lactococcus lactis* ssp. *lactis* strain 7962 wildtype.
- B *Lactococcus lactis* ssp. *lactis* strain NZDRI 5191.
- C *Lactococcus lactis* ssp. *lactis* strain NZDRI 5201.

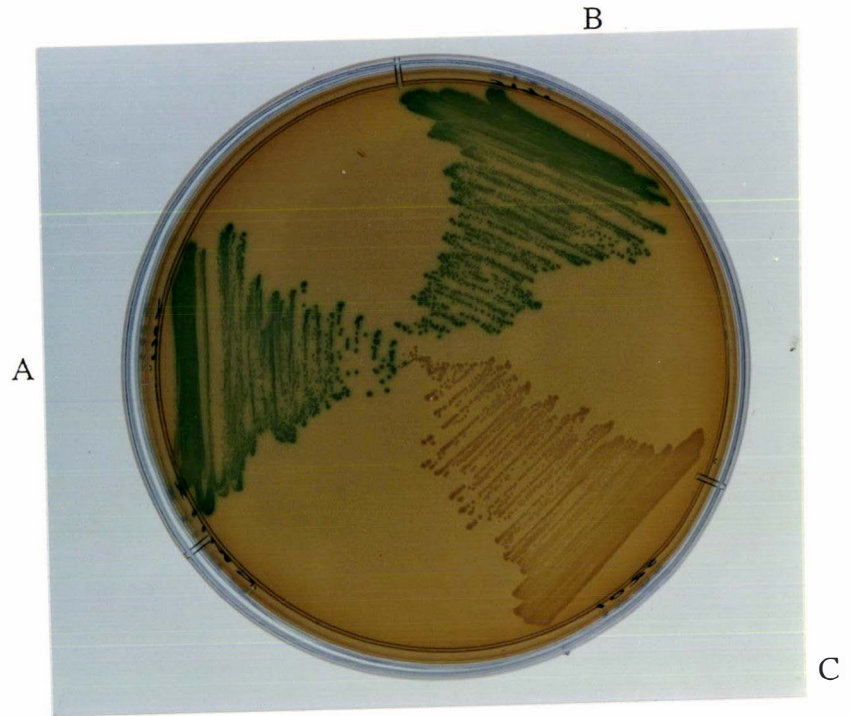
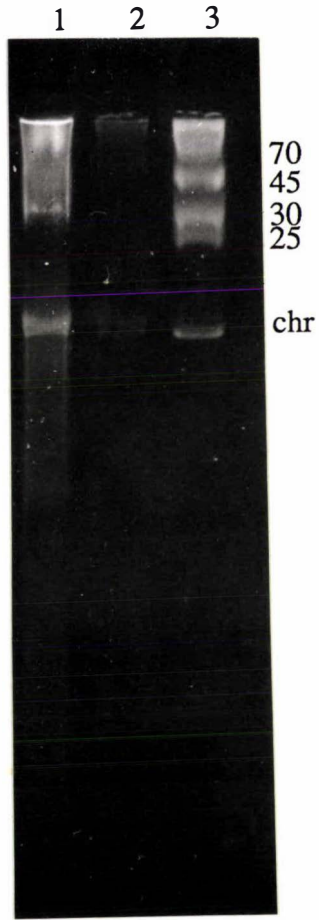


Figure 10

Plasmid profiles of *Lactococcus lactis* ssp. *lactis* strain ATCC 7962 wildtype and plasmid cured derivatives.

Plasmid DNA was isolated using the method of Anderson and McKay as described in section 2.5.1 and Table 13. (size of plasmid is in kb)

- | | |
|--------|---|
| Lane 1 | <i>Lactococcus lactis</i> ssp. <i>lactis</i> strain NZDRI 5191. |
| Lane 2 | <i>Lactococcus lactis</i> ssp. <i>lactis</i> strain NZDRI 5201. |
| Lane 3 | <i>Lactococcus lactis</i> ssp. <i>lactis</i> strain ATCC 7962 wildtype. |



4.3 Construction of a physical map of pDI3

4.3.1 Restriction enzyme digestion of pDI3

Plasmid pDI3 was prepared from strain NZDRI 5191 according to the protocol of Anderson and McKay and further purified by ultracentrifugation in a CsCl density gradient as described in Table 13, sections 2.5.2 and 2.7.1, respectively. The purity and concentration of the DNA was determined as described in section 2.8. Samples of DNA (about 0.5 μg) were digested in a total volume of 50 μl containing 5 μl of 10x restriction enzyme buffer, 5 μl of acetylated BSA and 1 Unit of the restriction enzyme as described in section 2.12.1. The pDI3 digests were separated by gel electrophoresis at 20 volts for 16 h., stained and photographed as described in section 2.9. The molecular weight of all the linear DNA fragments cleaved by the enzymes were determined by comparing the mobilities of the fragments with *Hind*III cut λ DNA run under the same electrophoretic conditions as described in section 2.10.

The DNA was first digested with a range of restriction endonucleases to identify which enzymes would be the most suitable for mapping. These pDI3 digests are shown in Figure 11. The most suitable enzymes appeared to be *Bam*HI (frame A lane 2), *Pst*I (frame A; lane 4 or frame B lane 6) and *Sal*I (frame A lane 5 or frame B lane 7) which gave 2, 7 and 6 fragments, respectively. Although restriction enzyme *Eco*RI cleaved pDI3 into 15 linear fragments (Figure 11 frame A lane 3 and Figure 11 frame B lane 4) it was also chosen for other purpose (see later sections 4.4, 4.5, and 4.6). The 4.2 kb fragment observed in the *Pst*I digest is probably a doublet (lane 4 in Figure 11 frame A and lane 6 in Figure 11 frame B, as discussed later in section 4.3.4) and an additional 0.3 kb fragment is present in the *Sal*I digest as well as in the *Eco*RI digest (see later in section 4.3.4). Single and double digests of pDI3 cut with *Bam*HI, *Eco*RI, *Pst*I, and *Sal*I were separated by electrophoresis at 1.5 V/cm in an overnight gel (section 2.9) to determine the sizes of the various fragments produced (Figure 11 frame A and Figure 12). The size and number of fragments generated from these digestions are summarized in Table 14. The size of the *Bam*HI fragments from lane 2 Figure 11 frame A was also introduced in this Table. The number of fragments observed in the double digests were

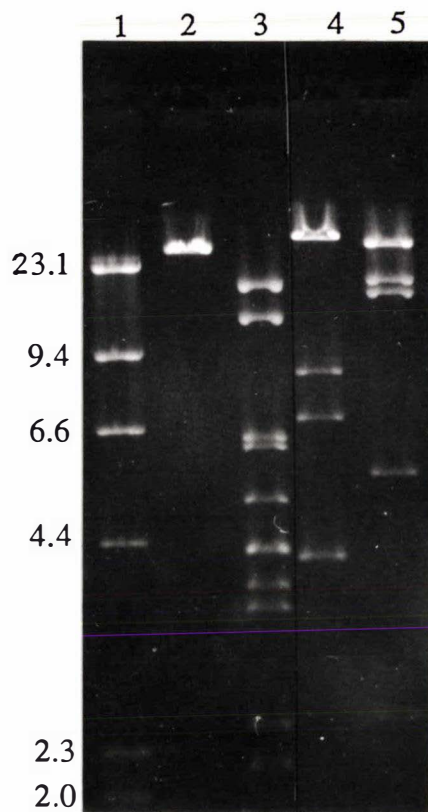
Figure 11

Restriction enzyme digests of pDI3.

pDI3 was isolated and purified as described in sections 2.5.2 and 2.7.1, respectively. Digests were carried out and separated by electrophoresis as described in sections 2.12.1 and 2.9, respectively.

frame A: Gel photograph of various restriction enzyme digests of pDI3 separated on a 0.5% agarose gel: *Bam*HI (lane 2); *Eco*RI (lane 3); *Pst*I (lane 4) and *Sal*I (lane 5). Lane 1 is a *Hind*III digest of λ DNA (fragment sizes are in kb)

frame B: Gel photograph of various restriction enzyme digests of pDI3 separated on a 0.7% agarose gel: *Bgl*II (lane 2); *Cla*I (lane 3); *Eco*RI (lane 4); *Hind*III (lane 5); *Pst*I (lane 7) and *Sal*I (lane 7). Lane 1 is a *Hind*III digest of λ DNA (fragment sizes are in kb). Lane 8 is BRL's 1 kb DNA ladder standards.



B

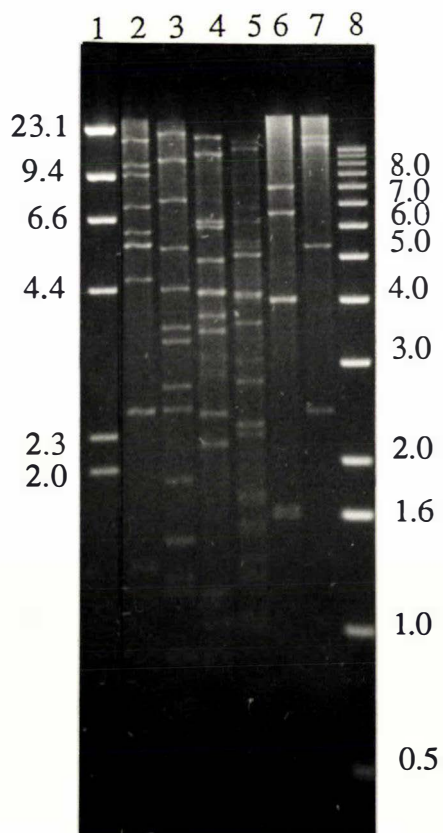


Figure 12

Single and double restriction enzyme digests of pDI3.

Digests were carried out and separated by electrophoresis on a 0.7% agarose gel as described in sections 2.12.1 and 2.9, respectively.

Gel photograph of various restriction enzyme digests of pDI3: *EcoRI* (lane 2); *PstI*; (lane 3); *SalI* (lane 4); *BamHI/EcoRI* (lane 5); *BamHI/PstI* (lane 6); *BamHI/SalI* (lane 7); *EcoRI/PstI* (lane 8); *EcoRI/SalI* (lane 9) and *PstI/SalI* (lane 10). Lane 1 is a *HindIII* digest of λ DNA (fragment sizes are in kb)

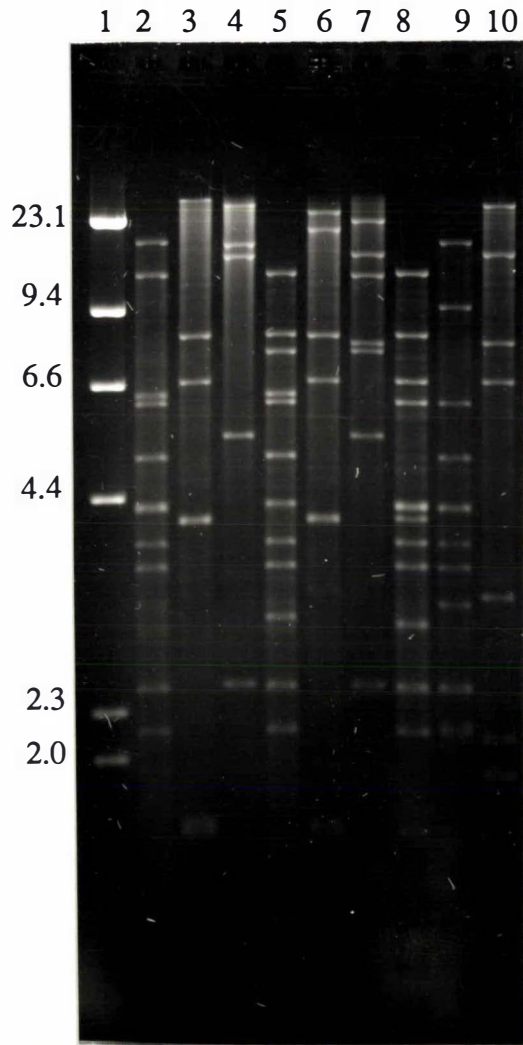


Table 14 Fragment sizes generated by single and double digestions of pDI3 with *Bam*HI, *Eco*RI, *Pst* I, and *Sal* I

Bam	Eco	Pst	Sal	Bam/Eco	Bam/Pst	Bam/Sal	Eco/Pst	Eco/Sal	Pst/Sal
(n=2) ²	(L. 2) ¹ (n=15)	(L. 3) (n=7)	(L. 4) (n=6)	(L. 5) (n=17)	(L. 6) (n=9)	(L. 7) (n=8)	(L. 8) (n=22)	(L. 9) (n=21)	(L. 10) (n=13)
36.5 ³	16.0	43.4	32.2	11.7	25.4	20.6	11.7	15.8	29.5
33.5	11.7	8.3	15.9	8.2	18.0	13.6	8.3	9.4	13.6
	6.4	6.7	13.6	7.8	8.2	11.6	6.7	6.3	8.2
	6.3	4.2a	5.5	6.4	6.7	8.0	6.3	5.0	6.7
	5.0	4.2b	2.5	6.3	4.2a	7.9	4.3a	4.3	3.2
	4.3a ⁴	1.7	0.3	5.0	4.2b	5.5	4.3b	4.2	2.3
	4.3b	1.6		4.3	1.7	2.5	4.2	3.8	1.9
	3.8			3.8	1.6	0.3	3.8	3.6	1.6
	3.6			3.6	0.1		3.6	3.2	1.1
	2.5			3.0			3.0	2.5a	1.0
	2.2			2.5a			2.5	2.5b	0.6
	1.3			2.2			2.2	2.3	0.2
	1.2			1.3a			1.6	2.2	0.1
	1.1			1.3b			1.3a	1.25	
	0.3			1.2			1.3b	1.2	
				1.1			1.2	1.1	
				0.3			1.1	0.7	
							0.9	0.3	
							0.8	0.2	
							0.4	0.1	
							0.3	0.05	
							0.2		

¹ Lane number as in Figure 12 except *Bam* HI which has been introduced from the other gel

² n is the predicted number of fragments generated in each digest

³ The fragment sizes in the range from 0.5kb to 23kb were determined experimentally. Fragments > 23kb were calculated by subtracting the size of all fragments < 23kb from 70kb. The very small fragments (i.e. < 0.5kb) were determined from the physical map of pDI3

⁴ a and b indicate similar sized fragments in digest shown

L = Lane, Bam = *Bam*HI, Eco = *Eco*RI, Pst = *Pst* I, and Sal = *Sal* I

less than predicted from the single digest results, presumably due to the small size of the fragments generated. Seven bands were observed from the *Bam*HI/*Sa*II double digests where eight were expected and similarly eight bands were observed from the *Bam*HI/*Pst*I digest where nine were expected and ten bands were observed from *Pst*I/*Sa*II where thirteen were expected. The estimates of the largest fragments are probably inaccurate due to the poor resolution in this size range. Based on the summation of fragment sizes generated after the restriction enzyme digests, we estimated pDI3 to be about 70 kb in size (Table 14). Using the data generated from the single and double enzyme digests it is possible to predict whether a particular restriction enzyme fragment will be cut with another enzyme. A summary of predicted restriction enzyme sites is shown in Table 15.

4.3.2 Molecular cloning of pDI3 fragments

As part of the strategy to construct a physical map of pDI3 various fragments of this plasmid were cloned using the shotgun approach. An aliquot (1µg) of pDI3 was digested with an appropriate restriction enzyme and the digestion checked on a minigel (sections 2.12.1 and 2.9) to confirm the digest had gone to completion. The digest was purified by phenol/chloroform extraction, precipitated and resuspended in water as described in sections 2.12.1, 2.7.2, and 2.7.3, respectively. Plasmid vector DNA (0.5 µg) was linearized with the same restriction enzyme and dephosphorylated as described in sections 2.12.1 and 2.12.2. The pDI3 digests were ligated with the vector DNA (section 2.12.3) and were transformed into *E. coli* host strain JM109 either by heat shocking competent cells prepared in CaCl₂ or electroporation as described in sections 2.16.1 and 2.16.2, respectively. The transformants were grown overnight at 37°C on an appropriate selective medium (section 2.2). Potential recombinants were screened for insert DNA by digesting the plasmid DNA prepared by the rapid boil mini-preparation method described in section 2.6.1 with the appropriate restriction enzyme (section 2.12.1).

Table 15 Predicted restriction enzyme sites in *EcoRI*, *PstI* and *SalI* fragments of pDI3 as determined by examining the double digest of each

<i>EcoRI</i> (lane 2) ¹	<i>BamHI</i> (lane 5)	<i>PstI</i> (lane 8)	<i>SalI</i> (lane 9)
16.0	+ ²	+	+
11.7	-	-	+
6.4	-	+	+
6.3	-	-	-
5.0	-	+	-
4.3a	-	-	+
4.3b	+	-	-
3.8	-	-	-
3.6	-	-	-
2.5	-	-	-
2.2	-	-	-
1.3	-	-	+
1.2	-	-	-
1.1	-	-	-
0.3	ND ³	ND	ND

<i>PstI</i> (lane 3)	<i>BamHI</i> (lane 6)	<i>EcoRI</i> (lane 8)	<i>SalI</i> (lane 10)
43.4	+	+	+
8.3	+	-	+
6.7	-	-	-
4.2a	-	-	+
4.2b	-	+	+
1.7	-	+	+
1.6	-	-	-

<i>SalI</i> (lane 4)	<i>BamHI</i> (lane 7)	<i>EcoRI</i> (lane 9)	<i>PstI</i> (lane 10)
32.2	+	+	+
15.9	+	+	+
13.6	-	+	-
5.5	-	+	+
2.5	-	-	+
0.3	-	+	+

1 lanes indicated are those shown in Figure 12

2 A + indicates the presence of at least one site for that particular enzyme in the fragment shown in the outer column

a and b indicate doublet

3 Not determined

A number of cloning experiments were carried out using different restriction enzyme digests of pDI3. These were as follows:

(i) *EcoRI* digests of pDI3 ligated with pBR328 (Experiment 1)

EcoRI digests of pDI3 were ligated with *EcoRI* linearized pBR328 and transformed into *E. coli* JM109. A number of Ap^rCm^rTc^r recombinants were identified and the plasmid digests from these recombinants are shown in Figure 13 frame A. In this experiment several *EcoRI* fragments of pDI3 were successfully cloned including the 2.5 kb fragment (lane 2), the 3.6 kb fragment (lane 3), the 1.2 kb fragment (lane 4), one of the 4.3 kb fragments (4.3a; lane 5), and the 3.8 kb fragment (lane 6). These plasmids were designated pSY102, pSY103, pSY101, pSY105, and pSY104, respectively. Subsequent work (see sections 4.5, 4.6, and 4.7) showed that pSY105 harboured the 4.3a kb *EcoRI* insert fragment from pDI3.

(ii) *PstI* digests of pDI3 ligated with pBR322 (Experiment 2)

PstI digests of pDI3 were ligated with *PstI* linearized pBR322 and transformed into *E. coli* JM109. A number of Ap^sTc^r recombinants were identified and plasmid digests from these recombinants are shown in Figure 13 frame B. In this experiment several *PstI* fragments of pDI3 were successfully cloned including the 1.7 kb fragment (lane 2), one of the 4.2 kb fragments (4.2b; lane 3), the 6.7 kb fragment (lane 4), and the 8.3 kb fragment (lane 5). These plasmids were designated pSY201, pSY202, pSY203 and pSY204, respectively. Subsequent work (see sections 4.3.3 and 4.3.4) showed that pSY202 harboured the 4.2b kb *PstI* insert fragment from pDI3.

(iii) *SalI* digests of pDI3 ligated with pBR322 (Experiment 3)

SalI digests of pDI3 were ligated with *SalI* linearized pBR322 and transformed into *E. coli* JM109. A number of Tc^sAp^r recombinants were screened. In this experiment one *SalI* fragment was successfully cloned and found to contain the 5.5 kb *SalI* fragment (Figure 13 frame B lane 6). The recombinant plasmid was designated pSY302.

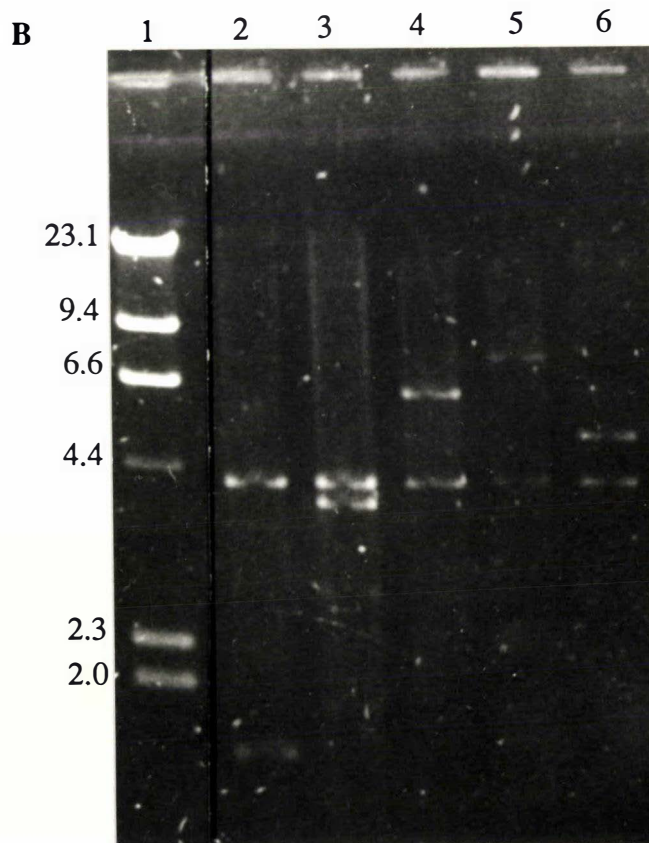
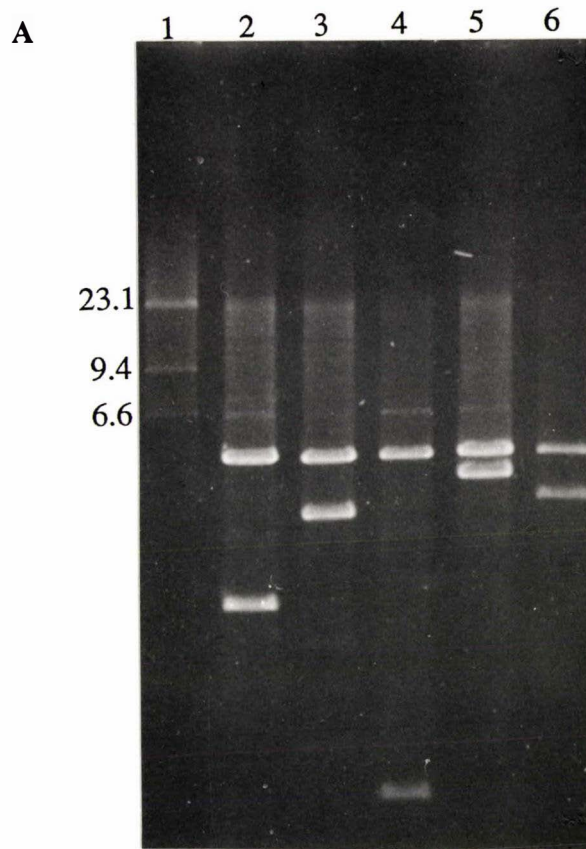
Figure 13

Restriction enzyme analysis of cloned fragments of pDI3.

DNA was isolated from each clone by the method described in section 2.6.1. Digests were carried out and separated by electrophoresis on a 0.7% agarose gel as described in sections 2.12.1 and 2.9, respectively.

frame A: Gel photograph of *EcoRI* digests of the recombinant plasmids; pSY102 (lane 2); pSY103 (lane 3); pSY101 (lane 4); pSY105 (lane 5) and pSY104 (lane 6). Lane 1 is a *HindIII* digest of λ DNA (fragment sizes are in kb).

frame B: Gel photograph of *PstI* digests of the recombinant plasmids; pSY201 (lane 2); pSY202 (lane 3); pSY203 (lane 4); pSY204 (lane 5) and lane 6 is a *SalI* digest of pSY302. Lane 1 is a *HindIII* digest of λ DNA (fragment sizes are in kb).



(iv) *SalI* digests of pDI3 ligated with pSA3 (Experiment 4)

SalI digests of pDI3 were ligated with *SalI* linearized pSA3 and transformed into *E. coli* JM109. A number of Tc^rCm^r transformants were screened. One recombinant was identified and found to contain the 2.5 kb *SalI* fragment of pDI3 (data not shown). This recombinant plasmid was designated pSY301.

(v) *HindIII* digests of pDI3 ligated with pFX1 (Experiment 5)

HindIII digests of pDI3 were ligated with *HindIII* linearized pFX1 and transformed into *L. lactis* ssp. *lactis* plasmid-free strain LM0230 as described in section 2.16.3. A number of Cm^r recombinant were screened. One recombinant was identified to contain the 3.7 kb *HindIII* fragment of pDI3 (data not shown). The recombinant plasmid was isolated as described in section 2.5.1 and designated pSY401.

Confirmation that these fragments were derived from pDI3 was also carried out by hybridizing Southern blots of these recombinant plasmids with [α -³²P]dCTP labelled pDI3 (sections 2.13, 2.14, and 2.12.4). All insert fragments from these plasmids hybridized to pDI3 (data not shown) confirming that they were all derived from pDI3.

To verify restriction enzyme sites in the cloned pDI3 fragments, restriction endonuclease analyses of the insert DNAs were then carried out. The recombinant plasmid DNA was isolated and digested with the restriction enzymes that were used for the construction of the physical map i.e. *Bam*HI, *Pst*I, and *Sal*I. The analysis and localization of the various restriction sites in the cloned pDI3 fragments was carried out with reference to known restriction sites on the physical map of the cloning vector. In the cases depicted below the map of pBR322 and pBR328 were used (see Appendix I and II). The predicted restriction sites contained in various cloned pDI3 fragments are illustrated in Table 16.

