Application of Molecular Biology in the Evaluation of *Lactobacillus plantarum* Strains as Silage Inoculants.

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I herby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Fiona Duffner Date: 18:9:93

To my parents and Lar.

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Abstract

Methods to distinguish between strains of *Lactobacillus plantarum* were investigated with the aim of analysing strain heterogeneity in grass silage. Chromosomal restriction endonuclease patterns, rDNA fingerprints and plasmid profiling were applied to isolates from five different well preserved grass silages and a number of dominant strains were identified. Ribotyping proved to be a most useful technique for strain differentiation using restriction enzymes *Eco*RI and *Bam*HI to achieve optimum results. Plasmid profiling complemented the information obtained from ribotyping while chromosomal restriction endonuclease analysis proved to be too cumbersome for routine use.

Having demonstrated that *L. plantarum* effected improved forage preservation when applied at an inoculation rate of $1x10^6$ cfu/g grass, a number of *L. plantarum* strains were investigated for their suitability as grass silage inoculants. A novel assay was designed to assess the competitiveness of each *L. plantarum* strain when co-inoculated individually with the internal standard strain, *L. plantarum* DCU101. The plasmid based strain specific DNA probe, pGB100, was used to enumerate the proportion of *L. plantarum* DCU101 in each treatment over the fourteen day ensilage period. The results demonstrated that competitiveness and dominance do occur within the silo and the most competitive strain, *L. plantarum* B2, was used in further silo trials to establish its usefulness as a bacterial silage inoculant.

The PCR amplification of the V1 and V6 variable regions of 16S rRNA genes from *L. plantarum* was investigated for its usefulness in obtaining species and genus specific probes. Direct sequencing from PCR was investigated but failed due to the small size of the PCR fragment (about 100bp) which resulted in its rapid renaturation. The cloned PCR amplified V1 and V6 regions from three strains of *L. plantarum* were sequenced and analysed using hybridisation experiments and computer assisted alignments with sequences extracted from the GenBank and EMBL databases. On alignment of the V1 and V6 regions of thirty species of *Lactobacillus* and a variety of Gram-negative and Gram-positive genera, three *L. plantarum* specific oligonucleotide sequences were identified. The specificity of these selected oligonucleotides was confirmed using the BLAST alignment programme.

Chapter 1.
Introduction

1. Introduction

The essential role of Lactic Acid Bacteria (LAB) in fermentation technology is the inhibition of spoilage bacteria. In addition, both stereoisomers of lactic acid taste quite acceptably and are easily metabolised by the human body. In the earlier days of mankind, spontaneous lactate fermentation improved the keeping qualities and the hygienic status of collected food. The process was gradually improved and "domesticated" as civilisation proceeded. The microbiological and biochemical foundation of lactate fermentation only became known however in the last century. As a result, it became possible to control this process scientifically and to apply it to modern food technology.

The term, lactic acid bacteria, has become widely accepted to represent Gram-positive, non-sporing, aerotolerant bacteria, whose main fermentation product from carbohydrate is lactate. This functionally defined but distantly related group of bacteria is composed primarily of the genera *Lactococcus, Lactobacillus, Leuconostoc* and *Pediococcus* [Stackebrandt & Teuber, 1988].

Phylogenetically, they are members of the *Clostridium-Bacillus* subdivision of Gram-positive eubacteria and evolved as individual lines of descent about 1.5 to 2 billion years ago when the earth passed from an anaerobic to aerobic environment. Most of the lactic acid bacteria are mesophilic organisms with optimum growth temperatures between 25-40°C and extremes of 5° and 50°C and optimum pH of 5.5-5.8. *Lactobacillus* and *Pediococcus* species are generally more tolerant of low pH and many will grow at pH 4.0 or less [Kandler & Weiss, 1986; Garvie, 1986]. In contrast, below pH 4.5, *Lactococcus* and *Leuconostoc* species do not grow or do so very slowly [Garvie, 1986].

LAB are involved in the manufacture of fermented foods from raw materials such as milk, meat, vegetables and cereals and are becoming increasingly important as silage additives in agriculture. Table 1.1 illustrates the predominant species of LAB involved in some of their diverse applications to date.

The LAB have also recieved the attention of researchers because of their supposed beneficial attributes to human health. On ingestion,

Lactobacillus acidophilus has been shown to survive and establish in the complex ecosystem of the intestine [Kleeman & Klaenhammer, 1982; Hood & Zottola, 1988] and has been reported to possess anti-carcinogenic [Goldin & Gorbach, 1984] and hypocholesterolemic properties [Gilliland *et al.*, 1985; Gilliland & Walker, 1990]. Gilliland and Walker (1990) indentified several possible health or nutritional benefits from some species of lactic acid bacteria including improved nutritional value of some foods, control of intestinal infections, improvement of lactose utilization in persons classified as lactose malabsorbers, inhibitory actions toward some forms of cancer and control of serum cholesterol levels.