Table 16 Restriction enzyme sites identified in the cloned pDI3 fragments used to construct a physical map of pDI3

	<i>Bam</i> HI	<i>Eco</i> RI	<i>Pst</i> I	<i>Sal</i> I
Clone				
pSY105	-	-	-	+
pSY201	-	+	-	+
pSY202	-	+	-	+
pSY203	-	-	-	-
pSY204	+	-	-	+
pSY301	-	-	+	-
pSY302	-	+	+	-

+ indicates the presence of at least one restriction site for the restriction enzyme indicated in the column for the particular cloned pDI3 fragment

- indicates the absence of the restriction enzyme site in the column for the particular cloned pDI3 fragment

The following information was obtained from this analysis. Firstly, none of the cloned *Pst*I fragments contained *Bam*HI restriction sites except the 8.3 kb *Pst*I fragment of pSY204 in which one internal *Bam*HI site was discovered. Secondly, one internal *Sal*I restriction site was found in each of the cloned *Pst*I fragments except for the 6.7 kb *Pst*I fragment of pSY203. Thirdly, the cloned 4.2 kb fragment of pSY202 also contained three *Eco*RI sites. Fourthly, the cloned 5.5 kb *Sal*I fragment of pSY302 contained 1 internal *Pst*I restriction site. Finally, one internal restriction site of *Pst*I was found in the cloned 2.5 kb *Sal*I fragment of pSY301.

4.3.3 DNA hybridizations to pDI3 digests for the construction of the physical map of pDI3

Hybridization studies based on DNA/DNA sequence homology between two different fragments in the same region is another approach that can be used to determine the arrangement of restriction fragments. As a result, an overlap region can be determined to set up the fragment array of the entire plasmid.

The first approach was to use the cloned *Pst*I fragments of pDI3 as DNA probes. These included the 1.7 kb *Pst*I fragment from pSY201; the 4.2 kb *Pst*I fragment from pSY202; the 6.7 kb *Pst*I fragment from pSY203; and the 8.3 kb *Pst*I fragment from pSY204. Each of these fragments was isolated and purified as described in section 2.11 and then radioactively labelled with [α -³²P]dCTP by the random primer method (section 2.12.4). These DNA probes were used to hybridize to a Southern blot of various single and double digestions of pDI3 using the restriction enzymes; *Bam*HI, *Eco*RI, *Pst*I, and *Sal*I. The DNAs were transferred to a nylon membrane as described in section 2.13. All hybridizations were carried out under high stringency conditions (65°C in 3x SSC) as mentioned in section 2.14. The hybridization results for each of these 4 probes are shown in Figure 14 to Figure 17 and a summary of the DNA bands that hybridized and the intensity of hybridization is summarized in Table 17.

The hybridization results between pDI3 digests and the 1.7 kb *Pst*I fragment from pSY201 are illustrated in Figure 14 frame B and summarized in Table 17. The pDI3

Figure 14

Hybridization of the 1.7 kb *Pst*I fragment from pSY201 to pDI3 digests.

Transfer of DNA samples was carried out from a 0.7% agarose gel onto a nylon membrane as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out under high stringency conditions (3x SSC at 65°C). Autoradiography was carried out using an X-ray film (Cronex) as described in section 2.14.

frame A: Gel photograph of various restriction enzyme digests of pDI3, *Eco*RI (lane 2); *Pst*I (lane 3); *Sal*I (lane 4); *Bam*HI/ *Eco*RI (lane 5); *Bam*HI/*Pst*I (lane 6); *Bam*HI/*Sal*I (lane 7); *Eco*RI/*Pst*I (lane 8); *Eco*RI/*Sal*I (lane 9) and *Pst*I/*Sal*I (lane 10). Lane 1 is a *Hind*III digest of λ DNA (fragment sizes are in kb).

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [α -³²P]dCTP-labelled 1.7 kb *Pst*I fragment from pSY201.

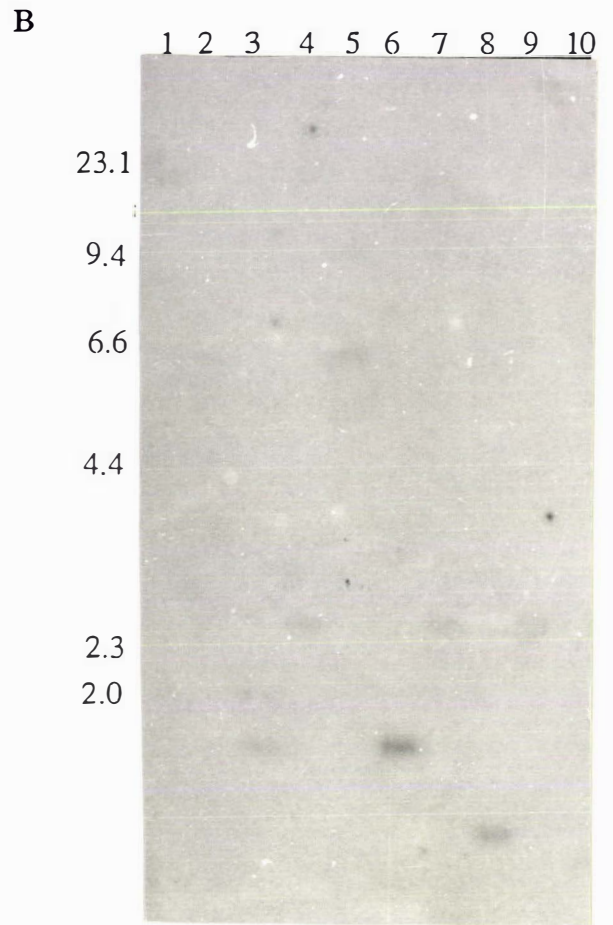
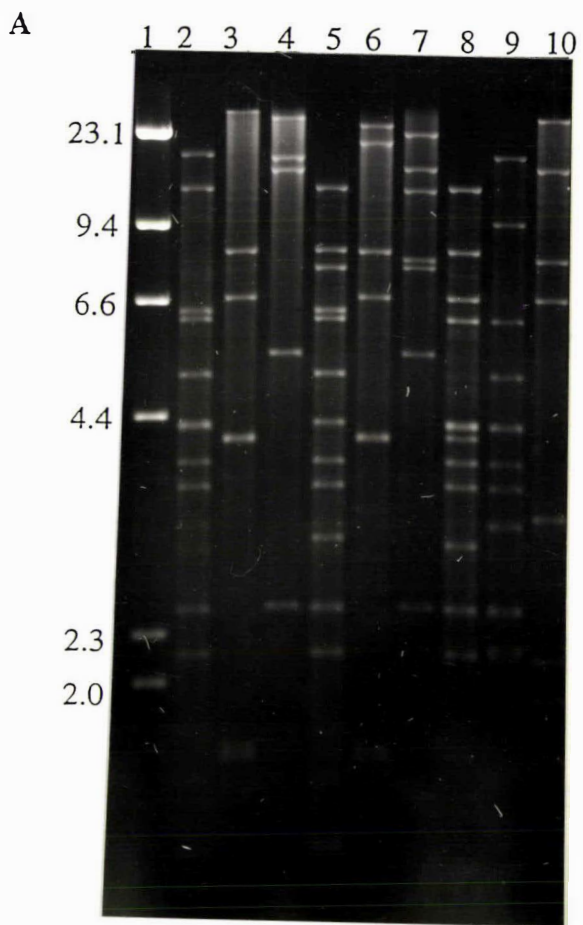


Table 17 Summary of hybridization results used to construct a physical map of pDI3

pDI3 Digests									
	Eco (L. 2)	Pst (L. 3)	Sal (L. 4)	Bam/Eco (L. 5)	Bam/Pst (L. 6)	Bam/Sal (L. 7)	Eco/Pst (L. 8)	Eco/Sal (L. 9)	Pst/Sal (L. 10)
Probe Pst									
1.7	6.4++	1.7+++	2.5+++	6.4++	1.7+++	2.5+++	1.3+++	2.5+++	1.1+++
4.2 b	16.0- 6.4+ 3.6- 1.3++ 1.2+++	43.4- 4.2b+++ 15.9+	32.2- 5.5+++	8.2- 6.4++ 3.6- 1.3+ 1.2+++	18.0- 4.2b+++	20.6- 13.6- 8.0+ 5.5+++	3.6- 1.3a+++ 1.2+++ 0.9+++ 0.8+++	15.8- 3.6- 3.2+++ 1.25+++ 1.2+++	3.2+++ 1.0-
6.7	16.0+++ 11.7+	8.3- 6.7+++	15.9+++	11.7- 7.8+++	25.4- 8.2- 6.7+++	11.6+ 7.9+++	11.7++ 8.3- 6.7+++	15.8+++ 9.4++	6.7+++
8.3	16.0+++	8.3+++ 6.7-	15.9+++	8.2+++	8.2+++	8.0+++ 6.7-	8.3+++	15.8+++ 6.7-	8.2+++ 6.7-
Probe Sal									
2.5	6.4+++	4.2a++ 1.7+++	2.5+++	6.5+++	4.2a++ 1.7+++	2.5+++	4.2a++ 1.2+++	2.5+++	1.9+++
5.5	6.4+++ 1.3+ 1.2++	4.2a+++ 4.2b+++	5.5+++	6.4+++ 1.3++ 1.2++	4.2a+++ 4.4b+++	5.5++	1.3a+++ 1.2++	3.2+++ 1.25++ 1.2+++	3.2+++ 2.3-
13.6	16.0+ 11.7+++ 4.3a++ 3.8- 3.6-	43.4+ 8.3+ 6.7-	13.6+++	11.7+++ 8.2+ 7.8- 4.3a+++ 3.8- 3.6-	25.4+++ 18.0+ 8.2++ 6.7++	13.6+++ 11.6+ 8.0++	11.7+++ 8.3+ 6.7- 4.3a+++ 3.8- 3.6-	15.8- 9.4+++ 4.3a+++ 3.8- 3.6-	13.6+++ 8.2- 6.7-
15.9	16.0+++ 11.7++	8.3+++ 6.7+	15.9+++	8.3+++ 7.9-	25.4- 18.0- 8.2+++	20.6- 13.6+ 11.6+ 8.0+++	11.7- 8.3+++ 6.7+	15.8+++ 9.4-	29.5++ 13.6++ 8.2+++ 6.7-
32.2	11.7- 6.3- 5.0++ 4.3b++ 3.8+++ 2.5+B	43.4+++ 1.7+++	32.2+++	11.7+ 6.3+++ 5.0++ 4.3b++ 3.8+++ 2.5++	25.4+++ 18.0+++ 1.7+++	20.6+ 11.6+++	11.7+ 6.3+++ 5.2++ 4.3b++ 3.8+++ 2.5++	3.8+++ 29.5+++	

- to +++ indicates relative intensity of the hybridization band from 1 to 4 which are compared within each experiment but not between experiments

L = Lane, Bam = *Bam*HI, Eco = *Eco*RI, Pst = *Pst*I and Sal = *Sal*II

fragments that hybridized strongly to the 1.7 kb *PstI* fragment from pSY201 included the 6.4 kb *EcoRI* fragment (lanes 2 and 5), the 1.7 kb *PstI* fragment (lanes 3 and 6), the 2.5 kb *SalI* fragment (lanes 4, 7, and 9), the 1.3 kb *EcoRI/PstI* fragment (lane 8), and the 1.1 kb *PstI/SalI* fragment (lane 10).

The hybridization results between pDI3 digests and the 4.2b kb *PstI* fragment from pSY202 are illustrated in Figure 15 frame B and summarized in Table 17. The pDI3 fragments that hybridized strongly to the 4.2b kb *PstI* fragment from pSY202 included the 6.4 kb *EcoRI* fragment (lanes 2 and 5), the 1.3 kb *EcoRI* fragment (lanes 2, 5, and 8) and the 1.2 kb *EcoRI* fragment (lanes 2, 5, and 8), the 4.2b kb *PstI* fragment (lanes 3 and 6), the 5.5 kb *SalI* fragment (lanes 4 and 7), the 3.2 kb *EcoRI/SalI* fragment (lane 9), the 1.25 kb *EcoRI/SalI* fragment (lane 9), the 3.2 kb and the 1.0 kb *PstI/SalI* fragments (lane 10).

The hybridization results between pDI3 digests and the 6.7 kb *PstI* fragment from pSY203 are illustrated in Figure 16 frame B and summarized in Table 17. The pDI3 fragments that hybridized strongly to the 6.7 kb *PstI* fragment from pSY203 included the 16.0 kb *EcoRI* fragment (lane 2), the 6.7 kb *PstI* fragment (lanes 3, 6, 8 and 10), the 15.9 kb *SalI* fragment (lane 4), the 7.8 kb *BamHI/EcoRI* fragment (lane 5), the 7.9 kb *BamHI/SalI* fragment (lane 7) and the 15.8 kb *EcoRI/SalI* fragment (lane 9).

The hybridization results between pDI3 digests and the 8.3 kb *PstI* fragment of pSY204 are illustrated in Figure 17 frame B and summarized in Table 17. The pDI3 fragments that hybridize strongly to the 8.3 kb *PstI* fragment from pSY204 included the 16.0 kb *EcoRI* fragment (lane 2), the 8.3 kb *PstI* fragment (lanes 3 and 8), the 8.2 kb *BamHI/EcoRI* fragment (lane 5), the 15.9 kb *SalI* fragment (lane 4), the 8.2 kb *BamHI/PstI* fragment (lane 6), the 8.0 kb *BamHI/SalI* fragment (lane 7), the 15.8 kb *EcoRI/SalI* fragment (lane 9), the 8.2 kb *PstI/SalI* fragment (lane 10), and the 8.3 kb *PstI* fragment from pSY204 (lane 12).

Figure 15

Hybridization of the 4.2 kb *Pst*I fragment from pSY202 to pDI3 digests.

Transfer of the DNA samples was carried out from a 0.7% agarose gel onto a nylon membrane as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out under high stringency conditions (3x SSC at 65°C). Autoradiography was carried out using an X-ray film (Cronex) as described in section 2.14.

frame A: Gel photograph of various restriction enzyme digests of pDI3, *Eco*RI (lane 2); *Pst*I (lane 3); *Sal*I (lane 4); *Bam*HI/*Eco*RI (lane 5); *Bam*HI/*Pst*I (lane 6); *Bam*HI/*Sal*I (lane 7); *Eco*RI/*Pst*I (lane 8); *Eco*RI/*Sal*I (lane 9) and *Pst*I/*Sal*I (lane 10). Lane 1 is a *Hind*III digest of λ DNA (fragment sizes are in kb).

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [α -³²P]dCTP-labelled 4.2 kb *Pst*I fragment from pSY202.

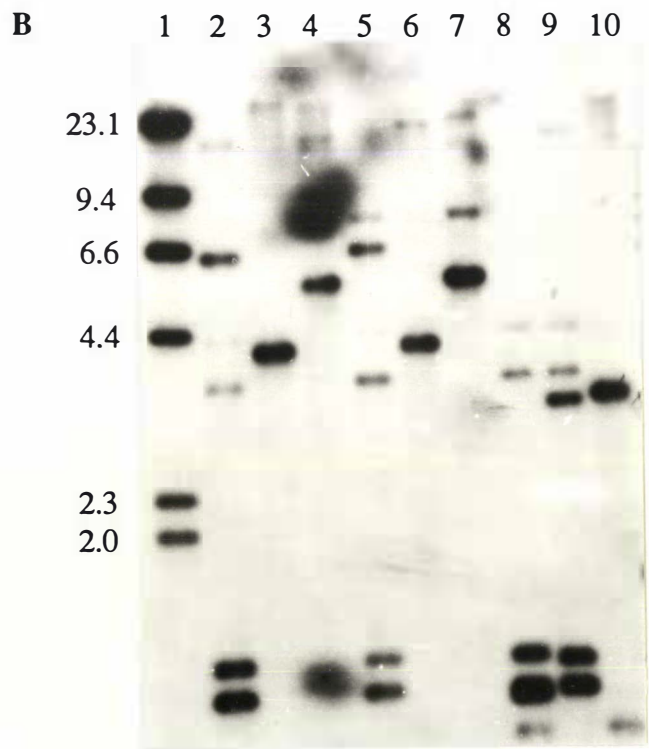
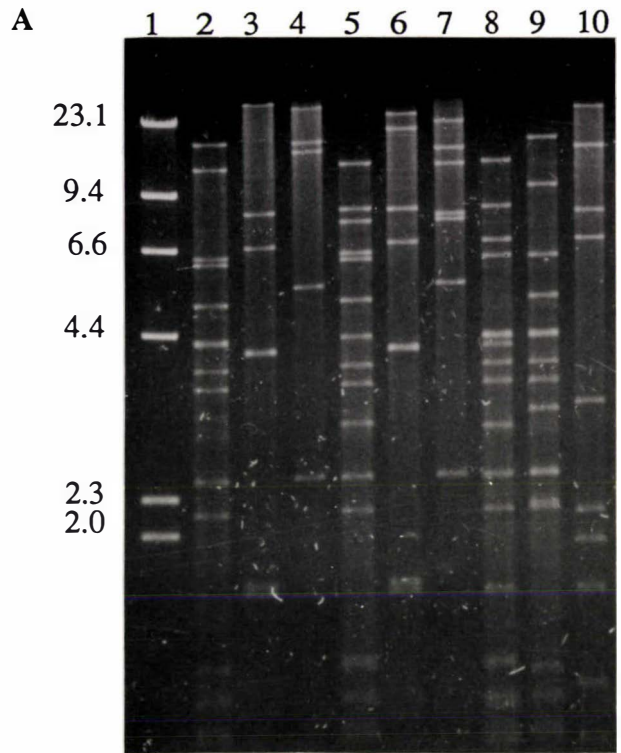


Figure 16

Hybridization of the 6.7 kb *Pst*I fragment from pSY203 to pDI3 digests.

Transfer of the DNA samples was carried out from a 0.7% agarose gel onto a nylon membrane as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out under high stringency conditions (3x SSC at 65°C). Autoradiography was carried out using an X-ray film (Cronex) as described in section 2.14

frame A: Gel photograph of various restriction enzyme digests of pDI3, *Eco*RI (lane 2); *Pst*I (lane 3); *Sal*I (lane 4); *Bam*HI/ *Eco*RI (lane 5); *Bam*HI/*Pst*I (lane 6); *Bam*HI/*Sal*I (lane 7); *Eco*RI/*Pst*I (lane 8); *Eco*RI/*Sal*I (lane 9) and *Pst*I/*Sal*I (lane 10). Lane 1 is a *Hind*III digest of λ DNA (fragment sizes are in kb).

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [α -³²P]dCTP-labelled 6.7 kb *Pst*I fragment from pSY203.

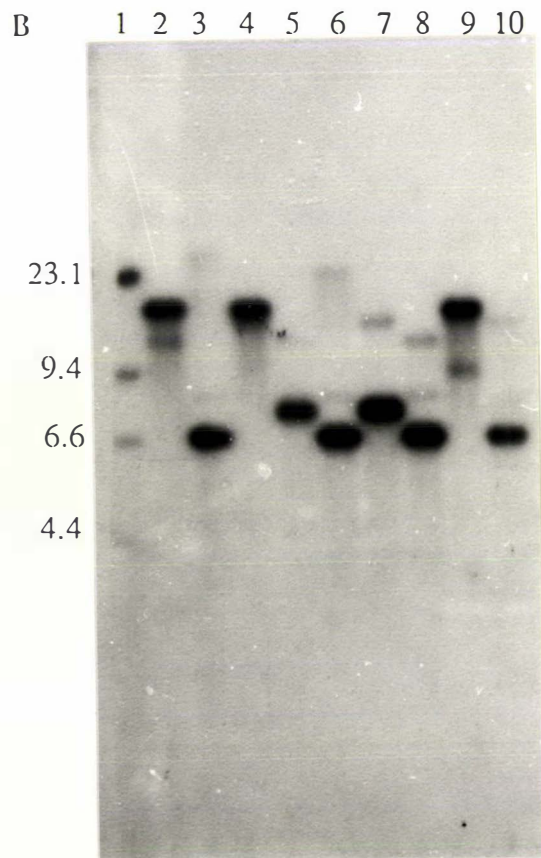
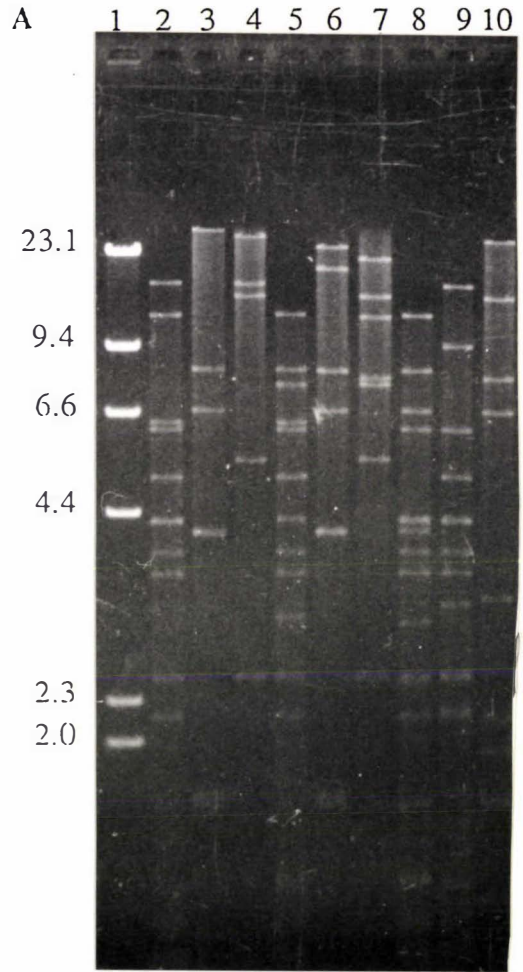


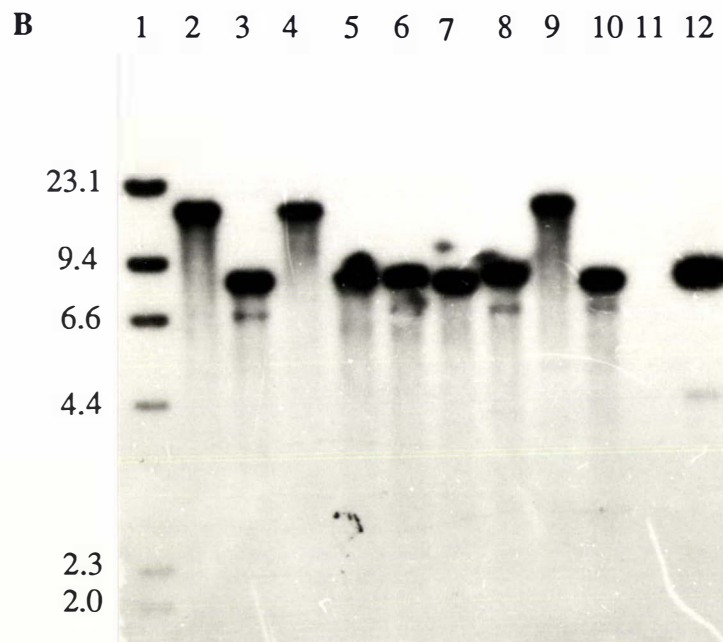
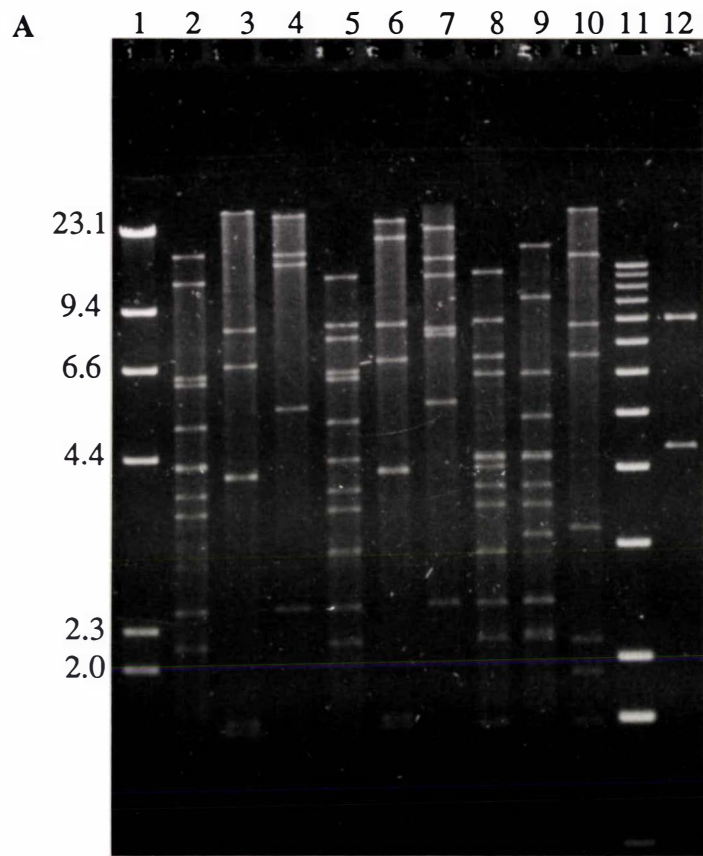
Figure 17

Hybridization of the 8.3 kb *Pst*I fragment from pSY204 to pDI3 digests.

Transfer of the DNA samples was carried out from a 0.7% agarose gel onto a nylon membrane as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out under high stringency conditions (3x SSC at 65°C). Autoradiography was carried out using an X-ray film (Cronex) as described in section 2.14.

frame A: Gel photograph of various restriction enzyme digests, *Eco*RI (lane 2); *Pst*I (lane 3); *Sal*I (lane 4); *Bam*HI/ *Eco*RI (lane 5); *Bam*HI/*Pst*I (lane 6); *Bam*HI/*Sal*I (lane 7); *Eco*RI/*Pst*I (lane 8); *Eco*RI/*Sal*I (lane 9) and *Pst*I/*Sal*I (lane 10). Lane 1 is a *Hind*III digest of λ DNA (fragment sizes are in kb).

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [α -³²P]dCTP-labelled 8.3 kb *Pst*I fragment from pSY204.



The second approach to confirm the arrangement of the *Pst*I fragments on the map of pDI3 was to use *Sal*I fragment of pDI3 as a probe. The various *Sal*I fragments were each isolated, purified from pDI3 *Sal*I digests as described in section 2.11 and labelled with [α - 32 P]dCTP (section 2.12.4) and used as a probe to a Southern blot of pDI3 digests similar to the Southern blot used in the first approach (i.e. when using a cloned *Pst*I fragment as a probe). The hybridization conditions were also performed at high stringency conditions (i.e. 65°C in 3x SSC) as described in section 2.14.

The hybridization results from the 2.5 kb, 5.5 kb, 13.6 kb, 15.9 kb, and 32.2 kb *Sal*I fragment probes are shown in Figures 18, 19, 20, 21, and 22, respectively. A summary of the fragments that hybridized and the intensity of the hybridization is shown in Table 17.

The hybridization results between pDI3 digests and the 2.5 kb *Sal*I fragment from pDI3 are illustrated in Figure 18 frame B and summarized in Table 17. The pDI3 fragments that hybridized strongly to the 2.5 kb *Sal*I fragment included the 6.4 kb *Eco*RI fragment (lanes 2 and 5), the 4.2a kb *Pst*I fragment (lanes 3, 6, and 8; see later in section 4.3.4), the 1.7 kb *Pst*I fragment (lanes 3 and 6), the 2.5 kb *Sal*I fragment (lanes 4, 7, and 9), the 1.3 kb *Eco*RI/*Pst*I fragment (lane 8), and the 1.9 kb *Pst*I/*Sal*I fragment (lane 10).

The hybridization results between pDI3 digests and the 5.5 kb *Sal*I fragment from pDI3 are illustrated in Figure 19 frame B and summarized in Table 17. The pDI3 fragments that hybridized strongly to the 5.5 kb *Sal*I fragment included the 6.4 kb *Eco*RI fragment (lanes 2 and 5), the 1.3 kb *Eco*RI fragment (lanes 2, 5, and 8), the 1.2 kb *Eco*RI fragment (lanes 2, 5, 8, and 9), the 4.2 kb *Pst*I doublets (i.e. both 4.2a and 4.2b kb *Pst*I fragments see later in section 4.3.4; lanes 3 and 6), the 5.5 kb *Sal*I fragment (lanes 4 and 7), the 3.2 kb *Eco*RI/*Sal*I fragment (lane 9), the 1.25 kb *Eco*RI/*Sal*I fragment (lane 9), the 3.2 and the 2.3 kb *Pst*I/*Sal*I fragments (lane 10).

The hybridization results between pDI3 digests and the 13.6 kb *Sal*I fragment from pDI3 are illustrated in Figure 20 frame B and summarized in Table 17. The pDI3 fragments that hybridized strongly to the 13.6 kb *Sal*I fragment included the 11.7 kb *Eco*RI

Figure 18

Hybridization of the 2.5 kb *SaII* fragment from pDI3 to pDI3 digests.

Transfer of the DNA samples was carried out from a 0.7% agarose gel onto a nylon membrane as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out under high stringency conditions (3x SSC at 65°C). Autoradiography was carried out using an X-ray film (Cronex) as described in section 2.14.

frame A: Gel photograph of various restriction enzyme digests of pDI3, *EcoRI* (lane 2); *PstI* (lane 3); *SaII* (lane 4); *BamHI/EcoRI* (lane 5); *BamHI/PstI* (lane 6); *BamHI/SaII* (lane 7); *EcoRI/PstI* (lane 8); *EcoRI/SaII* (lane 9) and *PstI/SaII* (lane 10). Lane 1 is a *HindIII* digest of λ DNA (fragment sizes are in kb).

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [α -³²P]dCTP-labelled 2.5 kb *SaII* fragment from pDI3.

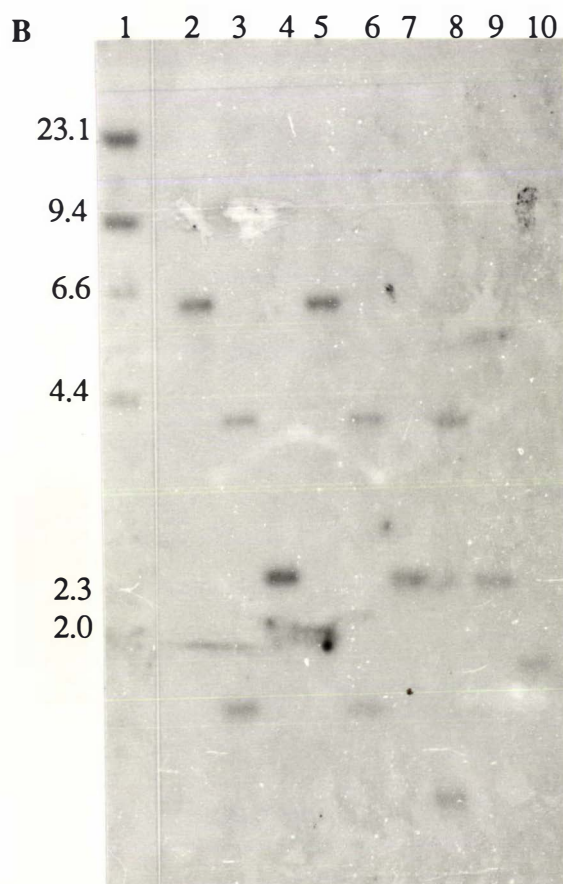
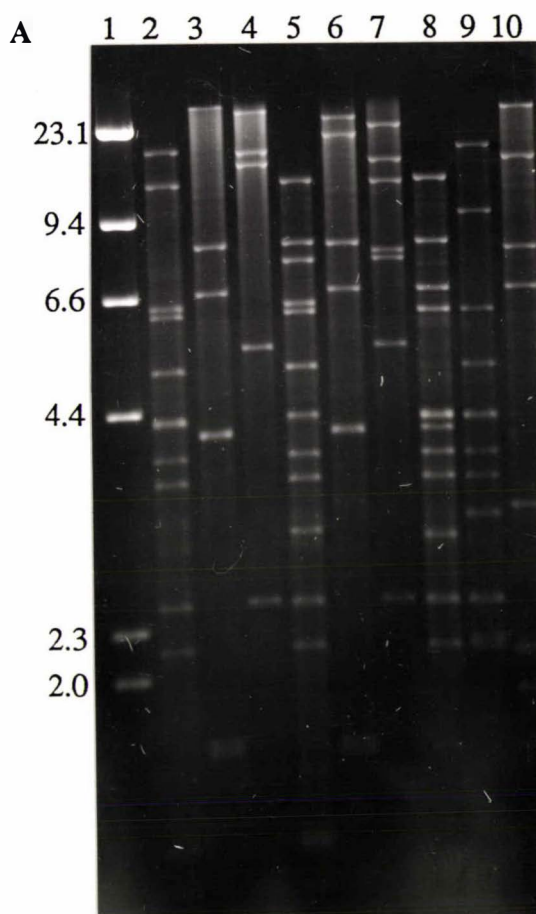


Figure 19

Hybridization of the 5.5 kb *SalI* fragment from pDI3 to pDI3 digests.

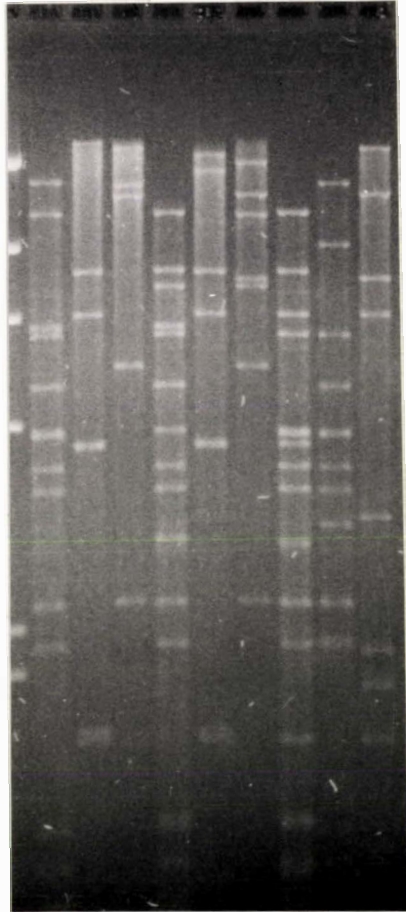
Transfer of the DNA samples was carried out from a 0.7% agarose gel onto a nylon membrane as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out under high stringency conditions (3x SSC at 65°C). Autoradiography was carried out using an X-ray film (Cronex) as described in section 2.14.

frame A: Gel photograph of various restriction enzyme digests of pDI3, *EcoRI* (lane 2); *PstI* (lane 3); *SalI* (lane 4); *BamHI/ EcoRI* (lane 5); *BamHI/PstI* (lane 6); *BamHI/SalI* (lane 7); *EcoRI/PstI* (lane 8); *EcoRI/SalI* (lane 9) and *PstI/SalI* (lane 10). Lane 1 is a *HindIII* digest of λ DNA standard (fragment sizes are in kb).

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [α -³²P]dCTP-labelled 5.5 kb *SalI* fragment from pDI3.

A 1 2 3 4 5 6 7 8 9 10

23.1
9.4
6.6
4.4
2.3
2.0



B 1 2 3 4 5 6 7 8 9 10

23.1
9.4
6.6
4.4
2.3
2.0

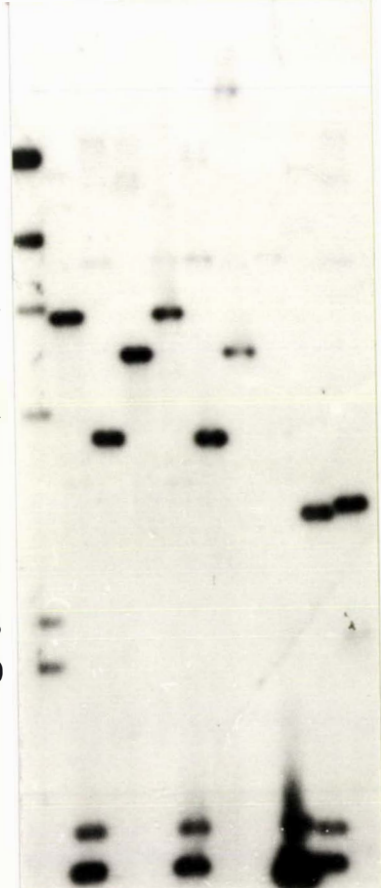


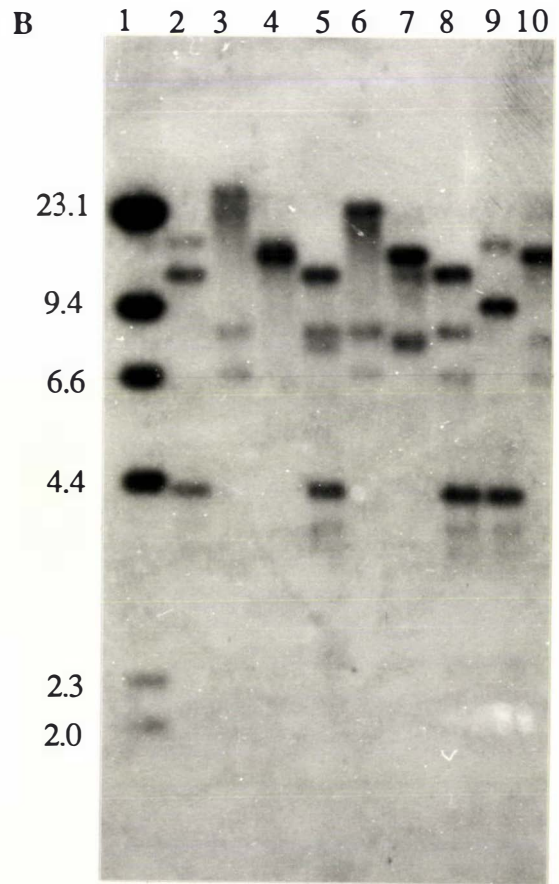
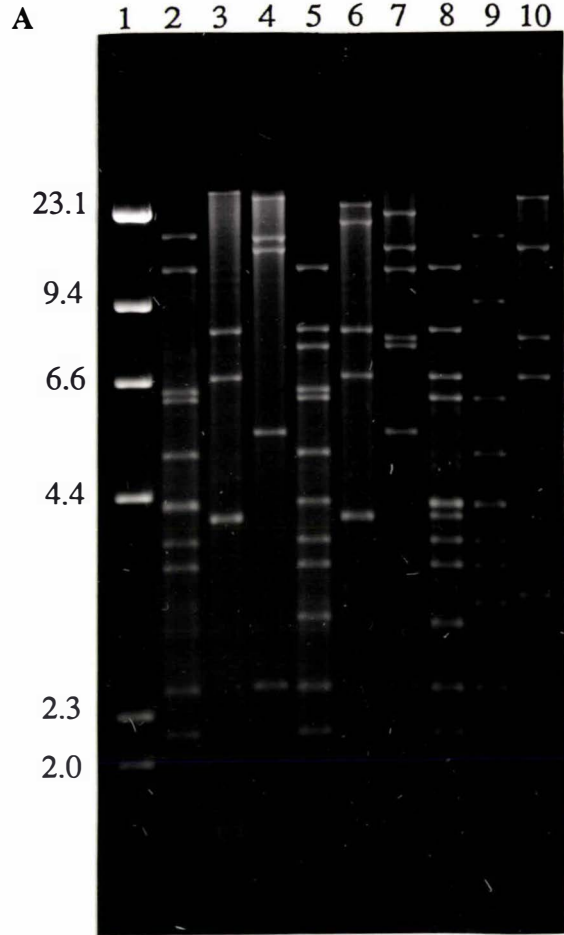
Figure 20

Hybridization of the 13.6 kb *SaII* fragment from pDI3 to pDI3 digests.

Transfer of the DNA samples was carried out from a 0.7% agarose gel onto a nylon membrane as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out under high stringency conditions (3x SSC at 65°C). Autoradiography was carried out using an X-ray film (Cronex) as described in section 2.14.

frame A: Gel photograph of various restriction enzyme digests of pDI3, *EcoRI* (lane 2); *PstI* (lane 3); *SaII* (lane 4); *BamHI/ EcoRI* (lane 5); *BamHI/PstI* (lane 6); *BamHI/SaII* (lane 7); *EcoRI/PstI* (lane 8); *EcoRI/SaII* (lane 9) and *PstI/SaII* (lane 10). Lane 1 is a *HindIII* digest of λ DNA (fragment sizes are in kb).

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [α -³²P]dCTP-labelled 13.6 kb *SaII* fragment from pDI3.



fragment (lanes 2, 5 and 8), the 4.3a kb *EcoRI* (i.e. one of the 4.3 kb *EcoRI* fragments see later in sections 4.3.4, 4.5, 4.6, and 4.7; lanes 2, 5, and 8), the 13.6 kb *SalI* fragment (lanes 4, 7 and 10), the 25.4 kb *BamHI/PstI* fragment (lane 6), the 9.4 and the 4.2 kb *EcoRI/SalI* fragments (lane 9).

The hybridization results between pDI3 digests and the 15.9 kb *SalI* fragment from pDI3 are illustrated in Figure 21 frame B and summarized in Table 17. The pDI3 fragments that hybridized strongly to the 15.9 kb *SalI* fragment included the 16.0 kb *EcoRI* fragment (lane 2), the 8.3 kb *PstI* fragment (lanes 3 and 8), the 15.9 kb *SalI* fragment (lane 4), the 8.2 kb *BamHI/EcoRI* fragment (lane 5), the 8.2 kb *BamHI/PstI* fragment (lane 6), the 8.0 kb *BamHI/SalI* fragment (lane 7), and the 8.2 kb *PstI/SalI* fragment (lane 10).

The hybridization results between pDI3 digests and the 32.2 kb *SalI* fragment from pDI3 are illustrated in Figure 22 frame B and summarized in Table 17. The pDI3 fragments that hybridized strongly to the 32.2 kb *SalI* fragment included the 3.8 kb *EcoRI* fragment (lanes 2, 5, 8 and 9), the 43.4 kb *PstI* fragment (lane 3), the 1.7 kb *PstI* fragment (lanes 3 and 6), the 32.2 kb *SalI* fragment (lane 4), the 25.4 kb *BamHI/PstI* fragment (lane 6), the 11.6 kb *BamHI/SalI* fragment (lane 7), and 29.5 kb *PstI/SalI* fragment (lane 10).

4.3.4 Data assembly for the construction of a physical map of pDI3

Using the information generated from the single and double restriction enzyme digests of pDI3 (Figures 11 and 12 and Tables 14 and 15), the restriction sites in the cloned pDI3 fragments (Table 16) and the corresponding hybridization results (Figures 14 to 22 and Table 17) a physical map for this plasmid was assembled.

The restriction enzymes that were used to construct this map were *BamHI*, *PstI*, and *SalI*. Some of the *EcoRI* fragments were also mapped. Table 17 and Figure 12 show the number and size of fragments generated by single and double digestion of pDI3 with the restriction enzymes: *BamHI*, *PstI*, and *SalI*. This plasmid was found to contain two

Figure 21

Hybridization of the 15.9 kb *SaII* fragment from pDI3 to pDI3 digests.

Transfer of the DNA samples was carried out from a 0.7% agarose gel onto a nylon membrane as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out under high stringency conditions (3x SSC at 65°C). Autoradiography was carried out using an X-ray film (Cronex) as described in section 2.14.

frame A: Gel photograph of various restriction enzyme digests of pDI3, *EcoRI* (lane 2); *PstI* (lane 3); *SaII* (lane 4); *BamHI/EcoRI* (lane 5); *BamHI/PstI* (lane 6); *BamHI/SaII* (lane 7); *EcoRI/PstI* (lane 8); *EcoRI/SaII* (lane 9) and *PstI/SaII* (lane 10). Lane 1 is a *HindIII* digest of λ DNA (fragment sizes are in kb).

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [α -³²P]dCTP-labelled 15.9 kb *SaII* fragment from pDI3.

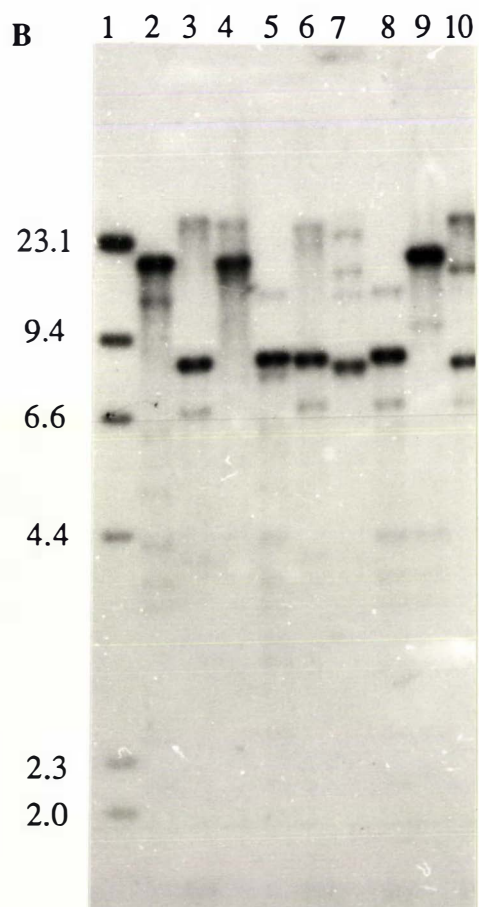
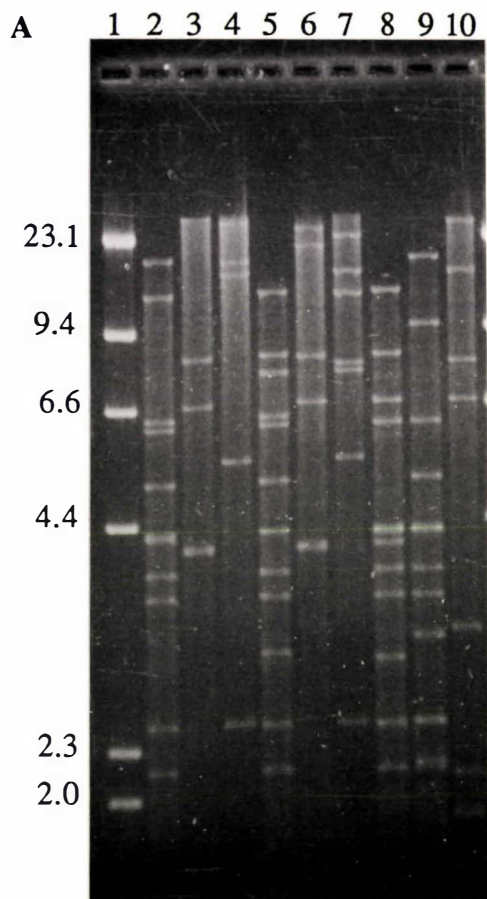


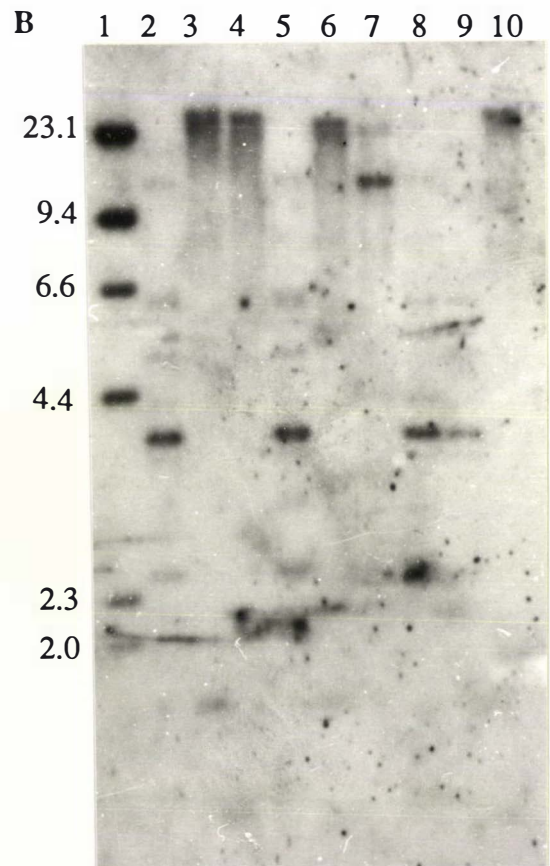
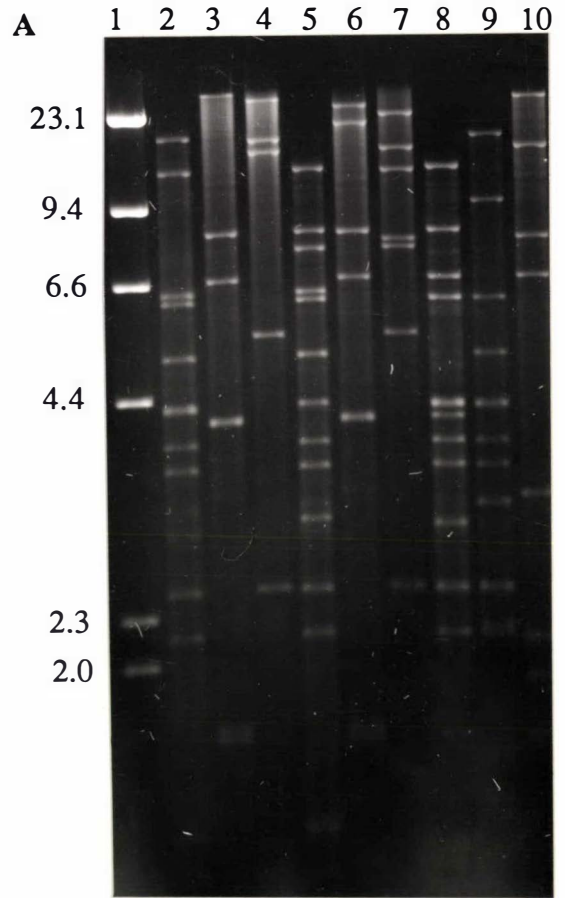
Figure 22

Hybridization of the 32.2 kb *SalI* fragment from pDI3 to pDI3 digests.

Transfer of the DNA samples was carried out from a 0.7% agarose gel onto a nylon membrane as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out under high stringency conditions (3x SSC at 65°C). Autoradiography was carried out using an X-ray film (Cronex) as described in section 2.14.

frame A: Gel photograph of various restriction enzyme digests of pDI3, *EcoRI* (lane 2); *PstI* (lane 3); *SalI* (lane 4); *BamHI/EcoRI* (lane 5); *BamHI/PstI* (lane 6); *BamHI/SalI* (lane 7); *EcoRI/PstI* (lane 8); *EcoRI/SalI* (lane 9) and *PstI/SalI* (lane 10). Lane 1 is a *HindIII* digest of λ DNA (fragment sizes are in kb).

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [α -³²P]dCTP-labelled 32.2 kb *SalI* fragment from pDI3.



*Bam*HI sites according to the number of fragments generated after double digestion with *Bam*HI. Seven fragments were generated after the DNA had been cleaved with *Bam*HI and *Sal*I. One of the *Bam*HI sites was situated within the 32.2 kb *Sal*I fragment to give the 20.6 and 11.6 kb *Bam*HI/*Sal*I fragments. The other *Bam*HI site was located within the 15.9 kb *Sal*I fragment, to give *Bam*HI/*Sal*I fragments of 7.9 and 8.0 kb.

The position of the two *Bam*HI sites was determined after a consideration of the followings results. According to the hybridization experiments, it was demonstrated that the 32.2 kb *Sal*I fragment had homology to the 43.4, 1.6, and 1.7 kb *Pst*I fragments as well as the 11.6 and the 20.6 kb *Bam*HI/*Sal*I fragments (see Figure 22). This was confirmed by the homology between the 1.7 kb *Pst*I from pSY201 and the 32.2 kb *Sal*I.