Gerritse *et al.* (1990) reported the use of *Lactobacillus* species indigenous to the gastrointestinal tract as safe live vector systems for the oral delivery of antigens. *Lactobacillus* strains have been shown to activate peritoneal macrophages and mononuclear phagocytes [Perdigon *et al.*, 1986a,b] and they show low intrinsic immunogenicity [Carlsson & Bratthall, 1985] in comparison with other microorganisms. These results demonstrate the feasibility of using orally administered antigen-*Lactobacillus* as a future approach to vaccination. Additional interest has focussed on the use of certain lactic acid bacteria as probiotics to improve the growth and performance of domestic livestock [Juven *et al.*, 1991].

The lactic acid bacteria are therefore of major economic importance and considerable efforts are being made to improve techniques for culture identification and strain selection and to understand nutrition, metabolism, physiology and genetics with the aim of producing uniform, high quality products [Mc Kay & Baldwin, 1990].

Vegetable fermentations	**Bakery products**
Leuconostoc mesenteroides	Sourdough
Pediococcus pentosaceus	*Lactobacillus sanfrancisco*
Lactobacillus plantarum	*Lactobacillus brevis*
Meat and fish fermentations	*Lactobacillus plantarum*
Lactobacillus plantarum	*Lactobacillus fermentum*
Pediococcus acidilactici	Soda crackers
Beverages	*Lactobacillus delbrueckii*
Leuconostoc oenos	*Lactobacillus leichmannii*
Lactobacillus delbrueckii	*Lactobacillus casei*
Soy sauce	*Lactobacillus plantarum*
Lactobacillus delbrueckii	*Lactobacillus brevis*
Pediococcus soyae	**Fermented dairy products**
Probiotics in health	*Lactococcus lactis* ssp.*lactis*
Lactobacillus acidophilus	*L. lactis* ssp. *cremoris*
Lactobacillus casei	*L. lactis* ssp. *lactis* var. *diacetylactis*
Silage	*Leuconostoc mesenteroides* ssp. *cremoris*
Lactobacillus plantarum	*Leuconostoc lactis*
Pediococcus acidilactici	*Streptococcus thermophilus*
	Lactobacillus delbrueckii ssp. *bulgaricus*
	Lactobacillus helveticus
	Lactobacillus acidophilus
	Lactobacillus casei

Table 1.1. Varied use of the lactic acid bacteria and examples of predominant species utilised.

1.1 Historical classification of the LAB

The group name, Lactic Acid Bacteria, was created for bacteria causing fermentation and coagulation of milk and effectively began with the comparison by Hueppe (1884) of "Milchsäurebazillus" with *Bacterium acidi lactici* [Ingram, 1975]. Weigmann (1899a) defined LAB as those which produce milk acid from milk sugar and considered the relation to *B. acidi lactici* of a number of other bacteria which he eventually (1899b) divided into five groups, one of which contained aerobic proteolytic micrococci. Henneberg (1904) described 21 species from a wide variety of sources besides milk and noted that different optical forms of lactic acid might be produced. Weigmann (1905) then recognised two main groups related to *Lactococcus lactis* subsp. *cremoris* and *B. lactis aerogenes*. The LAB were associated with the coli-aerogenes group in the classification of Lehmann and Neumann (1907). Löhnis (1907) remarked on the chaos prevailing in this field and produced a grouping which begins to look more familiar, although it still included aerobic proteolytic micrococci, it excluded coli-aerogenes bacteria and the Gram-positive character of the group was established.

Rogers & Davis (1912) examined a large collection of LAB to determine whether fermentation of sugars, polyalcohols and polysaccharides might be of value in their classification. They demonstrated the ability of the LAB to attack a wide range of carbohydrates and recognised three broad groups, apart from the gelatin-liquefying micrococci which (they observed) rarely took part in the spontaneous souring of milk.

In 1914, Orla-Jensen presented a paper to the Sixth International Dairy Congress [Ingram, 1975] in which a physiological classification of the 'genuine lactic acid bacteria' was proposed, based first on the manner in which nutrients and energy sources were utilised and second, on the ability to utilise carbon and nitrogen sources [Ingram, 1975]. In the following decades, Orla-Jensen (1942) established a systematic order of LAB on the basis of morphological and cultural features; sources of energy and how this energy was generated, temperature tolerance, fermentation end products and optical isomer of lactic acid produced. The guidelines laid down by Orla-Jensen still

provide a number of rational criteria for the identification and classification of LAB. In his classification scheme, genera containing LAB were characterised as follows;

1. "*Thermobacterium*", *"Streptobacterium"* and "*Streptococcus*"; rod and sphere forms without catalase, which produce only traces of by-product in addition to lactic acid.

2. "*Betabacterium"* and "*Betacoccus"*; rod and sphere forms without catalase but as a rule produce detectable amounts of gas and other by-products in addition to lactic acid.

3. *"Microbacterium"* and *"Tetracoccus"*; rod and sphere forms with, as a rule, catalase.