The position of the 11.6 kb *Bam*HI/*Sal*I in the 43.4 kb *Pst*I was verified by the fragment profile (18.0, 13.6, 11.6 and 0.2 kb) of the 43.4 kb *Pst*I fragment. This fragment profile of the 43.4 kb *Pst*I was created after the double digestion of the 43.4 kb *Pst*I with *Bam*HI and *Sal*I (data not shown). This data demonstrates that one of the *Bam*HI sites is definitely located where the 11.6 kb *Bam*HI/*Sal*I was cleaved from the 32.2 kb *Sal*I fragment. The other *Bam*HI site is located in the 15.9 kb *Sal*I fragment to give the 7.9 and 8.0 kb *Bam*HI/*Sal*I fragments.

In addition, the 13.6 kb *Sal*I fragment of pDI3 was found to contain neither *Bam*HI nor *Pst*I sites by restriction analysis (Figure 12, Tables 14 and 15). Thus, both the 32.2 and 13.6 kb *Sal*I must be situated next to each other, with the 13.6 kb *Sal*I fragment internal to the 43.4 kb *Pst*I fragment and to the left of the 32.2 kb *Sal*I.

The right end of 32.2 kb *Sal*I was located within the 1.7 kb *Pst*I. The precise position of this *Sal*I site was determined from the size (1.1 and 0.6 kb) of the *Pst*I/*Sal*I fragments generated from the cleavage of the 1.7 kb *Pst*I fragment from pSY201 (Table 16). From the hybridization results (Figure 14 and Table 17) and the restriction analysis of the 1.6 kb *Pst*I fragment (Table 15) it was apparent that both the 1.6 and 1.7 kb *Pst*I fragments were located next to each other toward the right end of the 32.2 kb *Sal*I fragment, with the 1.6 kb *Pst*I fragment located between the 43.4 and 1.7 kb *Pst*I fragments.

To determine which of the two *PstI/SalI* fragments cleaved from the right end of the 32.2 kb *SalI* (i.e. the 1.1 kb *PstI/SalI* fragment or the 0.6 kb *SalI/PstI* fragment) which corresponds to the overlap region between the *PstI* and the *SalI* maps, the fragment profiles from the double digests of these two enzymes were analysed. The 2.5 kb *SalI* fragment hybridizes to the 1.7 kb *PstI* and 1.1 kb *PstI/SalI* fragments (Figure 18 and Table 17). This result confirms that the 1.1 kb *PstI/SalI* fragment is at the end of the 32.2 kb *SalI* fragment and that the 1.1 kb *SalI/PstI* fragment overlaps with the 2.5 kb *SalI* fragment. The 0.6 kb *SalI/PstI* fragment is then located next to the 1.1 *PstI/SalI* fragment to make up the 1.7 kb *PstI* fragment. Besides the 2.5 kb *SalI* fragment also hybridizes to the 1.9 kb *PstI/SalI* fragment (Figure 18 and Table 17). Therefore the 1.9 kb *PstI/SalI* fragment must be located next to the 0.6 kb *SalI/PstI* fragment to make up to the size of the 2.5 kb of *SalI* fragment.

Furthermore, the 2.5 kb *SalI* fragment also shares homology to one of the 4.2 kb *PstI* bands. The 4.2b kb *PstI* fragment showed homology to the 5.5 kb *SalI* fragment but not to the 2.5 kb *SalI* fragment (Figure 15 and Table 17). Therefore the 4.2 kb *PstI* fragment that showed homology to the 2.5 kb *SalI* fragment must be the 4.2a kb *PstI* fragment. Thus the other *SalI/PstI* fragment that makes up the 4.2a kb *PstI* fragment must be the 2.3 kb *SalI/PstI* fragment.

The 5.5 kb *SalI* fragment hybridizes to the 4.2 kb *PstI* doublet (Figure 19 and Table 17) and *SalI/PstI* fragments of 2.3 kb and 3.2 kb. When the 5.5 kb *SalI* fragment was digested with *PstI* enzyme two *SalI/PstI* fragments of 2.3 and 3.2 kb were generated (Tables 14 and 16). The presence of the *PstI* site in the 5.5 kb *SalI* fragment was also confirmed by restriction enzyme analysis of the cloned 5.5 kb *SalI* fragment in pSY302 (data not shown). Consequently, the 4.2a and 4.2b kb *PstI* fragments are adjacent to one another with the 2.3 kb *SalI/PstI* and the 3.2 kb *PstI/SalI* of the 5.5 kb *SalI* fragment overlapping with the *PstI* fragments of 4.2a and 4.2b, respectively.

Additionally, the cloned 4.2 kb *PstI* fragment b of pSY202 also hybridized to the 5.5 kb and 15.9 kb *SalI* fragments and to *PstI/SalI* fragments of 3.2 and 1.0 kb (Figure 15

and Table 17). Therefore the 15.9 kb *SalI* fragment is adjacent to the 5.5 kb *SalI* and has a 1.0 kb *PstI/SalI* overlap with the 4.2 b *PstI* fragment.

When the 15.9 kb *SalI* fragment was digested with *PstI* enzyme three fragments of 1.0, 6.7 and 8.2 kb were generated (Table 14). Both the cloned 8.3 and 6.7 kb *PstI* fragments (in pSY203 and pSY204, respectively) hybridized to the 15.9 kb *SalI* fragment, indicating that these are the fragments adjacent to the 4.2b kb *PstI* fragment. The arrangement of these two fragments (6.7 and 8.3 kb *PstI* fragments) was determined

by the position of the internal *BamHI* site. The 8.3 kb *PstI* was found to contain one *BamHI* site and one *SalI* site while the 6.7 kb *PstI* was found to contain neither. Thus the 6.7 kb *PstI* was located next to the 4.2b kb *PstI* fragment.

From the restriction analysis of the cloned 8.3 kb *PstI* fragment, its *BamHI* and *SalI* site was located at about 100 bp inside from the left end and at about 200 bp inside from the right end, respectively (Figure 23). This *BamHI* site resided within the 15.9 kb *SalI* fragment in a position that generates two *BamHI/SalI* fragments of 7.9 and 8.0 kb and *BamHI/PstI* fragments of 6.7, 8.2, and 0.1 kb. The 0.1 kb *PstI/BamHI* fragment was too small to be observed from the gel, thus explaining why eight *BamHI/PstI* were detected instead of the predicted nine (Table 14).

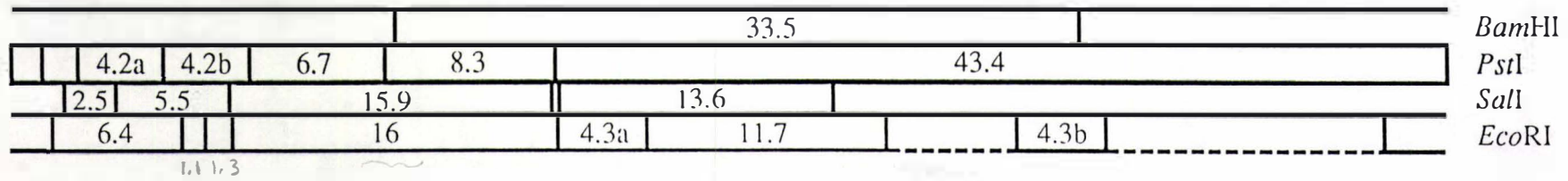
Following the arrangement of all the *SalI* fragments (32.2, 2.5, 5.5, 15.9, and 13.6 kb), the small *SalI* fragment of about 300 bp was predicted to be located between the 15.9 and 13.6 kb *SalI* fragments (Figure 23). This fragment was also too small to be visualized on the gel. The predicted linear and circular physical map of pDI3 for *BamHI*, *PstI*, and *SalI* is illustrated in Figure 23 and Figure 24, respectively.

Based on the acquired hybridization results for the *EcoRI* fragments of pDI3, the location of eight (i.e. the 16.0, 11.7, 6.4, 5.0, 4.3a, 4.3b, 1.3, and 1.1 kb *EcoRI* fragments) of the fifteen *EcoRI* fragments of pDI3 was also determined. The 5.0 kb *EcoRI* fragment is located on the 32.2 kb *SalI* fragment as it hybridized to the 32.2 *SalI* fragment (Figure 22 and Table 17). The 5.0 kb *EcoRI* fragment contains at least one

Figure 23

Restriction endonuclease map of pDI3 isolated from *Lactococcus lactis* ssp. *lactis* strain NZDRI 5191 demonstrating restriction sites of restriction enzymes: *Bam*HI, *Eco*RI, *Pst*I and *Sa*II.

a and b indicate the same size fragment (doublet) generated with the same restriction enzyme digest.



5 kb

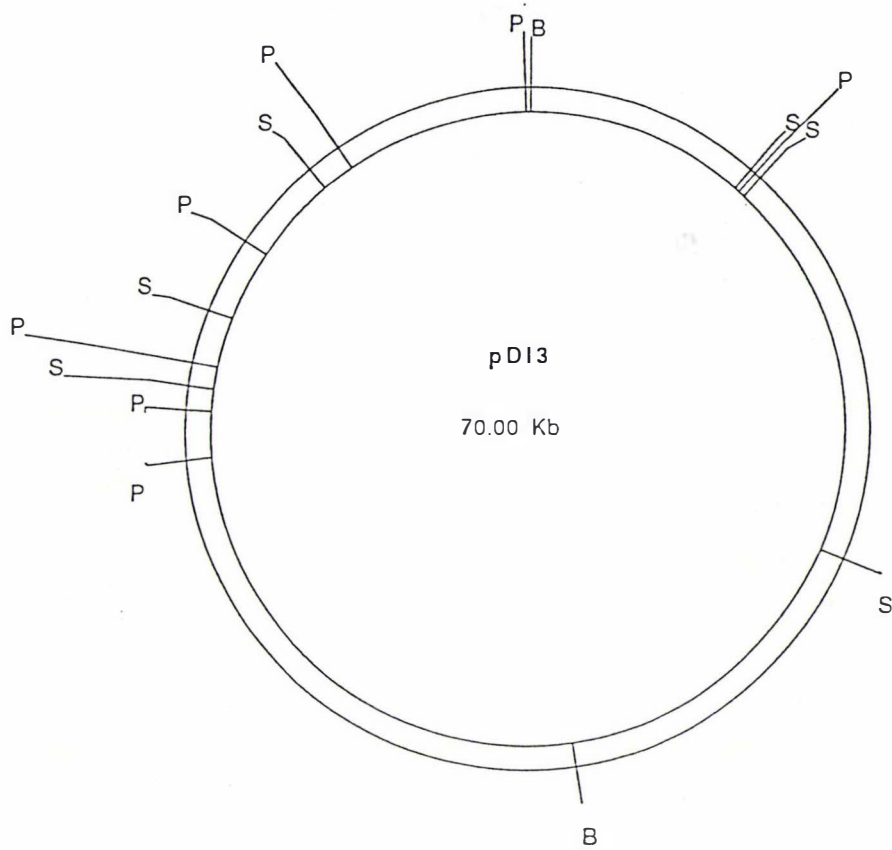
1, 1, 3

3.5

Figure 24

A physical map of pDI3 isolated from *Lactococcus lactis* ssp. *lactis* strain NZDRI 5191 incorporating *Bam*HI, *Pst*I and *Sal*I restriction sites.

B, P and S indicate *Bam*HI, *Pst*I and *Sal*I restriction sites, respectively.



internal *Pst*I site as it is affected by the *Pst*I enzyme to generate at least one fragment which is the 3.0 kb *Eco*RI/*Pst*I fragment (Figure 12, Tables 14 and 15). Hence the location of one end of the 5.0 kb *Eco*RI fragment must be in the 32.2 kb *Sal*I fragment toward the right end of the 43.4 *Pst*I fragment where the 3.0 kb *Eco*RI/*Pst*I fragment is generated.

The 1.6 kb *Pst*I fragment situated between the 43.4 kb *Pst*I fragment and the 1.7 kb *Pst*I fragment must be the internal *Pst*I fragment of the 5.0 kb *Eco*RI fragment as it is not affected by *Eco*RI enzyme (Figure 12 and Table 15). The location of the 5.0 kb *Eco*RI was also confirmed by the presence of one *Eco*RI site in the cloned 1.7 kb *Pst*I fragment in pSY201 (Table 16). As a result this *Eco*RI site must come from the other end of the 5.0 kb *Eco*RI fragment to give a 0.4 kb *Pst*I/*Eco*RI fragment and a 1.3 kb *Eco*RI/*Pst*I fragment (Figures 12 and 23, Table 14). The generation of the 1.3 kb *Eco*RI/*Pst*I fragment was also confirmed by hybridization result of the 2.5 kb *Sal*I fragment of pDI3 to the 1.3 kb *Eco*RI/*Pst*I pDI3 fragment (Figure 18 and Table 17).

The 6.4 kb *Eco*RI fragment hybridized to both *Pst*I fragments of 1.7 and 4.2b kb and *Sal*I fragments of 2.5 and 5.5 kb (Figures 14, 15 and 18, 19, respectively and Table 17). Therefore the 6.4 kb *Eco*RI fragment must be located as an adjacent fragment to the 5.0 kb *Eco*RI fragment with an overlap of the 1.3 kb *Eco*RI/*Pst*I fragment.

As shown in Table 15 the 4.2b kb *Pst*I fragment contains at least one *Eco*RI site. When the cloned 4.2 kb *Pst*I fragment b from pSY202 was digested with *Eco*RI enzyme four fragments: two *Eco*RI fragments of 1.3 and 1.2 kb and two *Eco*RI/*Pst*I fragments of 0.9 and 0.8 kb were generated (data not shown). The 0.9 kb *Pst*I/*Eco*RI fragment is the overlap between the 6.4 kb *Eco*RI fragment and the 4.2b *Pst*I fragment (Figure 23).

In addition, the 16.0 kb *Eco*RI fragment hybridizing to the 15.9 kb *Sal*I fragment (Figure 21 and Table 17) is adjacent to the 5.5 kb *Sal*I fragment with the overlap fragment of the 0.8 kb *Eco*RI/*Pst*I fragment mentioned above. This indicates that the 16.0 kb *Eco*RI fragment must also contain the internal *Bam*HI site located in the 15.9 kb *Sal*I fragment.

Consequently the 16.0 kb *EcoRI* fragment must be an adjacent fragment to the 6.4 kb *EcoRI* fragment with the overlap of the 4.2b kb *PstI* fragment.

The location of the 1.3 kb *EcoRI* fragment, one of the *EcoRI* fragments generated from the *EcoRI* digest of the cloned 4.2b kb *PstI* fragment is determined based on the evidence that pDI3 contains only one *EcoRI* fragment of 1.3 kb in size (lane 2 Figure 12 and Table 14). Additionally, this 1.3 kb *EcoRI* fragment is cleaved by restriction enzyme, *SaII* (Figure 12 and Table 15). The location of the 1.3 kb *EcoRI* is therefore confirmed at the site where the *SaII* site from the 5.5 kb *SaII* is internal to the 1.3 kb *EcoRI* fragment. Following the location of the 1.3 kb *EcoRI* fragment the other 1.2 kb *EcoRI* fragment generated from the *EcoRI* digest of the cloned 4.2b *PstI* fragment is determined as it does not contain any *SaII* site (Table 15).

The 13.6 kb *SaII* fragment hybridizes to both *EcoRI* fragments of 11.7 and 4.3a kb and *EcoRI/SaII* fragment of 9.4 kb (Figure 20 and Table 17). Thus, these two *EcoRI* fragments are adjacent to one another to make up the 13.6 kb *SaII* fragment. The location of the 11.7 kb *EcoRI* fragment was determined by the generation of the 9.4 kb *EcoRI/SaII* and the 2.3 kb *SaII/EcoRI* fragments after the double digest between restriction enzymes *EcoRI* and *SaII* of pDI3 (Figure 12, Tables 14 and 15). The 13.6 kb *SaII* fragment does not hybridize to the 2.3 kb *SaII/EcoRI* fragment therefore this 2.3 kb *SaII/EcoRI* fragment must be an overlap between the 13.6 kb *SaII* fragment and the 32.2 kb *SaII* fragment. Moreover, the arrangement of the 11.7 and 4.3a kb fragments of *EcoRI* is also confirmed by restriction analysis of the cloned 13.6 kb *SaII* fragment of pSY303 (Table 16 and see later in section 4.7).

According to the restriction analysis neither *BamHI* nor *PstI* sites were found within the cloned 4.3a kb *EcoRI* fragment from pSY105 but this fragment did contain one *SaII* site (data not shown). The 4.3a kb *EcoRI* fragment contains one *SaII* site to give 0.1 kb *EcoRI/SaII* and 4.2 kb *SaII/EcoRI* fragment (Table 15). Therefore the 4.3a kb *EcoRI* fragment must be located at the other end of the 13.6 kb *SaII* fragment where the 0.1 kb *EcoRI/SaII* and the 4.2 kb *SaII/EcoRI* fragment is generated.

As mentioned earlier, the 4.3a kb *EcoRI* contains no *BamHI* site. Furthermore, there are two *BamHI* sites in pDI3. One is located on the 15.9 kb *SalI* fragment or the 16.0 kb *EcoRI* fragment and the other one is located on the 32.2 kb *SalI* fragment. After the double digest of pDI3 with the restriction enzymes *BamHI* and *EcoRI*, two *EcoRI* fragments of pDI3, the 16.0 kb *EcoRI*, and one of the 4.3 kb *EcoRI* fragment (i.e. 4.3b) were cleaved. This indicates that the 4.3b kb *EcoRI* must contain the other *BamHI* site located on the 32.2 *SalI* fragment to give 3.0 kb *EcoRI/BamHI* and 1.3b kb *EcoRI/BamHI* fragments (Figure 12, Tables 14 and 15).

Although the 4.3b kb *EcoRI* fragment is located in the 32.2 kb *SalI* fragment the arrangement between the two generated *BamHI/EcoRI* fragments (1.3 and 3.0 kb) was not confirmed. Nevertheless the predicted location of all the eight *EcoRI* fragments are also included and illustrated in a linear map of pDI3 shown in Figure 23. The rest of the *EcoRI* fragments (i.e. 6.3, 3.8, 3.6, 2.5, 2.2, 1.1, and 0.3 kb) must all be located on the 32.2 kb *SalI* fragment but the arrangement of these *EcoRI* fragments was not determined.

4.4 Detection of the β -galactosidase gene of pDI3

4.4.1 Localization of the β -galactosidase gene of pDI3 using a heterologous probe.

The β -galactosidase (*cbg A*) gene of *Clostridium acetobutylicum* strain NCIB 2951 had been cloned and found to share some homology with the 4.3 kb *EcoRI* doublet of pDI3 (Hancock, 1988). To confirm the homology between the *cbgA* gene and pDI3, the 5.2 kb *EcoRI* fragment of pKH179 was isolated from an *EcoRI* digest of pKH179 using the method described in section 2.11 and labelled with [α -³²P]dCTP (section 2.12.4) and used as a heterologous DNA probe (CA1).

The DNA from pDI3 was digested to completion in separate digests with *EcoRI* and *SalI* (section 2.12.1). An *EcoRI* digest of pKH179 was used as a positive control for this experiment. All the digests were separated by electrophoresis as described in

section 2.9 and the resultant gel was Southern blotted as described in section 2.13. The hybridization was performed (section 2.14) at a moderate stringency (55°C, 3x SSC). The 5.2 kb *EcoRI* fragment of pKH179 showed some detectable homology with the 4.3 kb *EcoRI* doublet of pDI3 as shown in Figure 25 frame B. No hybridization was observed to the *SalI* digest of pDI3. However, there was a high background on this membrane.

4.4.2 Localization of β -galactosidase gene of pDI3 using an oligonucleotides probe.

A comparison study of the predicted amino acid sequences from the β -galactosidase gene sequences of the following species: *lacZ* gene of *E. coli* (Kalnins *et al.*, 1983), *ebg A* gene of *E. coli* (Stokes *et al.*, 1985), *lacZ* gene of *Klebsiella pneumoniae*, *kpn* (Buvinger *et al.*, 1985), *lacZ* gene of *Lactobacillus bulgaricus*, *lbu* (Schmidt *et al.*, 1989), and *cbgA* gene of *Clostridium acetobutylicum* (Hancock *et al.*, 1991) identified several highly similar regions. Using the conserved amino acid region at the positions 219 to 224 of the above deduced amino acid sequence a redundant 20-mer oligonucleotide mixture (CP20) was designed (as shown in Figure 26) to use as a probe.

The CP20 oligonucleotide mixture with 64 fold redundancy was synthesized and end labelled with [γ -³²P]ATP using T4 polynucleotide kinase, by the forward reaction described in section 2.15.1. The [γ -³²P]ATP end-labelled oligonucleotide probe (CP20) was purified from unincorporated [γ -³²P]ATP using the method described in section 2.15.1. A Southern blot of *PstI*, *EcoRI*, and *HindIII* digests of pDI3 and *EcoRI* digests of pKH179 was prepared and hybridized with the [γ -³²P]ATP end-labelled oligonucleotide mixture as described in sections 2.15.2 and 2.15.3, respectively. The hybridization conditions that were used are described in section 2.15.3. The 20-mer oligonucleotide probe, CP20 showed homology to the 4.3 kb *EcoRI* doublet (lane 3) and the 4.3 kb *HindIII* doublet (lane 5) of pDI3 as well as the 5.2 kb *EcoRI* (*cbgA*) fragment of pKH179 (lane 7) as shown in Figure 27 frame B. No clear hybridization signals were detected for the *PstI* digests of pDI3 (lane 2) and the *EcoRI* digests of the 43.4 kb *PstI* fragment of pDI3 (lane 4).

Figure 25

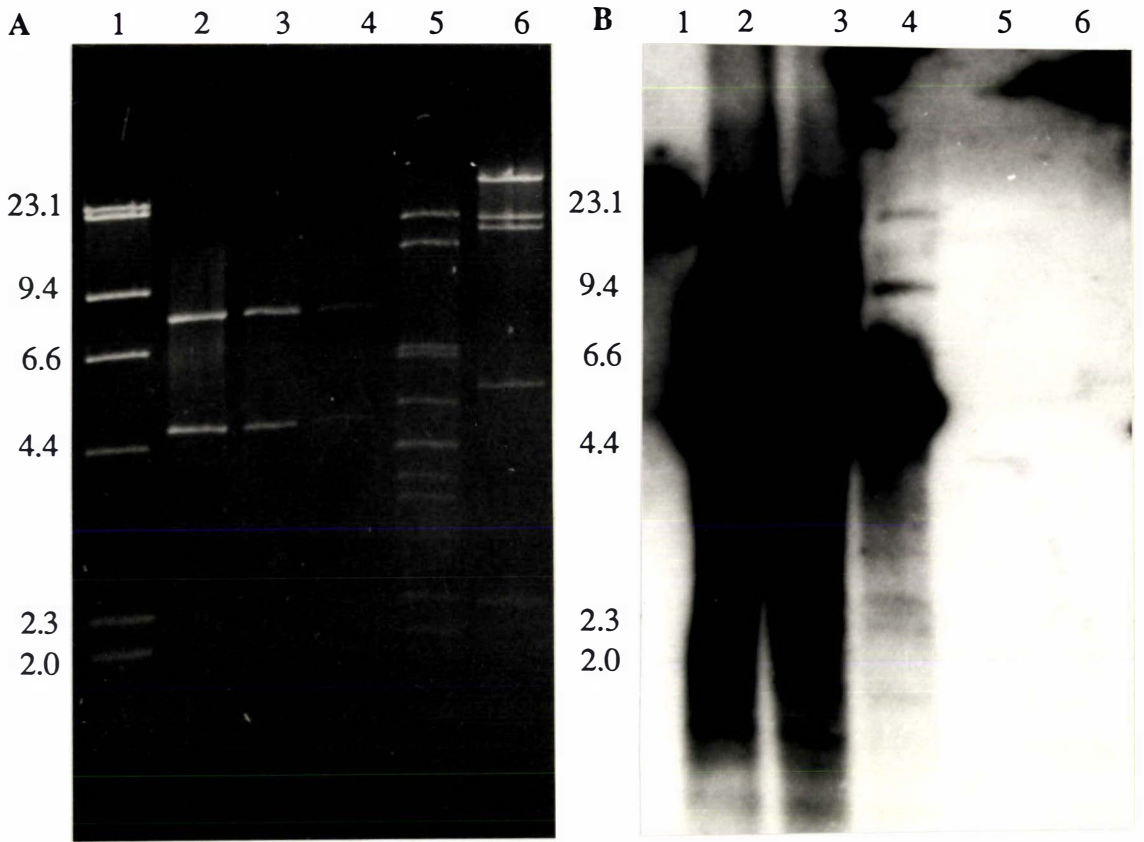
Hybridization of the 5.2 kb *EcoRI* fragment from pKH179 to pDI3 digests.

Transfer of DNA samples from 0.7% agarose gel onto a nylon membrane was carried out as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out under moderate stringency (3x SSC at 55°C). Autoradiography was carried out as described in section 2.14.

frame A: Gel photograph of: *EcoRI* digests of pKH179 (lanes 2-4); *EcoRI* digest of pDI3 (lane 5) and *SalI* digest of pDI3 (lane 6).

Lane 1 is a *HindIII* digest of λ DNA (fragment sizes are in kb),

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [α - 32 P]dCTP-labelled 5.2 kb *EcoRI* fragment from pKH179.



unlabeled?

Figure 26

The design of the oligonucleotide probe, CP20.

- A: Deduced amino acid sequences from a conserved region of the following β -galactosidase of: *cbgA* from *Clostridium acetobutylicum* (Hancock *et al.*, 1991), *lbu* from *Lactobacillus bulgaricus* (Schmidt *et al.*, 1989), *lacZ* from *Escherichia coli* (Kalnins *et al.*, 1983), *ebgA* from *Escherichia coli* (Stokes *et al.*, 1985) and *kpn* from *Klebsiella pneumoniae* (Buvinger *et al.*, 1985).
- B: The 20-mer oligonucleotide probe, CP20, with 64-fold redundancy, was designed based on the Gram positive bacterial β -galactosidase sequences (i.e. amino acid phenylalanine was chosen instead of methionine).

A

	E	D	Q	D	F	W	R	degeneracy
<i>(cbgA), Clostridium acetobutylicum</i>	GA ^A _G	GAT ^T _C	CAA ^A _G	GAT ^T _C	TT ^T _C	TGG ^A _C	AGX	64-fold
<i>(lbu), Lactobacillus bulgaricus</i>	GA ^A _G	GAT ^T _C	CAA ^A _G	GAT ^T _C	TT ^T _C	TGG ^A _C	AGX	64-fold
<i>(lacZ), Escherichia coli</i>	GA ^A _G	GAT ^T _C	CAA ^A _G	GAT ^T _C	ATG	TGG ^A _C	AGX	32-fold
<i>(ebgA), Escherichia coli</i>	GA ^A _G	GAT ^T _C	CAA ^A _G	GAT ^T _C	ATG	TGG	TGG	16-fold
<i>(kpn), Klebsiella pneumoniae</i>	GA ^A _G	GAT ^T _C	CAA ^A _G	GAT ^T _C	ATG	TGG ^A _C	AGX	32-fold

B

	E	D	Q	D	F	W	R	
5' <i>homotet</i> GA ^A _G	GAT ^T _C	CAA ^A _G	GAT ^T _C	TT ^T _C	TGG ^A _C	AG	3'	64-fold

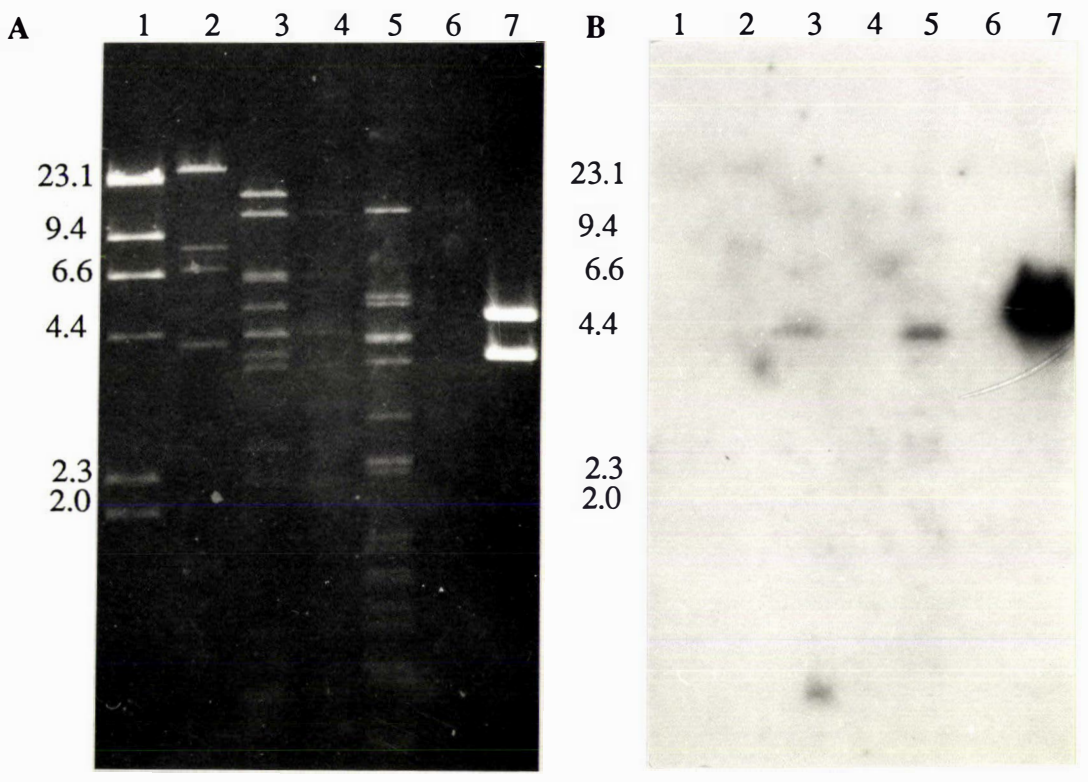
Figure 27

Hybridization of the 20-mer oligonucleotide probe, CP20, to pDI3 digests.

Transfer of DNA samples from a 0.7% agarose gel onto a nylon membrane was carried out as described in section 2.15.2. The oligonucleotide probe was labelled described in section 2.15.1. Hybridization and autoradiography were carried out as described in section 2.15.3.

frame A: Gel photograph of: *Pst*I digest of pDI3 (lane 2); *Eco*RI digest of pDI3 (lane 3); *Eco*RI digest of the 43.4 kb *Pst*I fragment of pDI3 (lane 4); *Hind*III digest of pDI3 (lane 5); blank (lane 6) and *Eco*RI digest of pKH179 (lane 7). Lane 1 is a *Hind*III digest of λ DNA (fragment sizes are in kb)

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [γ -³²P]ATP end-labelled oligonucleotide probe, CP20.



4.5. Molecular cloning of the 4.3 kb *EcoRI* doublet of pDI3

Given that the 4.3 kb *EcoRI* doublet of pDI3 hybridized to both the two probes used (CA1 and CP20) an attempt was made to clone both fragments. The 4.3 kb *EcoRI* doublet of pDI3 was isolated from an *EcoRI* digest of pDI3 (sections 2.12.1 and 2.11, respectively) and ligated to *EcoRI* cut and dephosphorylated pUC18 (sections 2.12.1, 2.12.2, and 2.12.3). The ligated DNA was transformed into *E. coli* MC1022 by electroporation as described in section 2.16.2.

One transformant was screened and designated PN1338. Plasmid DNA from this transformant was isolated as described in section 2.6.1. Recombinant plasmid DNA was digested with restriction enzyme *EcoRI* (section 2.12.1) and was found to contain an *EcoRI* insert of 4.3 kb. The recombinant plasmid was designated pSY115 and did not exhibit β -galactosidase activity.

4.6 Hybridization of a β -galactosidase oligonucleotide probe to the cloned 4.3 kb *EcoRI* fragments of pDI3.

Given that neither of the cloned 4.3 kb *EcoRI* fragments of both *E. coli* transformants (PN1337 and PN1338) expressed β -galactosidase activity, both plasmids were hybridized with a new oligonucleotide probe designed to a conserved region of deduced amino acid sequence from β -galactosidase sequences including the deduced amino acid sequences derived from *lacL*, the β -galactosidase gene from *Leuconostoc lactis* (David *et al.*, 1992). The oligonucleotide probe used in this experiment (BS13) was designed from the same region as that shown in Figure 26 except was two nucleotides shorter (i.e. the amino acid arginine was not included) to minimize the possibility of redundancy at the 3' end as shown in Figure 28.

This oligonucleotides mixture was also end labelled with [γ -³²P]ATP and purified as described in section 2.15.1 and used as a probe to hybridize to a Southern blot of the cloned fragment of pDI3 from strain PN1338 prepared as described in section

Figure 28 The design of the oligonucleotide probe, BS13

2.15.2. The 5.2 kb *EcoRI* fragment of pKH179 containing the *Clostridium acetobutylicum* β -galactosidase fragment (Hancock *et al.*, 1991) and the 7.0 kb *PstI* fragment of pCP2 containing *Streptococcus thermophilus*'s β -galactosidase fragment (C.J.Pillidge unpublished data) were included as positive controls.

The hybridization results of this experiment are shown in Figure 29 frame B. Strong hybridization was observed to the 5.2 kb *EcoRI* fragment of pKH179 (lanes 2 and 3) and the 7.0 kb *PstI* fragment of pCP2 (lanes 5 and 6). Weak hybridization was observed to the 4.3 kb *EcoRI* fragment of PN1338 (lane 7). No hybridization was detected to *EcoRI* digest of pDI3 (lane 4).

In addition, an experiment was carried out to confirm that both of the 4.3 kb *EcoRI* cloned fragments were derived from pDI3. The cloned 4.3 kb *EcoRI* fragment was isolated from the strain PN1338 (section 2.6.1), purified and labelled with [α -³²P]dCTP using the method described in sections 2.11 and 2.12.4. The labelled fragments was used as a probe to hybridize to a Southern blot (section 2.13) of *EcoRI* and *SalI* digests of pDI3. The hybridization was carried out at high stringency conditions (65°C, 3x SSC see also section 2.14).

The results of these hybridization experiments shown in Figure 30 confirmed that the 4.3 kb *EcoRI* fragment of PN1338 was derived from pDI3. The 4.3 kb *EcoRI* fragment of PN1338 also hybridized to *SalI* fragments of pDI3 (Figure 30 frame B lane 3) but which fragment hybridized (either the 13.6 kb *SalI* fragment or 15.9 kb *SalI* fragment) was not clear due to the poor resolution of these two bands. However, it can be assumed that the 4.3 kb *EcoRI* of PN1338 is the 4.3 kb *EcoRI* fragment a from the information obtained from the physical map of pDI3 (see section 4.3.4). Moreover, the location of the 4.3a kb *EcoRI* fragment on the pDI3 map helps to confirm that the hybridized *SalI* fragment of pDI3 to the 4.3a kb *EcoRI* is the 13.6 kb fragment.

Figure 29

Hybridization of the oligonucleotide probe, BS13 to cloned fragment of pDI3 and to β -galactosidase containing fragments from *Clostridium acetobutylicum* and *Streptococcus thermophilus*.

Transfer of DNA samples from a 0.7% agarose gel onto a nylon membrane was carried out as described in section 2.15.2. The oligonucleotide probe was labelled as described in section 2.15.1. Hybridization and autoradiography were carried out as described in section 2.15.3.

frame A: Gel photograph of: *Eco*RI digests of pKH179 (lanes 2-3); *Eco*RI digest of pDI3 (lane 4); *Pst*I digests of pCP2 (lanes 5-6) and *Eco*RI digest of pSY115 (lane 7). Lane 1 is a *Hind*III digest of λ DNA (fragment sizes are in kb).

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [γ -³²P]ATP end-labelled oligonucleotide probe, BS13.

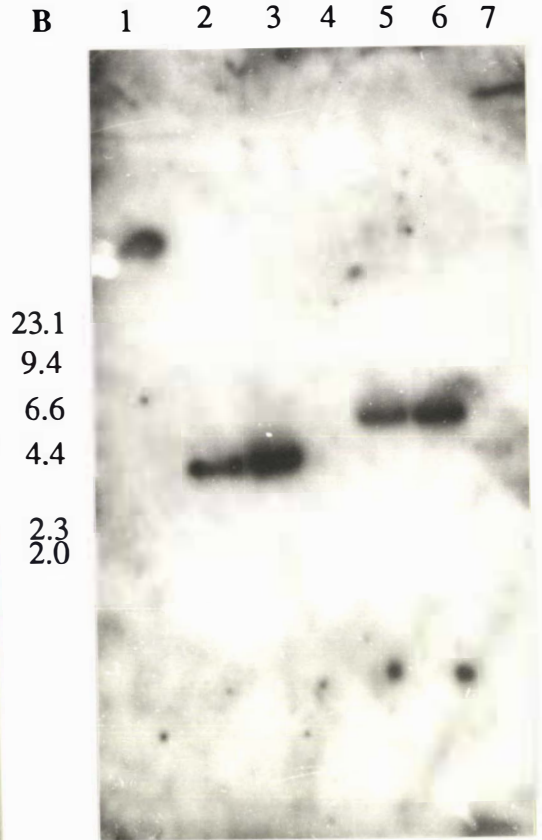
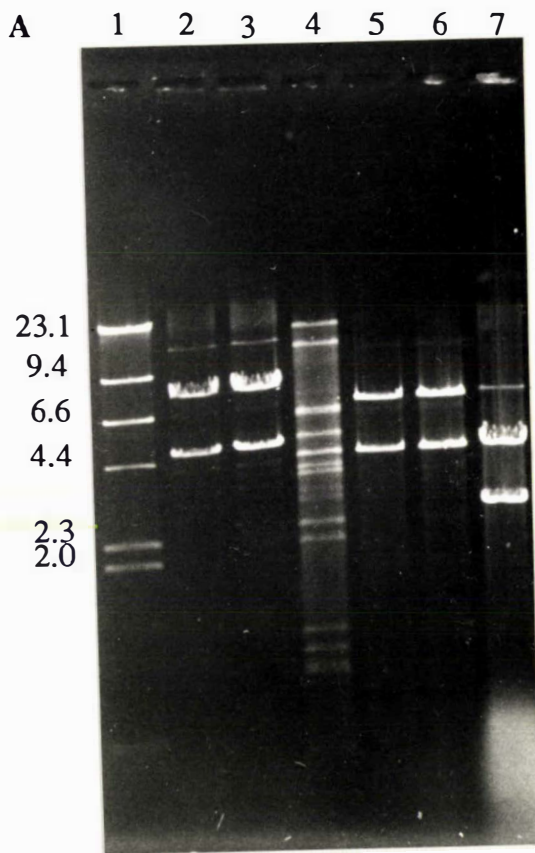


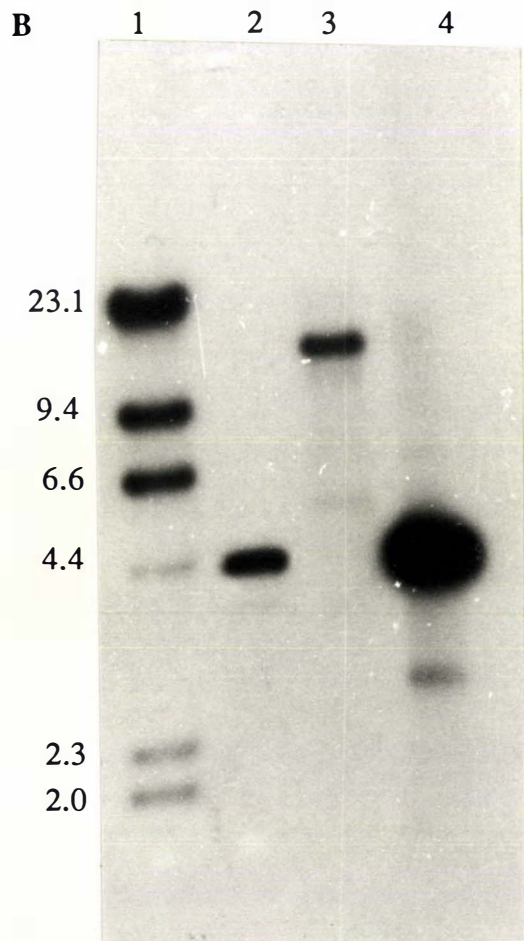
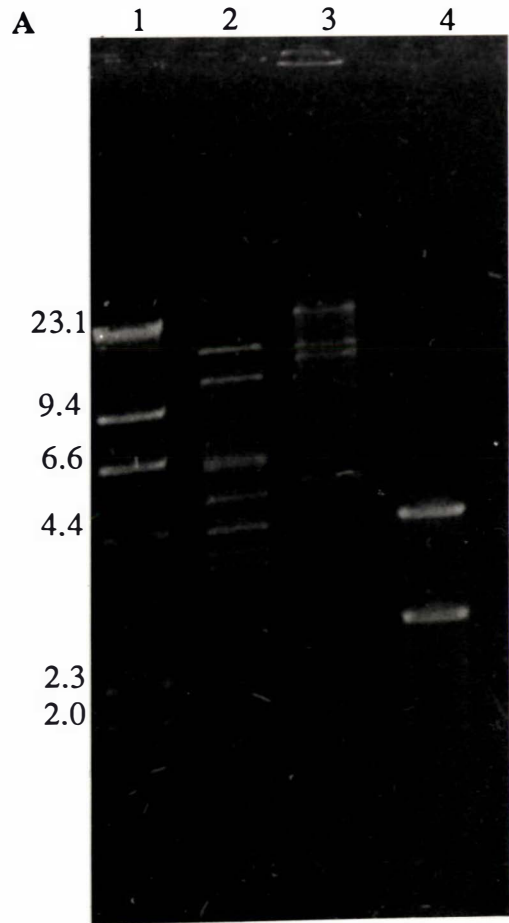
Figure 30

Hybridization of the 4.3 kb *Eco*RI fragment from PN1338 (pSY115) to pDI3 digests.

Transfer of DNA samples from 0.7% agarose gel onto a nylon membrane was carried out as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out under high stringency conditions (3x SSC at 65°C). Autoradiography was carried out using an X-ray film (Cronex) as described in section 2.14.

frame A: Gel photograph of: *Eco*RI digest of pDI3 (lane 2); *Sal*I digest of pDI3 (lane 3) and *Eco*RI digest of pSY115 (lane 4). Lane 1 is a *Hind*III digest of λ DNA (fragment sizes are in kb).

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [α -³²P]dCTP-labelled 4.3 kb *Eco*RI fragment from pSY115.



4.7 Molecular cloning of the other possible β -galactosidase fragments from pDI3

Due to the lack of β -galactosidase expression for the 4.3 kb *EcoRI* fragment of PN1338 (fragment 4.3a), other possible β -galactosidase fragments of pDI3 were analysed for further cloning experiments. Based on the hybridization result between the 4.3 kb *EcoRI* fragment of PN1338 and the *SalI* digest of pDI3 (Figure 30 frame B lane 3) and the location of this 4.3a kb *EcoRI* fragment on the pDI3 map, the 13.6 kb *SalI* fragment was the other possible β -galactosidase fragment to be cloned.

Due to the poor separation by electrophoresis of the fragments in this molecular weight range, both the 13.6 and 15.9 kb *SalI* fragments were isolated together from the gel, purified and ligated with *SalI* linearized pBR322 (sections 2.11, 2.7, and 2.12.3, respectively). The ligated DNA was used to transform *E. coli* JM109 as described in section 2.16.2. The transformants were screened and recombinant plasmids were isolated using the method described in section 2.6.1.

All clones analysed contained the 13.6 kb *SalI* fragment but none contained the 15.9 kb *SalI* fragment. The recombinant plasmid of 18.0 kb, designated pSY303, was isolated from the designated *E. coli* transformant PN1357. When pSY303 was digested with *SalI*, two fragments of DNA were obtained: a 4.4 kb fragment of vector DNA (pBR322) and a 13.6 kb *SalI* fragment of insert DNA from pDI3.

To confirm that the 13.6 kb *SalI* from PN1357 had come from pDI3, a Southern blot was prepared (2.13) with a sample of pSY303 digested with *SalI*, together with various digests of pDI3. This Southern blot was hybridized with the [α - 32 P]dCTP labelled 13.6 kb *SalI* fragment isolated from pDI3 (sections 2.14, 2.12.4, and 2.11, respectively). The hybridization was carried out under high stringency conditions (3x SSC at 65°C) as described in section 2.14. The hybridization results are illustrated in Figure 31 and summarized in Table 18.

Figure 31

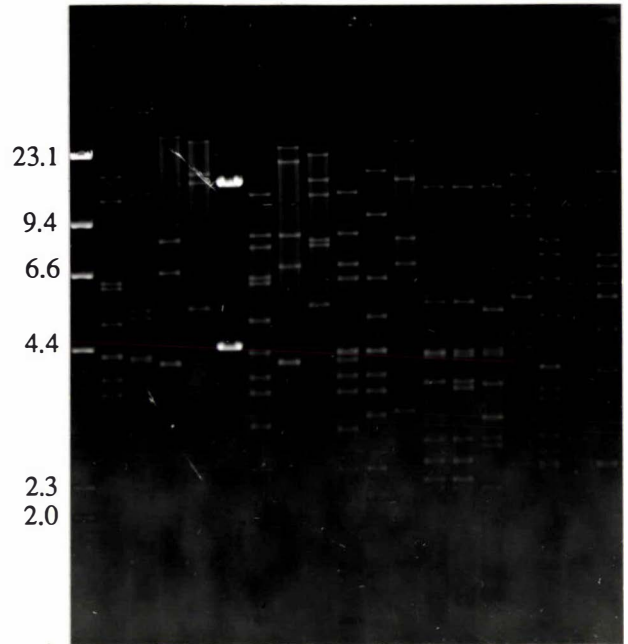
Hybridization of the 13.6 kb *SalI* fragment from pDI3 to a *SalI* digest of pSY303 from PN1357 and various digests of pDI3.

Transfer of the DNA samples was carried out from a 0.7% agarose gel onto a nylon membrane as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out at high stringency conditions (3x SSC at 65°C). Autoradiography was carried out using an X-ray film (Cronex) as described in section 2.14.

frame A: Gel photograph of various restriction enzyme digests of pDI3: *EcoRI* (lane 2); *HindIII* (lane 3); *PstI* (lane 4); *SalI* (lane 5); *BamHI/EcoRI* (lane 7); *BamHI/PstI* (lane 8); *BamHI/SalI* (lane 9); *EcoRI/PstI* (lane 10); *EcoRI/SalI* (lane 11); *PstI/SalI* (lane 12); *BamHI/HindIII* (lane 13); *PstI/HindIII* (lane 14); *SalI/HindIII* (lane 15); *BamHI/BglII* (lane 16); *BglIII/EcoRI* (lane 17); *BglIII/PstI* (lane 18) and *BglII/SalI* (lane 19). Lane 6 is a *SalI* digest of pSY303 from PN1357. Lane 1 is a *HindIII* digest of λ DNA (fragment sizes are in kb).

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [α -³²P]dCTP-labelled 13.6 kb *SalI* fragment from pDI3.

A 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19



B 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

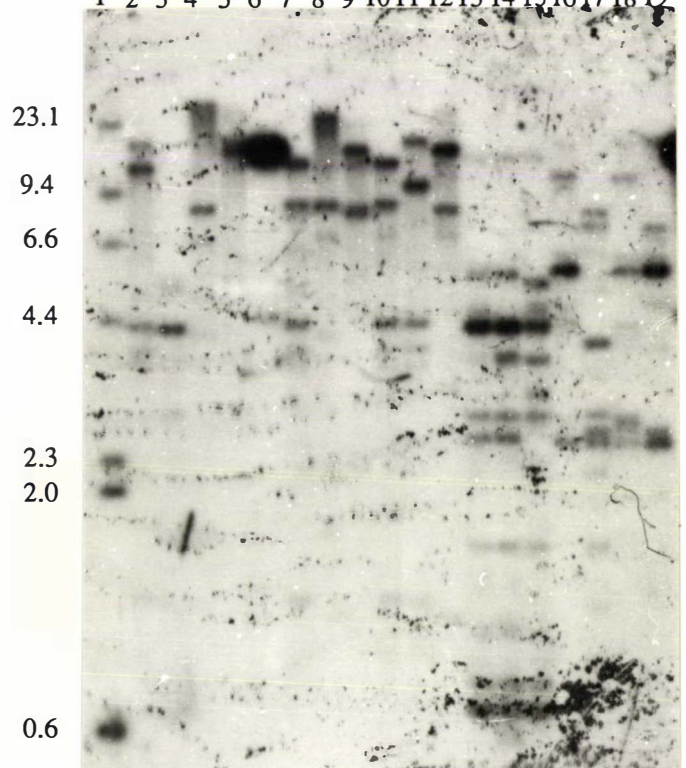


Table 18 Summary of the hybridization results between pDI3 digests and the 13.6 kb *SaI*I fragment of pDI3

pDI3 digests								
H (L. 3) ¹	pSY303 (L. 6)	B/H (L. 13)	P/H (L. 14)	S/H (L. 15)	B/Bg (L. 16)	E/Bg (L. 17)	P/Bg (L. 18)	S/Bg (L. 19)
4.3a+++ ²	13.6+++	5.5++	5.5++	5.3++	9.5++	8.3+	9.5+	6.8+
		4.3a+++	4.3a+++	4.3a+++	5.6a+++	6.8+	5.6a+++	5.6a+++
		2.9+	3.6++	3.6++	2.5+	4.1+++	2.8++	2.6+
		2.6+	2.9+	3.1++		2.8+	2.7++	2.5++
		1.8+	2.6+	2.9+		2.6++	2.5+	
		1.0-	1.8+	1.8+		2.5++		
		0.9+	1.0-	1.0-		1.8-		
		0.7++	0.9+	0.9+		1.5-		
			0.7++	0.7++				

¹ Lane numbers as shown in Figure 31

² - to +++ indicates the relative intensity of the hybridizing band from weak to strong which are compared within each digest not between digests

L = Lane, B = *Bam* HI, Bg = *Bgl*III, E = *Eco*RI, H = *Hin* dIII, P = *Pst*I and S = *Sa*II

The results obtained clearly indicated that the 13.6 kb *SalI* from PN1357 was derived from pDI3 (Figure 31 frame B lane 6). Moreover, the probe used in this experiment also showed strong homology to some other pDI3 fragments including the 4.3a kb *EcoRI* (see also sections 4.3.2 and 4.3.3), the 4.3a kb *HindIII* fragment from the 4.3 kb *HindIII* doublet, and the 5.6a kb *BglII* fragment from the 5.6 kb *BglII* doublet (Figure 31 frame B lanes 2, 3, and 16, respectively and also see later sections 4.9 and 4.10).

In addition to the 4.3 kb *EcoRI* fragment cloning as described in section 4.5, another 4.3 kb *EcoRI* fragment was also cloned as part of the pDI3 mapping work (see section 4.3.2). One of the recombinant plasmid resulting from the *EcoRI* shotgun cloning experiment (i.e. pSY105) was found to contain a 4.3 kb *EcoRI* fragment from pDI3 (section 4.3.2). To determine whether these two 4.3 kb *EcoRI* fragments (i.e. one from pSY105 another one from pSY115) were the same or different fragment, two approaches were used. Firstly, a hybridization between pSY105 *EcoRI* digest and the 13.6 kb *SalI* fragment from pDI3 was conducted.