Of the genera used by Orla-Jensen in his classification, only one, *Streptococcus* has retained its taxonomic rank. The genus names, "*Betacoccus*" and "*Tetracoccus*" have since been discarded and replaced by *Leuconostoc* and *Pediococcus* respectively, while the genera, "*Betabacterium*", "*Thermobacterium*" and "*Streptobacterium"* have been designated subgenera in the genus *Lactobacillus*. The *Microbacterium* have been designated to the family Corynebacteriaceae and form a distinct genus in Bergeys Manual of Systematic Bacteriology [Collins & Keddie, 1986].

1.1.2 Classification of the genus *Lactobacillus*

Buyze *et al.* (1957) segregated the lactobacilli into three physiological groups;

(a) the obligate heterofermenters which lack aldolase,

(b) the obligate homofermenters which lack glucose-6-phosphate and 6-phosphogluconate dehydrogenase and

(c) the facultative homofermenters which contain both dehydrogenases but preferentially dissimilate glucose via the Embden-Meyerhof-Parnas pathway.

Implicit in the use of such criteria is the belief that similarities in biochemical and physiological properties do in fact reflect natural affinities between organisms.

Bergeys Manual of Systematic Bacteriology arranges the species of *Lactobacillus* into the traditional three groups resembling Orla-Jensen's three genera without designating them as formal subgeneric taxa since they do not represent phylogenetically defined clusters. Although the majority of strains of each of the new groups agree with the original definitions of "thermobacteria", "streptobacteria" and "betabacteria", many of the recently described species do not fit these definitions. Hence, the following new definitions contain neither growth temperature nor morphology, the classical characteristics of Orla-Jensen's subgenera and are similar to the groups described by Buyze *et al.* (1957).

Group I, obligately homofermentative lactobacilli: hexoses are fermented almost exclusively to lactic acid by the Embden-Meyerhof pathway; pentoses or gluconate are not fermented. Rare reports on the pentose fermentation by particular strains of Group I should be reinvestigated.

Group II, facultatively heterofermentative lactobacilli: hexoses are fermented almost exclusively to lactic acid by the Embden-Meyerhof pathway or, at least by some species to lactic acid , acetic acid, ethanol and formic acid under glucose limitation; pentoses are fermented to lactic acid and acetic acid via an inducible phosphoketolase.

Group III, obligately heterofermentative lactobacilli: hexoses are fermented to lactic acid, acetic acid (ethanol) and CO_2; pentoses are fermented to lactic acid and acetic acid. In general, both pathways involve phosphoketolase. However, some species which probably possess other pathways for carbohydrate breakdown, but performing also a heterofermentation including the production of gas from hexoses, are tentatively also included in Group III. eg. *L. bifermentans*.

Group I harbours all the classical representatives of Orla-Jensens's thermobacteria and many related species. Group II contains the streptobacteria and many newly described species while Group III contains all the obligately heterofermentative gas-forming lactobacilli of Orla-Jensen's subgenus *Betabacterium* and several more recently described species. Table 1.2 lists the species in each group.

Group I	Group II	Group III
L. delbrueckii subsp. *delbrueckii*	*L. agilis*	*L. bifermentans*
L. delbrueckii subsp. *lactis*	*L. alimentarius*	*L. brevis*
L. delbrueckii subsp. *bulgaricus*	*L. bavaricus*	*L. buchneri*
L. acidophilus	*L. casei* subsp. *casei*	*L. collinoides*
L. amylophilus	*L. casei* subsp. *pseudoplantarum*	*L. confusus*
L. amylovorus	*L. casei* subsp. *rhamnosus*	*L. divergens*
L. animalis	*L. casei* subsp. *tolerans*	*L. fermentum*
L. crispatus	*L. coryniformis* subsp. *coryniformis*	*L. fructivorans*
L. farciminis	*L. coryniformis* subsp. *torquens*	*L. fructosus*
L. gasseri	*L. curvatus*	*L. halotolerans*
L. helveticus	*L. homohiochii*	*L. hilgardii*
L. jensenii	*L. maltaromicus*	*L. kandleri*
L. ruminis	*L. murinus*	*L. kefir*
L. salivarius	*L. plantarum*	*L. minor*
L. sharpeae	*L. sake*	*L. reuteri*
L. vitulinus		*L. sanfrancisco*
L. yamanashiensis		*L. vaccinostercus*
		L. viridescens

Table 1.2 *Lactobacillus* species grouped according to Kandler & Weiss (1986) from Bergey's Manual of Systematic Bacteriology.

LAB are nutritionally fastidious; they all require complex media for optimal growth. In synthetic media, all strains of lactic streptococci require amino acids such as isoleucine, valine, leucine, histidine, methionine, arginine and proline and vitamins such as niacin, Ca-pantothenate and biotin. While pantothenic acid and nicotinic acid are, with the exception of a few strains of lactobacilli, required by all species, thiamine is only necessary for the growth of the heterofermentative lactobacilli.