A Southern blot of an *EcoRI* digest of pSY105 and samples of pDI3 digests (*EcoRI*, *SalI*, *BglII*, *HindIII*, and *PstI*) were included and transferred (sections 2.12.1 and 2.13, respectively) as shown in Figure 32 frame A. The 13.6 kb *SalI* fragment isolated from pDI3 was labelled with [α - 32 P]dCTP (sections 2.11 and 2.12.4, respectively) and used as a probe to hybridize to the above Southern blot at 65°C in 3x SSC (section 2.14). The obtained hybridization result (Figure 32 frame B lane 3) showed that the 4.3 kb *EcoRI* from pSY105 also hybridized to the 13.6 kb *SalI* of pDI3. Moreover, the 13.6 kb *SalI* fragment from pDI3 also hybridized to one of the 5.6 kb *BglII* fragment from the 5.6 kb *BglII* doublet and one of the 4.3 kb *HindIII* fragment of the 4.3 kb *HindIII* doublet (Figure 32 frame B lanes 5 and 6, respectively and also see sections 4.9 and 4.10).

Secondly, a restriction enzyme analysis of the two clones was carried out as shown in Figure 33. Both of the cloned fragments shared the same restriction endonuclease profiles with common fragments of 1.6 and 2.7 kb *BglII/EcoRI* fragments (Figure 33

Figure 32

Hybridization of the 13.6 kb *SaII* fragment from pDI3 to various digests of pDI3 and an *EcoRI* digest of pSY105.

Transfer of the DNA samples was carried out from a 0.7% agarose gel onto a nylon membrane as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out under high stringency conditions (3x SSC at 65°C). Autoradiography was carried out using an X-ray film (Cronex) as described in section 2.14.

frame A: Gel photograph of various restriction enzyme digests of pDI3: *EcoRI* (lane 2); *SaII* (lane 4); *BglIII* (lane 5); *HindIII* (lane 6) and *PstI* (lane 7). Lane 3 is an *EcoRI* digest of pSY105. Lane 1 is a *HindIII* digest of λ DNA (fragment sizes are in kb).

frame B: Autoradiograph of a Southern blot of the above gel hybridized with the [α -³²P]dCTP-labelled 13.6 kb *SaII* fragment from pDI3.

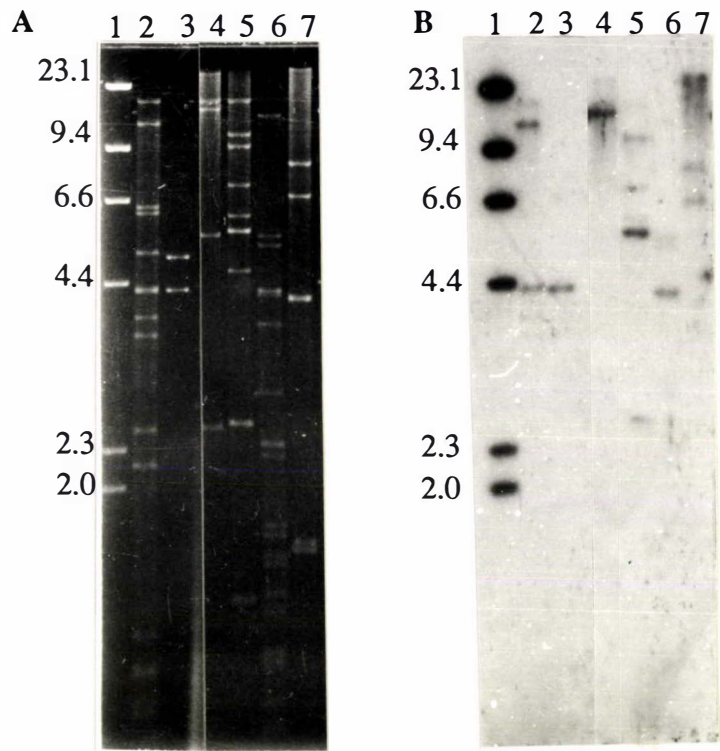
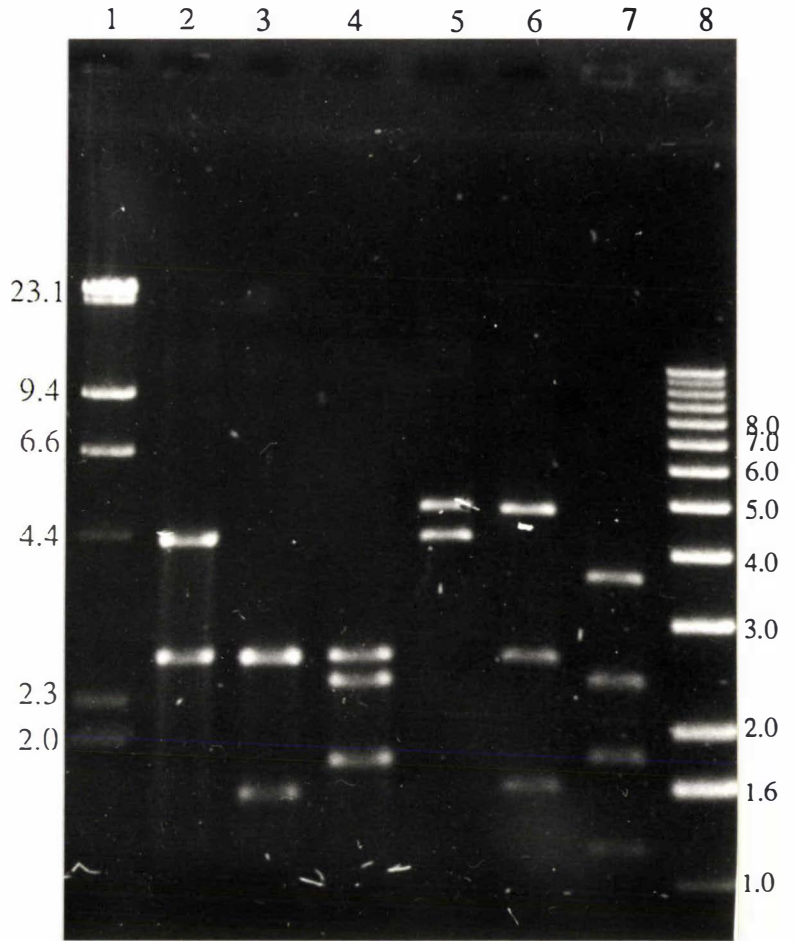


Figure 33

Restriction enzyme comparison of pSY105 and pSY115.

Gel photograph of various restriction enzyme digests of pSY115 and pSY105. Lanes 2-4 are the *EcoRI*, *EcoRI/BglII* and *ClaI/EcoRI* digests of pSY115, respectively. Lanes 5-7 are the *EcoRI*, *EcoRI/BglII* and *ClaI/EcoRI* digests of pSY105, respectively. Lane 1 is a *HindIII* digest of λ DNA (fragment sizes are in kb). Lane 8 is BRL's 1 kb DNA ladder standard.



lanes 3 and 6) and of 1.9 and 2.4 kb *ClaI/EcoRI* fragments (Figure 33 lanes 4 and 7). These two cloned 4.3 kb *EcoRI* fragments are therefore the same fragment and located in the 13.6 kb *SalI* fragment of pDI3. According to the restriction endonuclease map of pDI3 the location of the other 4.3 kb *EcoRI* (i.e. 4.3b *EcoRI* fragment) should be at the *BamHI* site located on the 32.2 kb *SalI* fragment of pDI3.

In conclusion, the 4.3 kb *EcoRI* doublet that showed some detectable homology to the β -galactosidase probes used in this study is actually the 4.3a kb *EcoRI* fragment. This fragment was successfully cloned into two different *E. coli* plasmid vectors (pSY115; pUC18 and pSY105; pBR328) and transformed into two different *E. coli* host strains (i.e. *E. coli* MC1022 to give PN1338 and *E. coli* JM109 to give PN1349). Neither of these clones showed detectable β -galactosidase activity.

4.8 Expression of *Lactococcus lactis* β -galactosidase in *E. coli*

Having successfully cloned the 13.6 kb *SalI* fragment which overlaps the 4.3 kb *EcoRI* fragment shown to hybridize to β -galactosidase probes (sections 4.4 and 4.6) experiments were initiated to see if this 13.6 kb *SalI* fragment would express β -galactosidase activity in *E. coli*. When strain PN1357 (i.e. *E. coli* JM109 containing pSY303) was plated on LB medium containing 0.1% (w/v) lactose, 40 μ g/ml of X-gal and 100 μ g/ml of ampicillin (LBLAX; sections 2.2 and 2.17) β -galactosidase activity was detected by formation of blue colonies after overnight incubation at 37°C (Figure 34 B). However, β -galactosidase activity of PN1357 was also found in LB medium containing 40 μ g/ml of X-gal, 200 μ g/ml of IPTG and 100 μ g/ml of ampicillin (LBAXI; sections 2.2 and 2.17) and LB medium containing 40 μ g/ml of X-gal and 100 μ g/ml of ampicillin (LBAX; sections 2.2 and 2.17). With respect to the inducer, the expression of the β -galactosidase was found to be constitutively expressed since IPTG was not required for the expression (see Table 19). However, 0.1% (w/v) lactose was added to all the media used in subsequent expression studies. Despite this observation lactose may produce some effect on the level of the β -galactosidase expression and stability of pSY303.

Figure 34

β -galactosidase expression of *E. coli* PN1357.

E. coli cultures were grown on LBLAX agar (section 2.2) overnight at 37°C.

- A *E. coli* host strain JM109
- B *E. coli* PN1357 demonstrating phenotypic segregation. Both blue and white colonies contained an intact pSY303.
- C *E. coli* PN1357 containing a deleted derivative of pSY303.

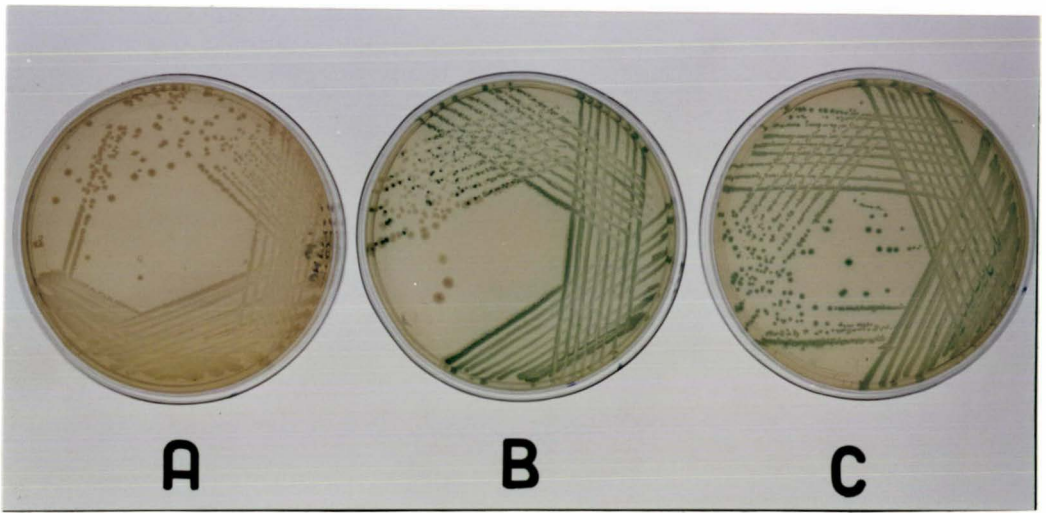


Table 19 Level of β -galactosidase activity of *Lactococcus lactis* ssp.*lactis* strain 7962 in permeabilized *E. coli* cells

Strains ²	β -galactosidase (U) ¹	
	+IPTG	-IPTG
DH5 α	3.5	0.1
LE392	182.3	ND ³
DH5 α /pUC18	175.9	115.7
DH5 α /pBR322	4.3	0.8
DH5 α /pSY303 (PN1356)	236.4	230.9
JM109	1.8	0.5
JM109/pBR322	2.4	0.5
JM109/pSY303 (PN1357)	271.4	242.2
XL-1 Blue	1.2	0.4
XL-1 Blue/pSY303 (PN1383)	221.6	238.7
PB2959 ⁴	0.0	0.0
PB2959/pSY303 (PN1394)	212.6	224.5

¹ Unit of β -galactosidase as described in section 2.17

² Cells were grown in AI medium containing glucose as the carbon source as described in section 2.2

³ ND = Not determined

⁴ Cells were grown in M56 medium containing glucose as the carbon source as described in section 2.17

The effect of glucose on the expression of the β -galactosidase activity was also studied by adding various concentration of glucose (i.e. from 0.05% (w/v) to 0.4% (w/v) glucose into the LBLAX agar (section 2.2). At these concentrations of glucose there was no detectable glucose effect on β -galactosidase expression in PN1357 as this culture still produced blue colonies.

pSY303 plasmid DNA isolated from PN1357 by the method described in sections 2.6.3 and 2.7.1 was transferred into other *E. coli* strains, DH5 α and XL-1 Blue by electroporation (see section 2.16.2) and the transformants obtained designated PN1356 and PN1383, respectively. *E. coli* strain PN1356 and PN1383 harboring pSY303 both expressed β -galactosidase activity as shown by the blue colonies formed on LBLAX medium (section 2.2 and 2.17) after overnight incubation period at 37°C (data not shown). Since all of these *E. coli* strains (i.e. JM109, DH5 α , and XL-1 Blue) contain the lacZ Δ M15 mutation either on the F' (JM109 and XL-1 Blue) or on the chromosome (DH5 α) the β -galactosidase activity observed could have been the result of α -complementation of the α -peptide of a natural *E. coli* β -galactosidase.

To confirm that the plasmid pSY303 encoded the entire β -galactosidase gene, this plasmid was purified and transferred by electroporation (sections 2.7.6 and 2.16.2) to *E. coli* PB2959, a strain that contains a deletion of the entire *E. coli* lac operon. Two μ l of pSY303 isolated from PN1357 (sections 2.6.1 and 2.7.6, respectively) was electroporated into 40 μ l cells of *E. coli* PB2959 (prepared as described in section 2.16.2). *E. coli* PB2959 harbouring pSY303 (designated PN1394) expressed the β -galactosidase as shown by the faint blue colonies in Figure 35 B. The blue colour developed after 2-3 days incubation at 37°C on M56 medium (M56LAX, section 2.2) supplemented with 0.1% (w/v) lactose, 40 μ g/ml X-gal and 100 μ g/ml ampicillin.

After testing for β -galactosidase activity by the plate assay the same cultures were biochemically assayed for β -galactosidase activity using a chromogenic substrate ONPG (o-nitro-phenyl- β -D-galactoside) as described in section 2.17. The assay was conducted in permeabilized *E. coli* host cells where ONPG was hydrolysed by β -galactosidase to galactose and a pale yellow colour of ONP (o-nitro-phenyl).

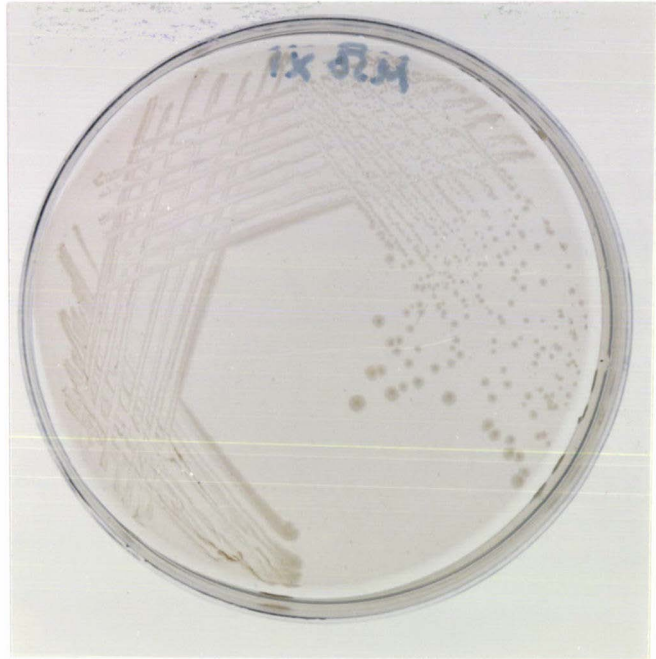
Figure 35

β -galactosidase expression of *E. coli* PB2959 containing pSY303.

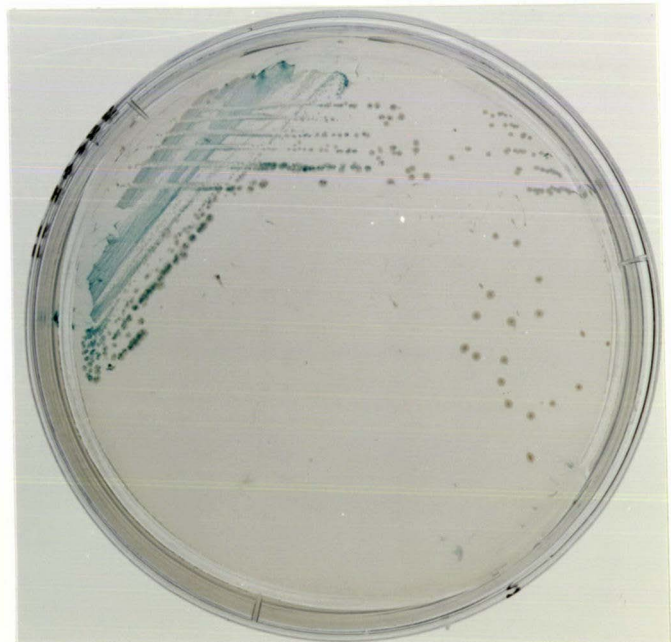
E. coli PB2959 was grown on M56LAX (section 2.2) at 37°C for 1-2 days.

- A: *E. coli* host strain PB2959
- B: *E. coli* PB2959 harbouring pSY303. The faint blue colonies contained intact pSY303, the dark blue colonies contained deleted derivatives of pSY303.

A



B



All *E. coli* cultures containing pSY303 exhibited a relatively high β -galactosidase activity as shown in Table 19. Each of the *E. coli* host strains harbouring pBR322 except *E. coli* PB2959 was used as a negative control where as *E. coli* DH5 α harboring pUC18 was used as a positive control. *E. coli* strain LE392 was also used as positive control as it is an *E. coli* wildtype strain possessing β -galactosidase activity by its own natural *E. coli lacZ*. The results showed that the level of β -galactosidase activity of all the studied *E. coli* host strains harbouring pSY303 were obviously higher than the level of the background activities of the host strains.

The expression of β -galactosidase activity is almost at the same level for all the four studied *E. coli* host strains. IPTG was observed to have no significant influence on β -galactosidase expression of pSY303 in all the studied *E. coli* host strains. This indicated that the β -galactosidase of *Lactococcus lactis* ssp. *lactis* strain NZDRI5191 was expressed constitutively in *E. coli*. It was also noticed that this β -galactosidase gene is neither induced by IPTG nor repressed by glucose.

A hybridization experiment was also carried out to confirm that the 13.6 kb *SalI* fragment of pSY303 from all the β -galactosidase positive *E. coli* strains was derived from pDI3. The hybridization was performed at high stringency conditions (3x SSC at 65°C) using the agarose gel purified 13.6 kb *SalI* fragment from pDI3 (section 2.11) as a [α -³²P]dCTP-labelled DNA probe (section 2.12.4).

A Southern blot of *SalI* digests of pSY303 isolated from PN1356, PN1357, PN1394, PN1383 including *SalI* digest of pDI3 as a positive control, was prepared as described in sections 2.12.1, 2.9, and 2.13, respectively. Results obtained from the hybridization indicated that all the β -galactosidase positive *E. coli* strains studied (i.e. blue colonies) were derived from the 13.6 kb *SalI* of pDI3 as shown in Figure 36 lanes 2-5. All of these experiments indicate that the 13.6 kb *SalI* fragment of pDI3 encodes a functional β -galactosidase.

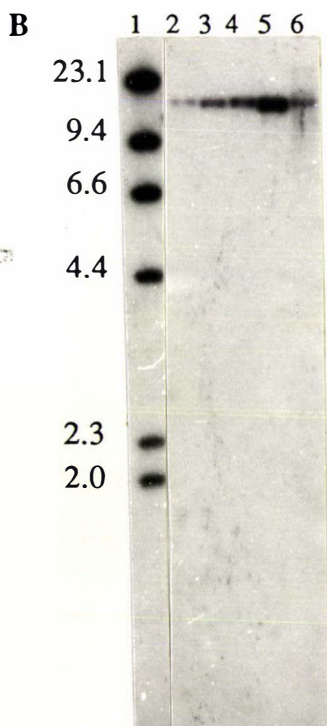
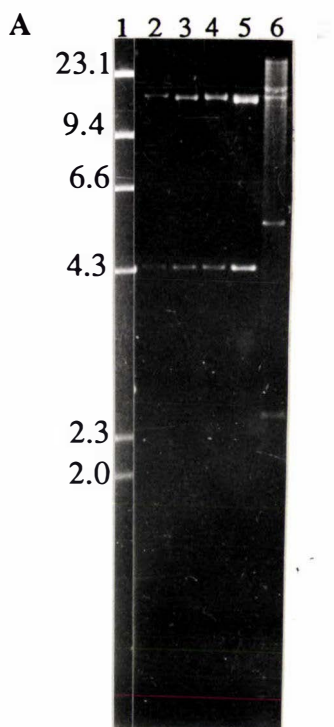
Figure 36

Hybridization of the 13.6 kb *SalI* fragment from pDI3 to the β -galactosidase positive *E. coli* strains.

Transfer of the DNA samples was carried out from a 0.7% agarose gel onto a nylon membrane as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out under high stringency conditions (3x SSC at 65°C). Autoradiography was carried out using an X-ray film (Cronex) as described in section 2.14.

frame A: Gel photograph of *SalI* digests of pSY303 isolated from β -galactosidase positive *E. coli* strains: PN1356 (lane 2); PN1357 (lane 3); PN1394 (lane 4); PN1383 (lane 5); and *SalI* digest of pDI3 (lane 6). Lane 1 is a *HindIII* digest of λ DNA (fragment sizes are in kb).

frame B: Autoradiograph of the above gel hybridized with the [α -³²P]dCTP-labelled 13.6 kb *SalI* fragment from pDI3.



4.9 The instability of pSY303 in *E. coli*

It was also found that the Lac phenotype of *E. coli* strains harbouring pSY303 was unstable. During subculturing of *E. coli* strains, segregated into white and blue colonies. This phenomenon was observed in all the *E. coli* host strains used in this study. A culture of *E. coli* JM109 containing pSY303 that is segregating for Lac⁻ (white colony) is shown in Figure 34 B.

Once white colonies were isolated they retained the Lac⁻ phenotype on further subculturing. The blue colonies, however, continued to segregate into white and blue colonies. Analysis of *Sa*I digests of plasmid DNA isolated from representative blue and white colonies revealed the presence of a 4.4 kb vector fragment (i.e. pBR322) and the 13.6 kb insert fragment. No differences were observed within the fragment profiles between plasmid DNA (pSY303) isolated from white and blue colonies (sections 2.6.1 and 2.12.1) when digested with restriction enzymes; *B*gIII, *C*laI, *E*coRI, and *H*indIII (data not shown).

The hybridization results between pDI3 digests and the cloned 13.6 kb *Sa*I insert fragment of pSY303 from PN1357 as shown in Figure 37 suggest that there may be some small repeat sequences on the 13.6 kb *Sa*I fragment of pSY303. This conclusion was made on the basis that the probe used also hybridized to some other fragments of pDI3 that are neither located in nor overlapped with the 13.6 kb *Sa*I fragment of pDI3 (see also Figures 23, 24, and 39). A Southern blot of various digests of pDI3 was prepared (section 2.12.1) and hybridized at high stringency (section 2.14, at 65°C in 3x SSC) with the [α -³²P]dCTP labelled 13.6 kb *Sa*I insert fragment of pSY303 from PN1357 (sections 2.11 and 2.12.4). The *Sa*I digests of pSY303 isolated from PN1357, from genomic DNA of *E. coli* JM109 were used as a positive control and a negative control, respectively (Figure 37 lanes 7 and 9). The results showed that the probe used hybridized to the 5.5 kb *Sa*I fragment; the 11.7, 8.2, 7.8, 4.3a, and 3.8 kb of *B*amHI/*E*coRI fragments; the 15.8, 9.4, 4.2, and 3.8 kb of *E*coRI/*Sa*I fragments; the

Figure 37

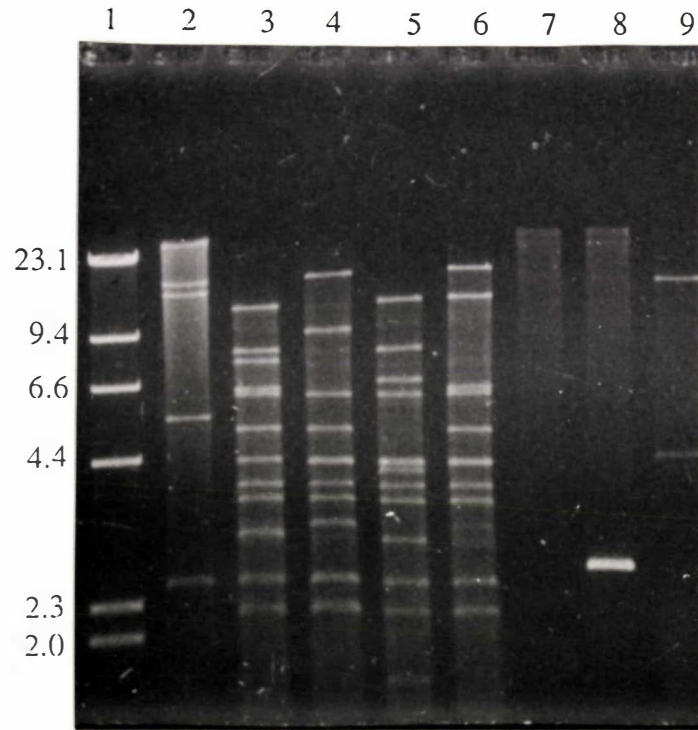
Hybridization of the 13.6 kb *SalI* insert of PN1357 to various digests of pDI3.

Transfer of the DNA samples was carried out from a 0.7% agarose gel onto a nylon membrane as described in section 2.13. The DNA probe was labelled as described in section 2.12.4. Hybridization was carried out under high stringency conditions (3x SSC at 65°C). Autoradiography was carried out using an X-ray film (Cronex) as described in section 2.14.

frame A: Gel photograph of various restriction enzyme digests of pDI3; *SalI* (lane 2), *BamHI/EcoRI* (lane 3), *EcoRI/SalI* (lane 4), *PstI/EcoRI* (lane 5), and *EcoRI* (lane 6). Lane 7 is a *SalI* digest of genomic DNA isolated from *E. coli* JM109. Lane 8 is a *SalI* digest of deleted derivatives of pSY303. Lane 9 is a *SalI* digest of pSY303. Lane 1 is a *HindIII* digest of λ DNA (fragment sizes are in kb).

frame B: Autoradiograph of a Southern blot of the above gel hybridized with [α -³²P]dCTP-labelled 13.6 kb *SalI* insert from pSY303.

A



B

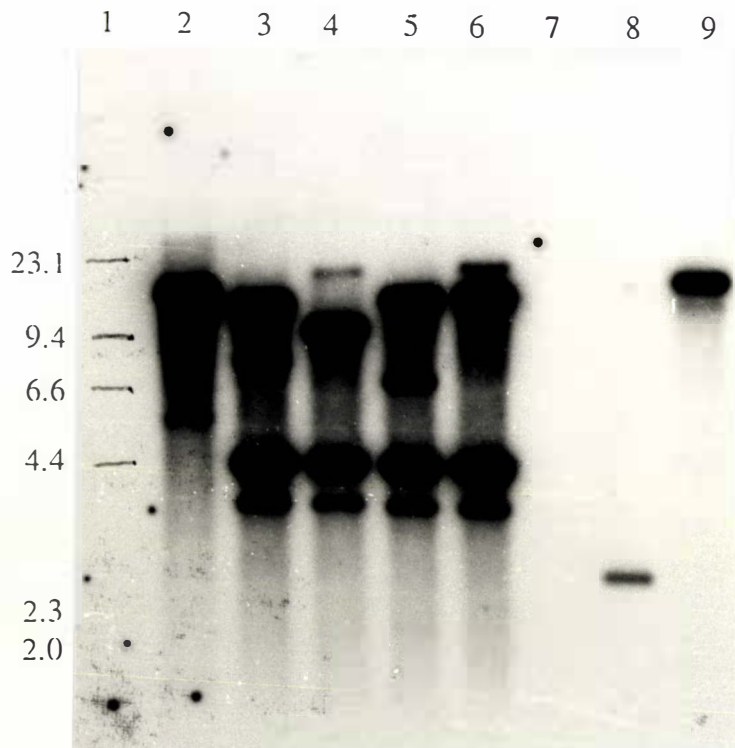


Figure 37

11.7, 6.7, 4.3a, and 3.8 kb of *PstI/EcoRI* fragments; and the 16.0, 11.7, 4.3a, and 3.8 kb *EcoRI* fragments (Figure 37 lanes 2, 3, 4, 5, and 6 and Table 20). The fragments of pDI3 which hybridized to the 13.6 kb *SalI* insert of pSY303 including the 5.5 kb *SalI*, the 8.2 kb *BamHI/EcoRI*, the 15.8 kb *EcoRI/SalI*, the 6.7 kb *PstI/EcoRI*, the 16.0 kb *EcoRI*, and the 3.8 kb *EcoRI* fragments indicate the possibility of small repeat sequence in the 13.6 kb *SalI* insert of pSY303 as neither of these fragments are located in or overlap with the 13.6 kb *SalI* of pDI3 (see also Figures 23, 24 and 39).

From the observations on the stability of pSY303 and its β -galactosidase expression in all the four *E. coli* strains studied the following conclusions can be made: intact pSY303 can be isolated from both white and blue colony of any of the strains and the higher frequency of phenotypic segregation can be found within the blue phenotypes. Among all the *E. coli* host strains used in this study, *E. coli* XL-1 Blue is a better host for the 13.6 kb *SalI* insert from pDI3 because pSY303 is found to be more stable in this host than the other hosts. Moreover, for this host the β -galactosidase activity expression results are much more consistent (i.e. the majority of *E. coli* XL-1 Blue strains harbouring intact pSY303 exhibit blue colonies).

4.10 Restriction endonuclease map of the 13.6 kb *SalI* insert of pSY303

pSY303, is a recombinant plasmid of 18 kb in size which resulted from the molecular cloning of the 13.6 kb *SalI* fragment of pDI3 into the *SalI* cloning site of pBR322. Its genotypic characteristics are $Tc^sAm^r \beta$ -gal⁺. A restriction endonuclease map of pSY303 was constructed using both restriction endonuclease analysis and the hybridization results obtained from sections 4.3.3 and 4.3.4. pSY303 was isolated and purified from an *E. coli* subclone PN1357 using the method described in sections 2.6.3 and 2.7.1, respectively. Its purity and concentration were determined as described in section 2.8. Samples of pSY303 (about 0.5 μ g) were subjected to both various single and double restriction enzyme digests in a total volume of 50 μ l containing 5 μ l of 10x restriction enzyme buffer, 1 Unit of the restriction enzyme as described in section 2.12.1. After

Table 20 Summary of hybridization results between pDI3 digests and the 13.6 kb SalI fragment of pSY303

pDI3 digests				
Sal (L. 2)	Bam/Eco (L. 3)	Eco/Sal (L. 4)	Eco/Pst (L. 5)	Eco (L. 6)
15.9+++	11.7+++	15.8+	11.7+++	16.0++
13.6+++	8.2+++	9.4+++	6.7+++	11.7+++
5.5++	7.8+++	4.3+++	4.3a+++	4.3a+++
	4.3a+++	4.2+++	3.8+++	3.8+++
	3.8+++	3.8+++		

Lane numbers as shown in Figure 39

+ to +++ indicates the relative intensity of the hybridizing band from weak to strong which are compared in each digest

L = Lane, Bam = *Bam*HI, Eco = *Eco*RI, Pst = *Pst*I and Sal = *Sal*I

the digestions had gone to completion, samples of digests were separated by gel electrophoresis at 20 Volts for 16 h., stained in ethidium bromide solution and photographed as described in section 2.9. The sizing of all the linear DNA fragments generated after the restriction enzyme digests were determined by comparing their mobilities with *Hind*III digested λ DNA run under the same electrophoretic conditions as described in section 2.10.

Restriction enzyme sites of interest in the insert 13.6 kb *Sal*I fragment were *Bgl*II, *Eco*RI and *Hind*III. Restriction enzymes; *Pst*I, *Sal*I as well as *Cla*I were used as the reference sites. Single digestions and double digestions among pairwise of these enzymes were carried out as shown in Figure 38. The number and size of the fragments generated from both single and double digestions of pSY303 are shown in Table 21. The restriction sites of the 13.6 kb *Sal*I insert of pSY303 were assigned in relation to the reference restriction sites from plasmid vector, pBR322 (see Appendix I).

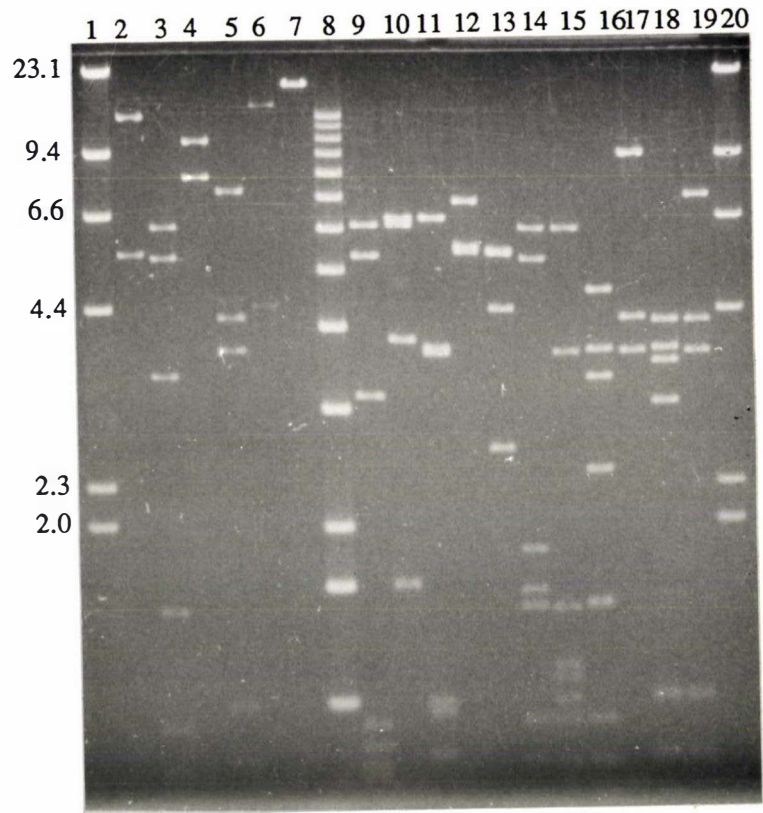
There is one *Pst*I site in pSY303 which comes from pBR322 as there is no internal *Pst*I site in the 13.6 kb *Sal*I of pDI3 (see also section 4.3.4). However, there are six *Cla*I fragments generated (Figure 38, lane 3) after *Cla*I digestion of pSY303. Therefore *Cla*I could not be used as a reference site. pSY303 was found to contain two restriction sites with restriction enzymes, *Bgl*II, *Eco*RI and *Sal*I. As there is no *Bgl*II site in pBR322, both of the two *Bgl*II sites observed actually come from the 13.6 kb *Sal*I insert to give a 12.4 and a 5.6 kb *Bgl*II fragment (Figure 38 and Table 21). Two *Eco*RI fragments of 10.1 and 7.9 kb were generated after *Eco*RI digestion of pSY303 (Figure 38 and Table 21). Since there is one *Eco*RI site contained in pBR322, the 13.6 kb *Sal*I insert should contain only one internal *Eco*RI site. *Sal*I digestion of pSY303 gave two linear fragments corresponding to the 13.6 kb insert and the 4.4 kb vector as shown in Figure 38 and Table 21. Six restriction sites were also found with restriction enzyme *Hind*III, one of which comes from pBR322. In addition, the hybridization results shown in Figures 31 and 32 also provide useful information in helping to locate the restriction sites in the 13.6 kb *Sal*I insert of pSY303.

Figure 38

Single and double restriction enzyme digests of pSY303.

pSY303 was isolated and purified as described in sections 2.6.3 and 2.7.1, respectively. DNA digests were carried out as described in section 2.12.1.

Gel photograph of pSY303 digests; *Bgl*III (lane 2); *Cla*I (lane 3); *Eco*RI (lane 4); *Hind*III (lane 5); *Sal*I (lane 6); *Pst*I (lane 7); *Bgl*III/*Cla*I (lane 9); *Bgl*III/*Eco*RI (lane 10); *Bgl*III/*Hind*III (lane 11); *Bgl*III/*Pst*I (lane 12); *Bgl*III/*Sal*I (lane 13); *Cla*I/*Eco*RI (lane 14); *Cla*I/*Hind*III (lane 15); *Cla*I/*Sal*I (lane 16); *Eco*RI/*Sal*I lane 17); *Hind*III/*Sal*I (lane 18); and *Hind*III/*Eco*RI (lane 19). Lanes 1 and 20 are *Hind*III digests of λ DNA (fragment sizes are in kb). Lane 8 is BRL's 1 kb DNA ladder size standards.



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Table 21 Fragment profiles generated from single and double digestion of pSY303 with various restriction enzymes

pSY303 digests											
Bg	E	H	S	P	Bg/E	Bg/H	Bg/P	Bg/S	E/S	H/S	H/E
L. 2 ¹	L. 4	L. 5	L. 6	L. 7	L. 10	L. 11	L. 12	L. 13	L. 17	L. 18	L. 19
(n=2) ²	(n=2)	(n=6)	(n=2)	(n=1)	(n=4)	(n=8)	(n=3)	(n=4)	(n=4)	(n=8)	(n=8)
12.4	10.1	7.5	13.6	18.0	6.4	6.5	6.7	5.6	9.4	4.3	7.4
5.6	7.9	4.3	4.4		6.0	3.8	5.7	5.3	4.2	3.8	4.3
		3.6			4.1	3.6	5.6	4.5	3.8	3.7	3.6
		1.0			1.5	1.0a		2.6	0.6	3.0	1.0
		0.9				1.0b				1.0	0.9
		0.7				0.9				0.9	0.5
						0.7				0.7	0.2
						0.5				0.6	0.1

¹ Lane number as shown in Figure 40

² n indicates number of the fragments generated in digest shown

a and b indicate the same size fragment generated after the digestion

L= Lane, Bg = *Bgl*II, E = *Eco*RI, H = *Hind*III, P = *Pst*I and S = *Sal*I

As a result the 13.6 kb *SalI* fragment of pSY303 should contain two *BglII* sites to generate an internal 5.6 kb *BglII* fragment (5.6a kb *BglII* fragment). As the 13.6 kb *SalI* fragment of pSY303 contains two *EcoRI* sites, one *EcoRI* site comes from pBR322 while the other site should be the site where the 4.2 kb *SalI/EcoRI* and the 9.4 kb *EcoRI/SalI* were generated.

Moreover, this *EcoRI* site within the 13.6 kb *SalI* was already located from the pDI3 map. This *EcoRI* site cleaves the 5.6a kb *BglII* fragment into a 4.1 kb *BglII/EcoRI* fragment and a 1.5 kb *EcoRI/BglII* fragment (Table 21). Therefore the 1.5 kb *BglII/EcoRI* fragment must be an overlap fragment between the 5.6 kb *BglII* and the 7.9 kb *EcoRI* fragment of pSY303 (i.e. the 4.2 kb *SalI/EcoRI* fragment plus 3.7 kb of *SalI/EcoRI* fragment pBR322).

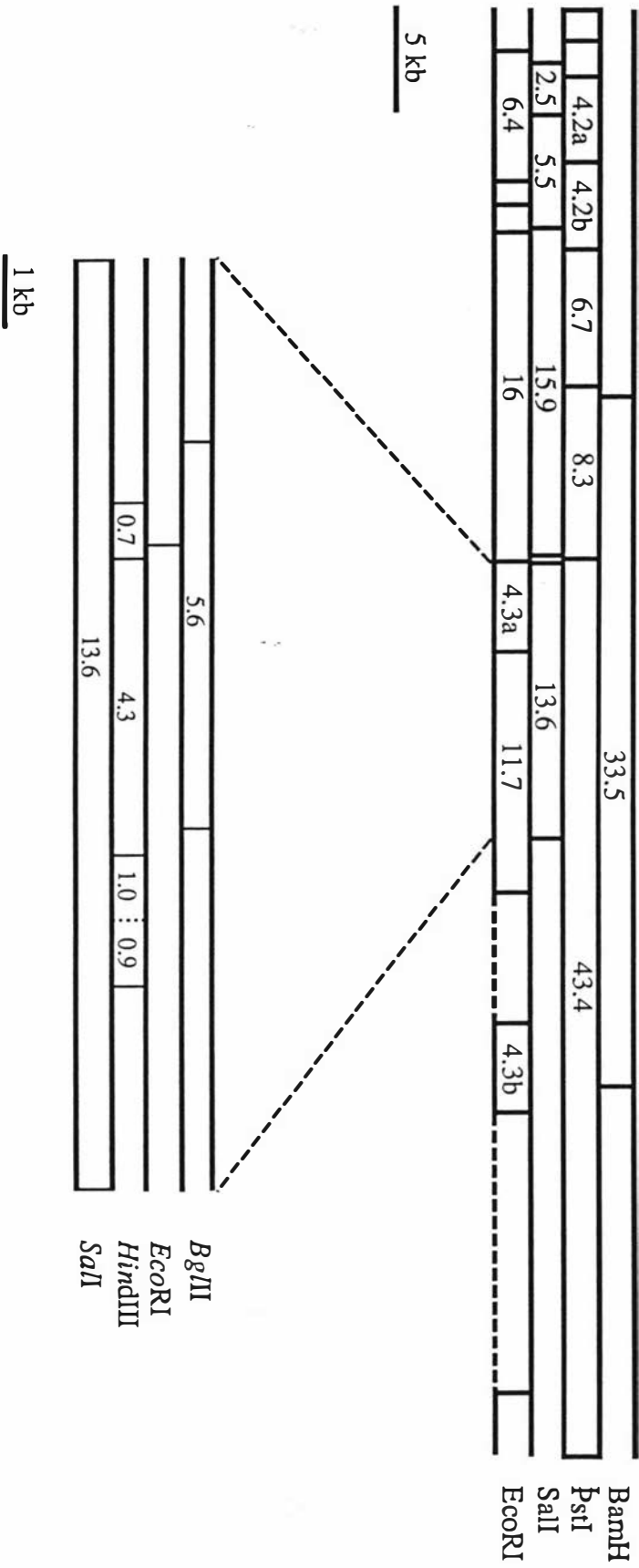
The 5.6a kb *BglII*, 4.3a kb *EcoRI*, and 4.3a kb *HindIII* are overlapping fragments, as they all show homology to the 13.6 kb *SalI* fragment of pDI3 as shown in Figure 31 and Figure 32. Thus both *BglII* sites and the *EcoRI* site in the 13.6 kb *SalI* of pSY303 were located as shown in Figure 39. One of the *BglII* sites is located within the 4.3a kb *HindIII* fragment to give a 3.8 kb *HindIII/BglII* fragment and 0.5 kb *BglII/HindIII* fragment.

However, this 4.3a kb *HindIII* fragment was not affected by the *EcoRI* site in the 13.6 kb *SalI* insert. The other *HindIII* fragment which was cut by restriction enzyme, *BglII* was the 7.5 kb *HindIII* fragment. This gave a 6.5 kb *HindIII/BglII* fragment and a 1.0 kb *BglII/HindIII* fragment (Table 21).

The *EcoRI* site contained in the 13.6 kb *SalI* cleaved the 0.7 kb *HindIII* fragment into a 0.5 kb *HindIII/EcoRI* fragment and a 0.2 kb *EcoRI/HindIII* fragment. The 7.5 kb *HindIII* fragment was also cut by the other *EcoRI* site of pSY303 to give a 7.4 kb *HindIII/EcoRI* fragment and a 0.1 kb *EcoRI/HindIII* fragment. As mentioned earlier, the 4.3a kb *HindIII* fragment is an overlap of both the 5.6a kb *BglII* and 4.3a kb *EcoRI* fragment. Therefore the 4.3a kb *HindIII* fragment is located as an adjacent fragment to the 0.7 kb *HindIII* fragment shown in Figure 39.

Figure 39

Restriction endonuclease map of the 13.6 kb *SalI* fragment containing the β -galactosidase gene region of pDI3 from *Lactococcus lactis* ssp. *lactis* strain NZDRI 5191 indicating the internal *BglII*, *EcoRI*, and *HindIII* restriction sites.



However, the arrangement between the 1.0 kb and 0.9 kb *Hind*III fragments contained in the 13.6 kb *Sal*I insert of pSY303 are not determined. The restriction map of the 13.6 kb *Sal*I insert of pSY303 incorporating *Bgl*III, *Eco*RI, *Hind*III, and *Sal*I sites in relation to the restriction map of pDI3 is illustrated in Figure 39.

4.11 Discussion

Lactococcus lactis ssp. *lactis* strain ATCC 7962 is an unusual lactococcal strain because of its high β -galactosidase activity and low phospho- β -galactosidase activity. In nature an individual lactococcal strain contains a range of different plasmids, the so-called plasmid profile, and this has been used for genetic identification of lactococcal strains. This natural characteristic of lactococci is also found in strain ATCC 7962, as Tsai and Sandine (1987) observed that this strain contains four natural plasmids, and this has been confirmed in this study.

There has been extensive lactococcal research in the area of plasmid profile and plasmid DNA isolation. This is due to a certain degree of difficulty encountered in plasmid isolation based on the fact that lactococci are Gram-positive bacteria and contain plasmid profiles that exist in a low copy number. As a result, the protocol of Anderson and McKay was established in 1983 and has been used as a basic method to prepare and isolate lactococcal plasmids and their plasmid profiles. The lactococcal plasmids are generally large in size and are associated with many important characteristics of this group of bacteria. This is the case for strain ATCC 7962 in which plasmid pDI3, which is 70 kb in size, was shown to be associated with its lactose metabolism by plasmid curing experiments (Davey, G.P. unpublished data). This was confirmed here as blue colonies were obtained on media containing a chromogenic β -galactosidase substrate, X-gal, for strain NZDRI 5191 (a plasmid cured derivative of ATCC 7962 wildtype containing only pDI3), and the wildtype strain of ATCC 7962, but white colonies were found for strain NZDRI 5201 (a plasmid-free derivative of strain ATCC 7962). Thus in this study, pDI3 was successfully isolated and prepared from strain NZDRI 5191 using the method of Anderson and McKay (1983), although the plasmid yield was not high which partly reflects the fact that pDI3 is a low copy number plasmid.

As pDI3 was found to be associated with β -galactosidase activity in lactococcal strain 7962, construction of a restriction endonuclease map of pDI3 was an important first initiative for this study, prior to isolating a fragment of pDI3 DNA encoding the β -galactosidase activity. Three strategies have been used, and these are discussed below. Problems that are found in the construction of a map, especially that of a lactococcal plasmid, are caused by the nature and the complexity of the plasmid. The difficulties that arose in the mapping of pDI1, a lactose plasmid of *L. lactis* ssp. *lactis* strain H1 (Stowell, 1985), were also encountered in this study when pDI3 was mapped.

Firstly, after primary screening for appropriate restriction enzymes from most of the common six basepair Type II restriction endonucleases, there were only a few that provided an appropriate number of restriction sites and suitable sizes to generate a map. There were two sites for *Bam*HI, six sites for *Pst*I, and five sites for *Sal*I. The other restriction enzymes that were used to digest pDI3 included *Ava*I, *Bcl*I, *Bgl*II, *Cla*I, *Eco*RI, *Eco*RV, and *Hind*III, but all cleaved pDI3 into too many fragments to make them suitable for mapping (see Figures 11 and 12, data not shown for *Ava*I, *Bcl*I, and *Eco*RV). The number of DNA fragments generated after restriction enzyme digests reflects the base composition of the pDI3 DNA. *L. lactis* ssp. *lactis* strain ATCC 7962 has been classified as a lactococcal strain, as it contains a G+C content of approximately 36% (Farrow and Garvie, 1979) and therefore restriction enzymes that recognize GC rich sequences would be most suitable for mapping. Even though the restriction enzyme *Eco*RI is not suitable for mapping, it was also included to provide additional fragments for subcloning, as is discussed later. Secondly, restriction endonuclease mapping relies on the accuracy of the determination of the molecular weight of generated DNA fragments, but this determination was found to be difficult here as many of the fragments were either too large or too small to obtain accurate size determinations.

It was found that pDI3 contained a number of doublets, such as the 4.2 kb fragments generated by the restriction enzyme, *Pst*I, the 4.3 kb fragments generated by the restriction enzyme, *Eco*RI, the 4.3 kb fragments generated by the restriction enzyme, *Hind*III, and the 5.6 kb fragments generated by the restriction enzyme, *Bgl*II. These doublets may be a result of pDI3 being AT-rich, as these restriction enzymes recognize

AT-rich sequences. Besides the difficulties caused by the doublets in pDI3 mapping, the presence of the doublets may be an interesting feature of pDI3, as they could indicate the presence of small repeat sequences. Despite the above difficulties, the molecular weight of pDI3 was confirmed to be 70 kb by addition of the molecular weights of all the fragments generated after each restriction enzyme digest.

As data obtained from the restriction enzyme digests of pDI3 were inadequate to construct a map, molecular cloning of pDI3 fragments and a hybridization approach were carried out. A number of pDI3 fragments were successfully cloned into high copy number *E. coli* plasmid vectors which allow a higher yield of the cloned DNA fragments. The cloned pDI3 fragments were used to confirm restriction sites contained in pDI3 and were used as DNA probes. This proved useful in providing further information for the mapping work, as well as for isolating DNA fragments encoding the β -galactosidase gene.

The hybridization approach involved the use of these cloned fragments and pDI3 fragments isolated directly from the gel as probes. These probes were used to hybridize to pDI3 digests. This approach was found to be a useful tool for constructing a map of pDI3, especially for confirmation of the arrangement of the restriction fragments. Following assembly of all the data obtained from these three strategies (restriction enzyme analysis, molecular cloning of various pDI3 fragments, and the hybridization approach) a physical map of pDI3 for the three restriction enzymes, *Bam*HI, *Pst*I, and *Sal*I was generated. Therefore characterization of this lactococcal plasmid, pDI3, was achieved, and this can be used as background genetic information for further study.

The localization of the β -galactosidase gene on pDI3 was one of the major obstacles in this study since there was a lack of a suitable probe. By definition closely related genes will share some DNA sequence homology. The localization of the β -galactosidase fragment of pDI3 was therefore carried out using probes based on the homology of this gene to the same gene in other closely related bacteria. In this study three probes were used. The first one was a heterologous probe from a 5.2 kb *Eco*RI fragment of *Clostridium acetobutylicum* that encodes the β -galactosidase gene, *cbgA*. This was

previously cloned and found to show weak homology to pDI3 (Hancock, 1988; Hancock *et al.*, 1991). The results obtained from the hybridization of this probe to pDI3 digests also indicated weak homology to the 4.3 kb *EcoRI* doublet of pDI3. The second probe was an oligonucleotide probe, CP20, designed from the highly conserved amino acid region derived from the β -galactosidase sequences of *lbu* from *Lactobacillus bulgaricus*, *cbgA* from *Clostridium acetobutylicum*, *lacZ* from *E. coli* and *kpn* from *Klebsiella pneumoniae*. CP20 was a mixture of 20-mer nucleotides with 64-fold redundancy and was found to show homology to the 4.3 kb *EcoRI* doublet as well as to a 4.3 kb *HindIII* doublet from pDI3. The third probe, BS13, was also designed from the predicted amino acid sequence derived from the β -galactosidase sequence of a more closely related bacterium to the lactococci, the *lacL* gene from *Leuconostoc lactis*, and the predicted amino acid sequences derived from other Gram-positive bacteria, including *lacZ* from *Streptococcus thermophilus*, *lacZ* from *Lactobacillus bulgaricus*, *cbgA* from *Clostridium acetobutylicum* and *lacZ* from *E. coli*. BS13 was a mixture of 18-mer nucleotides with 32-fold redundancy. Both CP20 and BS13 were designed from the same highly conserved region. In spite of the closely related bacterium, *Leuconostoc lactis*'s deduced amino acid sequence was included in the design of the BS13 oligonucleotide probe the deduced amino acids contained in the Gram-positive bacterial sequences were chosen in preference to those contained in the Gram-positive bacterial sequences (i.e. phenylalanine was chosen instead of the methionine present in *lacL*). According to the majority of the deduced amino acid sequence derived from the present β -galactosidase sequences derived from the same region, tryptophan was also chosen instead of the phenylalanine present in *lacL*, and arginine was excluded (see Figure 28). BS13 showed weak homology to the cloned 4.3 kb *EcoRI* fragment of PN1338.