The LAB are now characterised as consisting of Gram-positive, non-sporing, carbohydrate fermenting lactic acid producers, acid tolerant, of non-aerobic habit and catalase negative; typically they are non-motile and do not reduce nitrate [Ingram, 1975]. Almost all of these characteristics require qualification with the exception of the Gram reaction. The microaerophilic character is very variable and though the LAB are characteristically catalase negative, it has been known that some pediococci give a positive (pseudocatalase) reaction when grown on a low sugar source [Garvie, 1986]. LAB are typically regarded as non-motile yet there have been reports of motile strains especially from sources other than dairy products eg. meats [Harrison & Hansen, 1950; Deibel & Niven, 1958], fruit juice [Hays & Reister, 1952], cattle [Mann & Oxford, 1954] and fermentations [Vankova, 1957]. Again it is supposed that LAB do not reduce nitrate but it was demonstrated by Rogosa (1961) that this characteristic, when present, is suppressed by the high acidity developed when large concentrations of glucose are present; he suggested using 0.1% glucose instead of the usual 2% for this test. Using this technique, Spencer (1969) found 5 out of 12 isolates from meat to be nitrate reducers and Dempster (1972) found 3 out of 43 also to be positive.

1.1.3 The boundaries of the LAB group

The extent of the group seems to have been always somewhat vague and its boundaries are still uncertain corresponding with the uncertainty in the characteristic criteria by which it is defined.

That the LAB include not only the lactic streptococci (*Lactococcus lactis* and *Lactococcus raffinolactis*) but also the faecal streptococci of Group

D, has been generally accepted. The *Microbacterium* and *Bifidobacterium bifidum* included by Orla-Jensen in 1919 have since been rejected. Ingram (1975) suggested that *Erysipelothrix* should be included and the strict anaerobes, *Peptostreptococcus* and *Eubacterium* excluded. Despite such debates, the central position of the genera *Lactobacillus* - *Leuconostoc* - *Pediococcus* - *Streptococcus* has not been seriously undermined [Ingram, 1975].

Many of the LAB and related organisms have become highly specialised forms of life, as manifested by their complex nutritional requirements, lack of biochemical diversity and restricted habitats. Relatively few species appear to exhibit a significant degree of ubiquity in nature and those that do, also exhibit the greatest biochemical diversity [Buchanan & Gibbons, 1974]. The inability to utilise a large complement of organic substrates for growth, the absence of a functional electron transport system and the complex nutritional requirements necessary for their growth might be interpreted to mean that the LAB are relatively simple and primitive life forms which are evolving towards a greater degree of independence by the gradual acquisition of new characteristics. The alternative hypothesis is that this group of Gram-positive bacteria exchanged biochemical diversity and nutritional independence for a set of new properties that have enabled them to live successfully in close association with plants, animals and humans. If the extent of biochemical diversity is solely a function of genome size, then the latter hypothesis may be true as the DNA content of a *Streptococcus* is roughly equal to that of the more biochemically diverse *Achromobacter* [Lethbak *et al.*, 1970].

Environments which support the growth and proliferation of LAB are high in moisture and nutritionally rich and these conditions are also ideal for the growth of numerous other microorganisms, including pathogenic and spoilage species. The ability of the LAB to compete effectively in these environments is a direct result of their fermentative activities and production of a variety of antimicrobial compounds. Lactic acid bacteria are used and studied for their capacity to inhibit unwanted bacteria and thus increase the

shelf life of products [Piard & Desmazeaud, 1991, 1992]. To improve the ability of the lactic acid bacteria to dominate certain fermentations, the end products of metabolism and production of antimicrobial compounds must be understood.

1.2 Antagonistic activity of the lactic acid bacteria

The antimicrobial compounds produced by LAB include oxygen metabolites, end products of catabolism (organic acids, pH, diacetyl, acetaldehyde and D-isomers of amino acids), small antimicrobial substances (including reuterin) and bacteriocins. These have varying effects on lactic and non-lactic microorganisms competing in the same ecosystem. The possibility of exploiting this ability has provided the impetus for research on the potential use of LAB to improve food safety.

1.2.1 Oxygen metabolites

Lactic acid bacteria are called facultative anaerobes or aerotolerant and they catabolise sugars via fermentation. Fermentation is the metabolic process by which chemical energy is obtained from carbohydrates in the absence of molecular oxygen. All heterotrophic organisms ultimately obtain their energy from oxidation-reduction reactions. In fermentation, the electron transport chains do not intervene but in subsequent oxidation-reduction steps nicotinamide adenine dinucleotide (NAD) becomes the H^+ donor and acceptor. In aerobic conditions, NAD reacts with O_2 to form H_2O_2 or H_2O due to the action of various enzymes including NADH oxidase and peroxidase. With the exception of several isolated cases [Whittenbury, 1964; Kono & Fridovitch, 1983], LAB lack catalase because they cannot synthesize hemoporphyrins. Thus the LAB can only rid themselves of H_2O_2 by their NADH peroxidase. Depending on the activities of NADH oxidase and peroxidase, catalysing the formation and degradation of H_2O_2, [Anders *et al*, 1970], it follows that the concentration of H_2O_2 formed in aerobiosis is variable. When superoxide anions are present, hydroxyl radical formation occurs according to the

reaction;

$$O_2^{\cdot} + H_2O_2 \longleftrightarrow OH^{-} + OH^{\cdot} + O_2$$

[Gregory & Fridovitch, 1974]

Aerobic growth of LAB thus leads to the formation of 3 principle derivatives of O_2, all of which are vectors of "oxygen toxicity"; H_2O_2, $O_2^{\cdot}$ and $OH^{\cdot}$.

The toxicity of oxygen may result from the peroxidation of lipid membranes with a subsequent increased membrane permeability [Piard & Desmazeaud, 1991]. Free radicals and H_2O_2 can damage bacterial nucleic acids leading to irreversible alterations. H_2O_2 apparently causes breaks in the carbon-phosphate backbone of DNA, releasing nucleotides and preventing chromosome replication [Freese *et al.*, 1967]. Hydroxyl radicals can attack the methyl group of thymine and active molecular oxygen can react with guanine and cause breaks in the DNA strand [Di Mascio *et al.*, 1989].

Gram negative bacteria, due to their lipopolysaccharide layer, which traps active molecular oxygen [Dahl *et al.*, 1989], are less sensitive to the toxic effects of oxygen metabolites. However, Price and Lee (1970) concluded that H_2O_2 was responsible for the inhibition of *Pseudomonas*, *Proteus* and *Bacillus*, by *Lactobacillus plantarum*, while Dahiya and Speck (1968) reported the inhibition of *Staphlococcus aureus* and that the optimal H_2O_2 production was obtained at 5°C.

It has been suggested that LAB can adapt to oxygen. Condon *et al.* (1987), demonstrated that lactococci sensitive to H_2O_2, but exposed to a sublethal concentration of the compound became capable of growth in the presence of a lethal concentration of H_2O_2. He observed the simultaneous induction of NADH peroxidase and to a lesser extent that of NADH oxidase. The author suggested that this may be due to the induction of stress proteins - a phenomenon reported for *Salmonella typhimurium* under similar conditions [Morgan *et al.*, 1986].

In conclusion, oxygen metabolites may affect both lactic acid flora as well as undesirable species. However, construction of strains containing amplified NADH oxidase systems leading to excess production of H_2O_2 and

the simultaneous induction of resistance (possibly via stress proteins) to avoid the phenomenon of autoinhibition, could lead to the exploitation of these natural bacterial inhibitors.

1.2.2 **End products of metabolism**

Conversion of carbohydrates to lactate by the LAB may well be considered as the most important fermentation process employed in food technology [Kandler, 1983]. Lactic acid bacteria exhibit different pathways of carbohydrate fermentation with end products that are either exclusively lactate (homofermentative) or at least 50% lactate (heterofermentative). They differ in the mode of splitting the carbon skeleton, thus leading to different sets of end-products. Glycolysis, occurring in lactococci, pediococci and homofermentative lactobacilli, is characterised by the splitting of fructose 1,6-phosphate into 2 triose phosphate moieties which are further converted to lactate. Heterofermentation in leuconostocs and the betabacteria is initiated by the oxidation of glucose-6-phosphate to gluconate-6-phosphate followed by decarboxylation and splitting of the resulting pentose-5-phosphate into a C2 and C3 moiety. Thus equimolar amounts of CO_2, lactate and acetate or ethanol are formed from hexoses.

Pentoses are usually fermented by all heterofermentative lactic acid bacteria. Pentoses are taken up by specific permeases and converted to lactate and acetate according to the lower half of the 6-phospho-gluconate pathway [Gottschalk, 1979]. However, many strains of the streptobacteria, streptococci and the pediococci ferment pentoses readily although they exhibit homolactic fermentation of hexoses. Upon induction of the necessary enzymes by the pentose, fermentation proceeds via the same pathway as in heterofermentative organisms resulting in equimolar quantities of lactate and acetate. Thus streptobacteria, streptococci and pediococci are heterofermentative with respect to pentose fermentation.

Lactic acid, the final major product of carbohydrate metabolism by lactic acid bacteria is in equilibrium according to the reaction;

$$CH_3\text{-}CHOH\text{-}CHOH + H_2O \longleftrightarrow CH_3\text{-}CHOH\text{-}COO^- + H_3O^+$$

Since its pK_a is 3.86 and the initial intracellular pH is close to neutrality, most lactic acid is ionized. The bacteria must therefore actively eliminate excess lactate and protons to restore osmotic balance and intracellular pH. Aside from the actual pH values of the medium, the quantity of organic acids which are non-dissociated at that pH also plays a role in cell inhibition. This is due to the non-dissociated fraction diffusing accross the cell membrane and becoming ionized due to the intracellular pH. The cell membrane is impermeable to ionized hydrophilic acids, while non-ionized hydrophobic acids diffuse passively. Weak organic acids such as acetic acid ($pK_a = 4.76$) will thus be present at the same concentration on both sides of the membrane as the pH is reduced to 4.76. However, the intracellular medium, being close to neutrality, will favour the ionization of these molecules, thus inhibiting their diffusion back out through the membrane. This leads to an increase in their intracellular concentration with a subsequent reduction in pH. The decrease in intracellular pH can lead to decreased activity of metabolic enzymes and even their denaturation [Accolas *et al.*, 1980].