The following discussion is concerned with the degree of similarity between the β -galactosidase gene sequences that were used as probes in this study, and pDI3. There are two levels of similarity, the DNA sequence level and the amino acid sequence level. The amino acid sequence is derived from the prediction of amino acids from the DNA sequence. To date, at least twelve β -galactosidase genes from eubacteria have been cloned and eight have been sequenced, including the genes isolated from lactic acid bacteria (Smart *et al.*, 1993). The deduced amino acid sequences derived from the β -

galactosidase genes of the following bacteria, *cbgA* from *Clostridium acetobutylicum*, *lbu* from *Lactobacillus bulgaricus*, *lacL* and *lacM* (*lacLM*) from *Leuconostoc lactis*, *lacZ* from *Streptococcus thermophilus*, *lacZ* from *E. coli*, and *lacZ* from *Klebsiella pneumoniae* were used to design the probes in this study. The deduced amino acid sequence predicted from the β -galactosidase sequence of *Leuconostoc lactis* showed 36.2% similarity to the *cbgA* gene of *Clostridium acetobutylicum*, 33.3% similarity to the *lacZ* gene of *Streptococcus thermophilus*, 28.2% similarity to the *lbu* gene of *Lactobacillus bulgaricus* and 22.7% similarity to the *lacZ* gene of *E. coli* (David *et al.*, 1992). In addition, Schroeder *et al.* (1991) found that there was no detectable hybridization between all the tested strains of *Streptococcus thermophilus* carrying *lacZ* on a 7.0 kb *Pst*I fragment and DNA isolated from *L. lactis* ssp. *lactis* strain ATCC 7962 under high stringency conditions. This was confirmed by Smart *et al.* (1993), who found that there was no detectable hybridization between the β -galactosidase fragment of *Streptococcus thermophilus* and total DNA from other species of lactic acid bacteria under medium to high stringency conditions. They suggested that the complete lack of hybridization between these bacteria may be due to the fact that β -galactosidase is a genetically diverse enzyme. Despite the certain degree of similarity in the deduced amino acid sequences derived from the β -galactosidase genes, there is still a considerable diversity of β -galactosidase sequences, even among species of lactic acid bacteria, at the DNA sequence level. The finding of Schroeder *et al.* (1991) are also in agreement with this conclusion, that is, a greater degree of similarity in the deduced amino acid sequence between the *lacZ* gene of *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *E. coli* and *Klebsiella pneumoniae* was found, but a lesser degree of similarity at the DNA sequence level was observed.

Even though pDI3 contains a β -galactosidase gene, the high degree of genetic diversity of β -galactosidase genes indicates that it is not unexpected that the probes used in this study share only weak homology to pDI3. However there are some reasons to expect a certain degree of homology between the β -galactosidase gene of pDI3 from strain 7962 and the *lacLM* gene of *Leuconostoc lactis* as, from a taxonomic point of view, these two bacteria are phylogenetically related genera (Ludwig *et al.*, 1985) and their β -galactosidase genes are both plasmid-borne. Moreover, *lacLM* shared the highest degree

of similarity with the *cbgA* gene, which was used as one of the probes in this study and was also found to show weak homology to the 4.3 kb *EcoRI* doublet from pDI3 (i.e. 4.3a kb *EcoRI* fragment). Thus the high degree of similarity between the *lacLM* gene and the *cbgA* gene lends confidence that the weak homology found between the *cbgA* gene and the 4.3a kb *EcoRI* fragment is an indication that the 4.3a kb *EcoRI* fragment of pDI3 contains β -galactosidase sequence.

Attempts to amplify the β -galactosidase sequence products using the polymerase chain reaction (PCR) were made (Watt and Scott, unpublished data). Two primers were designed to two conserved regions of the deduced amino acid sequence derived from the available β -galactosidase genes according to David *et al.* (1992); BS13 (as previously discussed) at position 219-224, and BS 14 (a 17-mer with 32-fold redundancy) at position 607-613. PCR was performed using BS13 and BS14 as primers to DNA from pKH179 (a plasmid containing the *cbgA* gene) and genomic DNA from Gram-positive *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. A PCR product of about 1 kb was obtained from all three control DNA samples, but not from the DNA of strain ATCC 7962. The results obtained showed that the primers contained sequence homology to the control Gram-positive bacterial sequences used in this experiment but not to DNA from strain 7962. Therefore it can be concluded that the β -galactosidase sequence from strain 7962 is different from β -galactosidase sequences of those bacteria that were tested, at least at the DNA sequence level.

Despite only weak homology found between the 4.3 kb *EcoRI* doublet and the probes used in this study, this doublet was subsequently cloned into pUC18 and transformed into *E. coli* MC1022, but neither of the cloned 4.3 kb *EcoRI* fragments showed β -galactosidase activity. However, one of the cloned 4.3 kb *EcoRI* fragment from PN1338 was found to show weak homology to the BS13 probe. This indicated that the 4.3 kb *EcoRI* fragment shared DNA sequence homology to BS13, but did not contain the entire sequence needed for a fully functional β -galactosidase gene, or was missing the sequence necessary for expression in *E. coli*. Moreover, it was observed that in most cases cloned β -galactosidase genes located to a larger fragment (5.2 kb to 16 kb, Table 8), with a few exceptions, since the gene codes for a large protein molecule, with

molecular weights ranging from 100-120 MDa (see Table 9) and amino acids ranging from 1000-1030 residues (Table 9). Thus approximately 2.3 kb of DNA is required to code for a 100 Mda protein, and this does not include the other sequences necessary for the control of protein synthesis and its expression (Herman and McKay, 1986). Therefore it is likely that the cloned 4.3 kb *EcoRI* fragment of pDI3 is of insufficient size for β -galactosidase activity.

β -galactosidase expression in *E. coli* was found on the 13.6 kb *SalI* fragment of pDI3 after this fragment was cloned into pBR322, an *E. coli* plasmid vector. This resulted in a recombinant plasmid, designated pSY303. Thus this confirmed that the β -galactosidase gene from strain 7962 is associated with pDI3 and also is expressed in *E. coli*. This indicates that the promoter of this gene is functional in *E. coli*. The expression of β -galactosidase from strain 7962 in *E. coli* was expected, as many other genes from a Gram positive background, including β -galactosidase genes, are able to express in *E. coli* e.g. *cbgA* from *Clostridium acetobutylicum*, *lacZ* from *Streptococcus thermophilus*, and *lacLM* from *Leuconostoc lactis*, as shown in Tables 6, 7, and 8. As discussed earlier, pDI3 contains a number of doublets, such as the 4.3 kb *EcoRI* doublet, the 4.3 kb *HindIII* doublet, and the 5.6 kb *BglII* doublet, and it is interesting that one of each of these doublets is located within the 13.6 kb *SalI* fragment (i.e. 4.3a kb *EcoRI*, 4.3a kb *HindIII*, and 5.6a kb *BglII*, see Figure 39 and Table 21). The cloned 4.3 kb *EcoRI* fragment of PN1338 (i.e. the 4.3a kb *EcoRI* fragment), which showed weak homology to all the three probes used to localize the β -galactosidase gene from pDI3 of strain 7962, was thus contained in this cloned 13.6 kb *SalI* fragment. Therefore the cloned 13.6 kb *SalI* fragment of pDI3 contains DNA sequence that shows weak homology to other β -galactosidase sequences and is more likely to code for all the *lac* genes that are necessary for control of β -galactosidase expression from strain ATCC 7962 than the 4.3a kb *EcoRI* fragment itself.

The expression of β -galactosidase in this study was carried out in *E. coli* strains containing a deletion in the *lacZ* gene, *lacZ* Δ M15, providing the α -complementation system to the *lacZ* gene, which allows restoration of β -galactosidase expression when

the deletion is complemented. The results indicate that either an entire β -galactosidase gene from strain 7962 is being transcribed and is functional, or that the cloned sequence from strain 7962 is able to complement the *lacZ* Δ *M15* deletion of *E. coli* β -galactosidase by α -complementation. However, the 13.6 kb *Sa*II fragment of pDI3 was also found to express β -galactosidase when pSY303 was transferred into *E. coli* PB2959, a strain of *E. coli* that lacks the entire *lac* operon. This result suggests that the 13.6 kb *Sa*II fragment of pDI3 encodes a functional β -galactosidase gene, although, only faint blue colonies of *E. coli* PB2959 were observed after 2-3 days incubation.

β -galactosidase activity from pSY303 was found to be constitutive at a higher level when 0.1% (w/v) lactose was included in the media containing X-gal. This result supports biochemical studies showing that the synthesis of β -galactosidase enzyme from *L. lactis* ssp. *lactis* strain ATCC 7962 was fully inducible by adding 1.0% (w/v) of lactose into the production broth (Citti *et al.*, 1965 and McFeters *et al.*, 1967). β -galactosidase activity, as measured in permeabilized cells, was higher than background (i.e. higher than that in the host strain and that in the host containing only pBR322) in all the *Lac*⁺ *E. coli* strains containing pSY303, and was constitutive as IPTG was not required (Table 19). This constitutive expression is similar to that reported for *Streptococcus bovis* (Gilbert and Hall, 1987), *Clostridium acetobutylicum* (Hancock *et al.*, 1989), *Streptococcus thermophilus* (Herman and McKay, 1986, Schroeder *et al.*, 1991), *Lactobacillus bulgaricus* (Schmidt *et al.*, 1989, Leong-Morgenthaler *et al.*, 1991) and *Leuconostoc lactis* (David *et al.*, 1992).

In most cases glucose has some effect, being either repression or induction, on the *lac* operon (Jacob and Monod, 1961). Glucose usually represses expression of the *lac* operon by reducing the concentration of cAMP which results in inactivation of the catabolic activator protein (CAP), a protein that stimulates the transcription of the *lac* operon (Miller, 1978). Glucose was found to have no significant repression effect on the expression of β -galactosidase from the 13.6 kb *Sa*II insert from pDI3, since *E. coli* cells grown in the presence of glucose still produced active β -galactosidase. Glucose was also reported to have neither a repressing nor inducing effect on the *cbgA* gene

(Hancock, 1988; Hancock *et al.*, 1991). The lack of glucose repression of the *cbgA* gene in *E. coli* was proposed to be due to the lack of endogenous *Clostridium acetobutylicum* gene products which normally mediate the glucose effect and/or the failure to respond to the *E. coli* regulatory system (Hancock *et al.*, 1991).

The phenotypic expression of *E. coli* host strains containing pSY303 was found to be unstable after subculturing Lac⁺ *E. coli* strains (blue colonies on media containing X-gal). Phenotypic segregation of *E. coli* host strains containing pSY303 into blue (i.e. Lac⁺) colonies and white (i.e. Lac⁻) colonies was observed. The Lac⁻ colonies resulting from the segregation remained as stable Lac⁻ colonies throughout further subculturing. This phenotypic segregation was observed in all the studied *E. coli* host strains. However, *E. coli* host strain XL-1 Blue showed greater stability of the Lac⁺ phenotype. This could have been the result of differences in the genetic background of this *E. coli* host strain, which has been constructed to provide a more stable background for β -galactosidase selection (Bullock *et al.*, 1987). Experiments were carried out to investigate any difference between pSY303 isolated from Lac⁺ and Lac⁻ colonies from all the *E. coli* host strains. There was no difference between pSY303 from Lac⁺ colonies and pSY303 from Lac⁻ colonies, based on the restriction digest pattern.

There is a possibility that pSY303 contains some small repeat sequences. Evidence for small repeat sequences (i.e. repetitive DNA) in pSY303 was found when the cloned 13.6 kb *SalI* fragment isolated from pSY303 was labelled and used as a probe to various pDI3 fragments (Figure 37 and Table 20). More pDI3 fragments hybridized to the cloned 13.6 kb *SalI* fragment of pSY303 than when the 13.6 kb *SalI* fragment isolated directly from pDI3 was used as a probe to pDI3 fragments (see Table 17 and Figures 12, 31, and 32). The pDI3 fragments that showed homology to the cloned 13.6 kb *SalI* fragment isolated from pSY303, and were not located on or did not overlap the 13.6 kb *SalI* fragment, were the 15.9 and 5.5 kb *SalI* fragments, the 6.7 kb *PstI* fragment, the 16.0 and 3.8 kb *EcoRI* fragments, and possibly the 4.3b kb *EcoRI* fragment.

The small repeat sequences that may be contained in the pSY303 construct provide a possible explanation for the phenotypic segregation in β -galactosidase expression among the *E. coli* host strains used in this study. That is, it occurred as a result of a spontaneous mutational event caused by the small repeat sequences, as was found for the β -galactosidase gene of *Lactobacillus bulgaricus* (Mollet and Delley, 1990). There were at least two classes of mutation that were found within the β -galactosidase gene of *Lactobacillus bulgaricus*, but probably only one is relevant in the case of pSY303. This is the mutation that did not give noticeable differences in the restriction enzyme digest pattern of the mutant β -galactosidase gene (i.e. pSY303 isolated from Lac⁻ colonies) compared to the wildtype gene (i.e. pSY303 isolated from Lac⁺ colonies) because this mutation was caused by an event such as a transition, a transversion or a frameshift. These events give rise to nonsense and missense mutations within the gene, or which affect its promoter region.

4.12 Conclusion

In conclusion, the β -galactosidase gene of lactococcal strain 7962 was found to locate to a 13.6 kb *SalI* fragment of pDI3. It was cloned into an *E. coli* plasmid vector, pBR322 and expressed in Lac⁻ *E. coli* strains; JM109, DH5 α , and XL-1 Blue containing a deletion of *lacZ* (*lacZ* Δ M15), and in an *E. coli* strain PB2959 lacking the entire *lac* region. The resulting recombinant construct, pSY303, was found to be unstable. This instability was manifested as Lac⁺/Lac⁻ phenotypic segregation. This phenomenon may relate to small repeat sequences that have been proposed to be contained within the pSY303 construct.

CHAPTER 5: SUMMARY AND CONCLUSIONS

This thesis has fallen into two distinct parts. The first part was the work concerning the sequencing of the 4.4 kb *EcoRI* fragment that contained D-tagatose-1,6-bisphosphate aldolase. This protein is one of six proteins involved in lactose metabolism via the Lac-PEP:PTS system in the dairy lactococcal starter, *Lactococcus lactis* ssp. *cremoris* strain H2. Sequence analysis of two contigs, from the 4.4 kb *EcoRI* fragment, 401-36a and 401-46, showed that these contigs shared 95.7% homology to the DNA sequence of the *E. coli purM* gene, and 97.2% homology to the *E. coli purN* gene, respectively. These are chromosomally located genes responsible for purine biosynthesis in *E. coli* strain K12 (Smith and Duam III, 1986).

These results refute the original hypothesis that the previously subcloned 4.4 kb *EcoRI* fragment was derived from lactococcal DNA. Thus this work was unable to extend in any way our knowledge concerning lactose metabolism of the Lac-PEP:PTS system of the dairy lactococcal starter strain.

The aim of this study was therefore modified and became the second part of the study. The work for the second part of the study concentrated on lactose metabolism in an unusual lactococcal strain, *Lactococcus lactis* ssp. *lactis* strain ATCC 7962, with emphasis on molecular cloning of the β -galactosidase gene from this strain, and its expression.

The initial requirement for this study was the construction of a restriction endonuclease map of pDI3 with three restriction endonucleases: *Bam*HI, *Pst*I and *Sal*I. The size of pDI3 was confirmed to be 70 kb. The β -galactosidase gene of strain 7962 was confirmed to be associated with pDI3. Work investigating the homology between the β -galactosidase gene encoded by pDI3 and other β -galactosidase sequences, especially the β -galactosidase sequences from members of the lactic acid bacteria as well as the *cbgA* gene of *Clostridium acetobutylicum*, showed that there was only weak homology between pDI3 and these other β -galactosidase sequences. All the probes used in this study showed weak homology to the 4.3 kb *EcoRI* and 4.3 kb *Hind*III doublets from

pDI3. Amplification of the corresponding polymerase chain reaction products suggested that the β -galactosidase gene of strain 7962 contains β -galactosidase sequence which is different from the other β -galactosidase sequences tested.

This work also confirmed that the β -galactosidase activity of strain 7962 is associated with the plasmid, pDI3, as the 13.6 kb *SalI* fragment of pDI3 cloned into pBR322 and transformed into *E. coli* Lac⁻ strains JM109, DH5 α , and XL-1 Blue was found to express functional β -galactosidase both on media containing X-gal and from permeabilized cells using ONPG as a substrate. The β -galactosidase expression was found to be constitutive. No glucose repression was found at concentrations of 0.1% to 0.4% glucose (w/v). The expression of β -galactosidase from the 13.6 kb *SalI* fragment of pSY303 did not require IPTG for induction, but a higher level of expression was found when lactose was present in the media. The 13.6 kb *SalI* fragment expresses functional β -galactosidase, as when pSY303 was transferred to *E. coli* strain PB2959, which lacks the entire *lac* operon, a Lac⁺ phenotype was obtained.

In addition, a detailed restriction endonuclease map of the 13.6 kb *SalI* fragment was constructed and this confirmed that this fragment contained the 4.3a kb *EcoRI* fragment (i.e. one of the fragments from the 4.3 kb *EcoRI* doublet) which showed homology to all the probes used, and the 4.3a kb *HindIII* fragment (i.e. one of the fragments from the 4.3 kb *HindIII* doublet) that also showed homology to the oligonucleotide probe, CP20. The 13.6 kb *SalI* fragment also contained the 5.6a kb *BglII* fragment (one of the fragments from the 5.6 kb *BglII* doublet). As it contains those fragments that showed homology to the probes used in this study, and is larger than those fragments, it is possible that the 13.6 kb *SalI* fragment codes for a functional β -galactosidase gene.

As *L. lactis* ssp. *lactis* strain ATCC 7962 and *Leuconostoc lactis* are phylogenetically related genera and their β -galactosidase genes are both located on plasmids, the two β -galactosidase genes could be quite similar. David *et al.* (1992) reported that the plasmid-borne β -galactosidase gene sequence from *Lactobacillus casei* strain ATCC 393 showed 99% similarity to the plasmid-borne β -galactosidase gene (i.e. the *lacL* and *lacM* sequences) from *Leuconostoc lactis* strain NZ6009. David *et al.* (1992) also found that

the organization of the plasmid-borne genes involved in lactose metabolism in *Leuconostoc lactis* was considerably different to that described for the organization of the *lac* genes found in *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *E. coli*. Hence it is of interest to study further the organization of the *lac* genes responsible for controlling lactose metabolism in *L. lactis* ssp. *lactis* strain ATCC 7962 as the genetic control mechanism for its lactose utilization is still unknown. The control mechanism of the *lac* genes of strain 7962 may relate to the unusual characteristics of this particular lactococcal strain, which utilizes lactose via the lactose permease system more than the Lac-PEP:PTS system, even though the former system is considered a less effective way of utilizing lactose. A defect in the latter system makes this strain a slow lactose fermenter, although it was originally isolated from a milk sample (Crow and Thomas, 1984).

The work on the molecular cloning of the β -galactosidase gene from strain ATCC 7962 has provided data that may be utilized in further research involving a more detailed study of the β -galactosidase gene sequence from this lactococcal bacterium. It would be of interest initially to sequence this lactococcal β -galactosidase gene and to compare the DNA sequence, the deduced amino acid sequence, the degree of similarity, etc., to other β -galactosidase sequences, as to date a β -galactosidase gene from lactococci has not been sequenced. It would be interesting to know the number of open reading frames (ORF) that are contained in the sequence, the size of protein predicted from the ORF of this β -galactosidase sequence, the putative ribosome-binding site (RBS), *E. coli* transcription initiation (the -10 and the -35 region) sites, putative terminator sites, etc.

β -galactosidase expression of pSY303 in *E. coli* was found to exhibit phenotypic segregation. This phenomenon was found in all the *E. coli* host strains used in this study, but *E. coli* strain XL-1 Blue was found to be the most suitable host for the expression of β -galactosidase from strain 7962. Lac⁺ colonies, when going through subculturing, segregated into white and blue colonies. Once white colonies were isolated they retained the white phenotype (i.e. Lac⁻ phenotype) through further subculturing. The blue colonies, however, continued to segregate into white and blue

colonies. Analysis of plasmid DNA isolated from representative blue and white colonies revealed intact pSY303 and no difference was found in the restriction digest pattern of isolated pSY303.

A possible explanation for this phenomenon relate to evidence that suggest that pSY303 contains some small repeat sequences or repetitive DNA. The phenotypic segregation found among the studied *E. coli* host strains could have been the result of a mutational event caused by a transversion, a transition, or a frameshift at a hot spot of small repeat sequences, resulting in nonsense or missense codons within the β -galactosidase gene or that affect the promoter region. This type of mutation would not give a noticeable difference in the restriction digest pattern between the wildtype gene and the mutated gene.

Thus not only was the β -galactosidase gene from strain 7962 successfully cloned and then found to express in *E. coli*, but the phenotypic segregation proposed as a result of a mutational event has also been observed. This phenomenon could lead to future study on this particular DNA construct, especially of the small repeat sequences that are possibly contained in pSY303. This phenomenon is also probably due to the differences in the β -galactosidase sequence from strain 7962.

Therefore mechanisms resulting from possible small repeat sequences in pSY303: mutations within the gene or that affect the promoter region has been proposed to explain the phenotypic segregation of the pSY303 construct. Future study of the mechanism is vital, at least to stabilize the β -galactosidase gene isolated from strain 7962, as the gene has potential for use as a genetic marker in the development of a food-grade lactococcal cloning vector.

There are a number of lactococcal genes have been cloned into either *L. lactis* ssp. *lactis* or *E. coli*, as well as into some other heterologous hosts (Tables 6, 7, and 8). Many of these cloned lactococcal genes have commercial applications, such as improvement of flavour in the dairy products industry, carbohydrate utilization, inhibition of pathogenic microorganisms, resistance to lactococcal bacteriophages, etc. The development of

genetic transfer systems among lactococci, critical for introducing cloned DNA fragments into desired host strains, has also progressed during the last decade, especially the development of electroporation, which has provided relatively high transformation efficiencies in lactococci (sections 1.5.4.6 and 1.6). Thus DNA can now be cloned and transferred into commercial lactic starter strains, providing that appropriate cloning vectors are available. A number of lactococcal cloning vectors have been developed, but the majority still contain selective markers encoding resistance to antibiotics used in human medication and therefore are unsuitable for food-producing bacteria like lactococci. There are regulations concerning use of any recombinant DNA in dairy lactococcal strains and this is still restricted throughout the world. Ideally, a food-grade cloning vector used should be constructed from DNA derived from approved food producing microorganisms and should contain selective markers that do not compromise human drug therapy.

Therefore there are some possible applications for this cloned lactococcal β -galactosidase gene, e.g. to replace the *E. coli lacZ* marker in pFX3 (a lactococcal shuttle vector, Xu, *et al.*, 1991) with the β -galactosidase gene from strain ATCC 7962, or to incorporate the gene as another marker into pFM011 [a plasmid construct containing *Nis*^r (Nisin resistance) and allowing a selection for the *Nis*^r phenotype for cloning and has been proposed to use as a food-grade lactococcal vector, Froseth and McKay, 1991]

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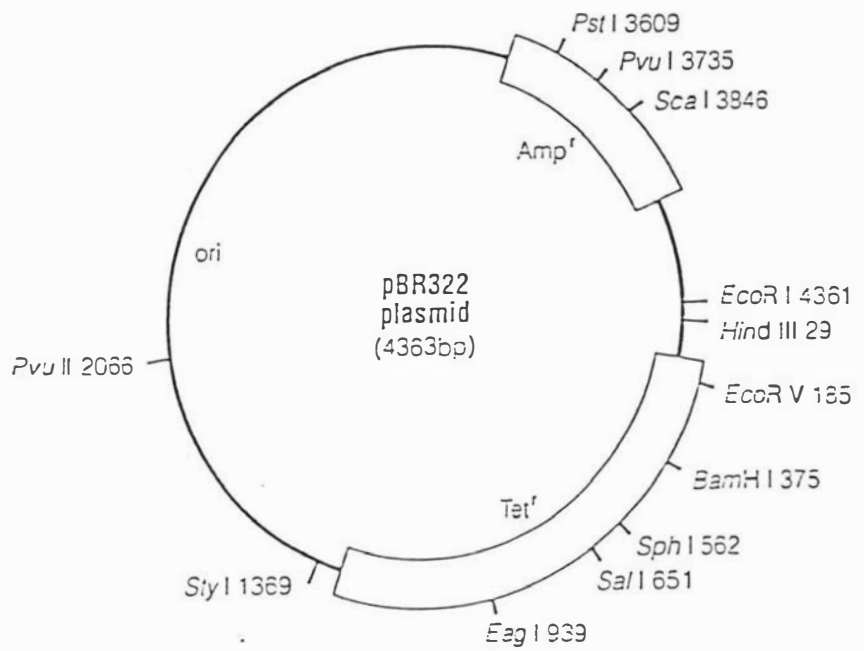
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Appendix I

Physical map of pBR322



Appendix II

Physical map of pBR328