The tolerance of metabolic enzymes and of transport systems to acid pH is variable depending on the strain. There are very few non-lactic bacteria capable of growing at pH values lower than the threshold pH of lactic acid bacteria (Table 1.3). Adams and Hall (1988) demonstrated that *Salmonella* were inhibited at pH values < 4.4 for lactic acid and < 5.4 for acetic acid. In light of their respective pK_a values of 3.86 and 4.76 which indicates the quantities of dissociated and non-dissociated forms, acetic acid becomes more toxic to the cells. Wong and Chen (1988) showed that *Bacillus cereus* is first progressively inhibited and then killed when co-cultured with various LAB. It was shown that growth completely stopped at pH values of 6.1, 6.0 and 5.6 in the cases of acetate, formate and lactate respectively and the major inhibitory power was attributed to acetate.

Production of organic acids serves two important antimicrobial functions for LAB. Organic acids in their undissociated form will passively traverse the cytoplasmic membrane causing a reduction in pH. At low pH, the overall competitive ability of the lactic acid bacteria is improved since they

are generally more tolerant to reductions in intracellular pH. Good lactic acidification should thus inhibit the growth of *Escherichia coli*, *Pseudomonas*, *Salmonella*, *Clostridium* and *Listeria monocytogenes*.

The more efficient utilization of this microbial inhibition system could include improved acid tolerance of the lactic acid bacteria perhaps once again through a thorough understanding of the lactic acid bacteria stress proteins. It would be of interest to design lactic acid bacteria which at the appropriate stage of the fermentation would switch their catabolism to heterofermentative pathways in order to profit from the high inhibitory power of acetic acid at low pH.

Table 1.3 Limit pH values allowing the initiation of growth of various organisms [Piard & Desmazeaud, 1991]

Gram negative bacteria	Limit pH
Escherichia coli	4.4
Klebsiella pneumoniae	4.4
Proteus vulgaris	4.4
Pseudomonas aeruginosa	5.6
Salmonella paratyphi	4.5
Salmonella typhi	4.0-4.5
Vibrio parahaemolyticus	4.8
Gram positive bacteria	
Bacillus cereus	4.9
Clostridium botulinum	4.7
Enterococcus sp.	4.8
Lactobacillus sp.	3.8-4.4
Micrococcus sp.	5.6
Staphylococcus aureus	4.0
Lactococcus lactis	4.3-4.8
Brevibacterium linens	5.6
Listeria monocytogenes	5.5

The final antimicrobial catabolic end product of interest is diacetyl. *Lactococcus lactis* subsp. *diacetylactis* and *Leuconostoc* species can use citrate in milk generating diacetyl as a by product from this metabolism [Cogan, 1980]. Jay (1982) published a thorough table of the inhibitory capacity of diacetyl indicating that it is lethal for Gram-negative bacteria and only bacteriostatic for Gram-positive bacteria. Diacetyl interferes with arginine utilisation by reacting with the arginine-binding protein of Gram-negative bacteria. Jay further suggested its use as an aseptic agent for surfaces and utensils. Its volatility would avoid contaminating the flavour of manufactured products in the food industry. Work is currently underway to construct hyperproducing diacetyl bacteria as the quantities of diacetyl produced during the milk fermentation are not high enough to be antibacterial [Piard & Desmazeaud, 1991].

1.2.3 **Antimicrobial compounds**

Aside from inhibiting factors resulting from carbohydrate metabolism, many lactic acid bacteria produce antibacterial substances including bacteriocins. Non-peptide antibacterial substances are distinguished from bacteriocins which have a proteinaceous active site. Branen *et al.* (1975) demonstrated that *Lactococcus diacetylactis* produced a small peptide of 100-300 Da active against *Pseudomonas fluorescens, Pseudomonas fragi, Pseudomonas putrefaciens* and *Esherichia coli*. Pulusani *et al.* (1979) successively extracted a material produced by *Streptococcus thermophilus* with methanol and then acetone (M-A extract) which reacted with ninhydrin and inhibited *Lactococcus lactis, Escherichia coli, Salmonella typhimurium, Shigella* spp., *P. fluorescens, Pseudomonas aeruginosa* and *Bacillus* species. Sikes and Hilton (1987) extended this study and demonstrated that the M-A extract was also active against *Clostridium perfringens, Staphylococcus aureus* and *Salmonella enteritidis*. Its partial purification led to the elution of a 700 Da substance with antibacterial activity [Pulusani *et al.*, 1979].

Considerable studies have led to the identification, purification and

characterisation of reuterin, an inhibitor produced by *Lactobacillus reuteri*, a bacterium found in the digestive tract of humans and animals. This broad spectrum inhibitor is produced when *L. reuteri* is grown anaerobically in the presence of glycerol [Talarico *et al.*, 1988]. Its spectrum of activity is exceptionally broad and includes gram positive and negative bacteria, yeasts, moulds and protozoa. Reuterin is a mixture of monomeric, hydrated monomeric and cyclic dimeric, 3-hydroxy-propionaldehyde [Talarico & Dobrogosz, 1989] and may interfere with ribonucleotide reductase, an enzyme involved in DNA synthesis. The wide spectrum of activity of this substance justifies further study, especially as regards the safety of reuterin and its value in the food and agriculture industry [Piard & Desmazeaud, 1992].

1.2.4 **Bacteriocins**

The lactic acid bacteria are well recognised for their production of bacteriocins [Klaenhammer, 1988]. Bacteriocins were first demonstrated in *E. coli* [Hardy, 1975] and later in gram positive bacteria [Tagg *et al.*, 1976]. Klaenhammer (1988) defined two classes of bacteriocins produced by lactic acid bacteria according to their spectrum of activity. One was composed of bacteriocins (nisins, pediocins) with a relatively broad spectrum of activity against gram positive bacteria and the other included bacteriocins active against bacteria taxonomically close to the producer. Bacteriocins active against gram negative bacteria have not yet been fully characterised and there is still doubt about whether these substances are bacteriocins or inhibitory agents [Mc Groarty & Reid, 1988].

In the case of lantibiotics, such as nisin produced by *Lactococcus lactis* subsp. *lactis*, the inhibitory action is generally against most gram positive bacteria. Lantibiotics are characterised by the presence of lanthionine and include subtilin, nisin and epidermin which all have a similar structure [Buchman *et al.*, 1988]. The broad spectrum activity of nisin, including action against *Listeria monocytogenes* [Benkerroum & Sandine, 1987; Harris *et al.*, 1989] and *Clostridium tyrobutyricum* [Hurst, 1981], has led to the authorisation of its use as a food additive in Europe [Hurst, 1981] and in the

US [FDA, 1988].

Other bacteriocins such as Lacticin A and B produced by different *Lactococcus delbrueckii* subsp. *lactis* [Toba *et al.*, 1991] strains exhibit a much narrower spectrum of activity inhibiting only *L. delbrueckii* subsp. *bulgaricus, L. delbrueckii* subsp. *lactis* and *L. delbrueckii* subsp. *delbrueckii.*

The high sensitivity of the LAB bacteriocins to metabolic proteolytic enzymes is very interesting with respect to food safety since it means that ingestion of bacteriocins will not alter digestive tract ecology and will not cause risks related to the use of common antibiotics. The heat tolerance of LAB bacteriocins is generally high [Davey & Richardson, 1981; Barefoot & Klaenhammer, 1984] and in all cases allows the bacteriocin to remain active after pasteurisation (63°C for 30 minutes or 72°C for 15 seconds). Most are stable at acid or neutral pH with the exception of nisin and lactostrepcins. Nisin becomes less soluble as the pH increases from 2.0 to 7.0 which is a considerable disadvantage for its use in non-acidic foods. This drawback may be overcome by genetically engineering more soluble forms resistant to neutral pH.

The mode of action of LAB bacteriocins remains as yet unclear. Their action is rapid as demonstrated by the decrease in viable population within several minutes following contact [Barefoot & Klaenhammer, 1983; Zajdel *et al.*, 1985; Piard *et al.*,1990] and their lethal effect is higher on exponentially growing cells than on stationary phase cells [Davey, 1981; Zajdel *et al.*, 1985]. Bacteriocins cause leakage of K^+, ATP and sometimes UV absorbing substances from target cells [Upreti & Hinsdill, 1975; Sahl & Brandis, 1983; Zajdel *et al.*, 1985; Bhunia *et al.*, 1991]. The loss of ATP and the efflux of K^+ ions leads to exhaustion of energy reserves in sensitive bacteria in an and eventually causes cell death. The involvement of membrane receptors or adsorption sites on the target cell surface is the subject of debate. The ability of nisin to create pores in liposomes clearly established that no membrane receptor is required for activity. Zajdel *et al.* (1985), in contrast, demonstrated that lactostrepcin 5 is ten times less effective against indicator cells previously treated with trypsin and totally inactive against protoplasts of

sensitive cells.

Considerable effort has recently been focussed on the understanding of the structure, genetic organisation and the mode of action of several bacteriocins [Piard & Desmazeaud, 1991]. The development of our knowledge of these substances should help direct genetic approaches for the design of more effective antimicrobial systems, more competitive and antagonistic starter cultures and consequently improved food and silage preservation. Bacteriocins which inhibit producer related strains could be used to favour the growth of an inoculant strain in competition with the natural flora. They could also be used to inhibit the development of lactic acid bacteria in certain alcoholic beverages, meat products and refined sugar, where their presence would be detrimental. The broader spectrum bacteriocins have an additional value since they combat certain pathogenic flora. These "natural" substances, biodegradable in the human digestive tract, are good candidates for replacing certain antibiotics used for pharmaceutical purposes.

Due to their fermentation end products and the generation of antimicrobial substances, the lactic acid bacteria can compete effectively in certain environments. As previously outlined, the lactic acid bacteria are used in a large number of commercial fermentations, one of which results in the preservation of crops as silage.

1.3.1 Silage

Silage, as defined by Whittenbury (1968) is the product formed when grass or other crops of sufficiently high moisture content, liable to spoilage by aerobic microorganisms, are stored anaerobically. It is an important winter feed for cattle in countries where there is a restricted growing season. The type of crop used for silage varies and while usually including grass, maize, legumes and grains, can include potatoes, cabbage, vegetable wastes and fish.

The primary objective of ensilage is to preserve the crop with the minimum loss of nutrients. Successful preservation is dependent upon the rapid achievement and maintainence of anaerobic conditions within the silo and sufficient production of acid to reduce the pH. The low pH decreases plant enzyme activity and combined with anaerobic conditions suppresses undesirable microorganisms such as enterobacteria, clostridia, yeasts and moulds. The lactic acid bacteria are central to the silage fermentation. The fermentation of crop water soluble carbohydrate (WSC) to acids, principally lactic acid, causes the decrease in pH which is detrimental for most spoilage species as outlined earlier (Table 1.3).

Spoilage Organisms in Silage

1.3.2 **Clostridia**

Clostridia are the anaerobic microorganisms most detrimental to the ensilage process. Of those isolated from silage, some clostridia will ferment lactic acid to butyric acid (*Clostridium butyricum, Clostridium paraputrificum* and *Clostridium tyrobutyricum*) while others ferment amino acids to amines and ammonia (*Clostridium bifermentans* and *Clostridium sporogenes*). The conversion of lactate to butyrate is the most common cause of pH reversal leading to a secondary growth of proteolytic clostridia, resulting in the spoilage of silage [Whittenbury, 1968]. Also these end products make silage less palatable to animals, reducing feed value [Neumark, 1967; Neumark & Tadmor, 1968]. *C. butyricum* and *C. tyrobutyricum*, in addition, are harmful for the preservation of cheeses subjected to medium and long term maturation periods [Grazia & Suzzi, 1984] and should be minimized in silage. The pH

at which growth of clostridia ceases is dependent on the water content of the silage. The wetter the silage, the lower the pH required to inhibit clostridia [Muck, 1988] but at a pH of lower than 4.0, clostridial activity essentially ceases [Mc Donald, 1981].

1.3.3 **Enterobacteria**

The enterobacteria isolated from silage include *Erwinia herbicola, E. coli, Hafnia alvei* and *Klebsiella* species and are undesirable because they compete with lactic acid bacteria for available WSC. The optimum pH for these bacteria is approximately 7.0 and as a result, they are usually only present at the onset of fermentation. The principal fermentation end product of the enterobacteria is acetic acid, however formic acid, succinate, ethanol, 2,3-butanediol, lactate, hydrogen and CO_2 may also result [Mc Donald, 1981]. A rapid reduction in pH inhibits the proliferation of enterobacteria. This inhibition may be underestimated at present as a quality criterium since enterobacteria usually cannot survive longer than 14 days in silages regardless of treatment. However they produce endotoxins which remain intact in the silage [Lindgren *et al.*, 1988]. Endotoxin is a component of the outer membrane of all gram negative bacteria and the active substance, lipid A, is part of the lipopolysaccharide complex. Ruminants are usually sensitive to this toxin [van Miert *et al.*, 1983] and so measures to reduce enterobacterial contamination should be taken.

1.3.4 **Yeasts and moulds**

Yeast growth during ensiling is undesirable as the fermentation end products (ethanol and CO_2) result in large dry matter losses (48.9%) with no reduction in pH. The majority of yeasts and moulds are aerobic organisms and in conjunction with *Bacillus* species, are involved in the aerobic degradation of silage. Aerobic deterioration of silage results in loss of nutritional components, a reduction in the preservation potential and an accumulation of degradation products, causing feed refusals by animals [Lindgren *et al.*, 1985]. Aerobic deterioration may be avoided by good ensiling practice but can

become a problem during feeding out. Once again, a rapid reduction in pH and the establishment of anaerobic conditions reduces the proliferation of yeasts and moulds. Hogg, (1991) reported the poisoning of cattle, fed silage which was contaminated with *Claviceps purpurea* (Ergot), a fungus widely distributed throughout the UK. Ergot can infect the seed heads of a variety of grasses and cereals and may cause gangrene if ingested by animals [Humphreys, 1988]. The silage had been made late in the autumn of 1989 from a field of permanent grass which had seeded before cutting. This fungus evidently survived the ensilage process so future developments of silage inocula should contain antifungal agents to prevent diseases associated with contaminated grass. Kalač and Woolford (1982) examined the area of animal health and silage ingestion extensively in their review.

1.3.5 *Listeria monocytogenes*

Silage has long been suspected as being a reservoir in the transmission of listeriosis in ruminants [Gray & Killinguer, 1966; Gray, 1960]. Because it is both an animal and human pathogen, there is concern that *L. monocytogenes* could survive in the silo or possibly multiply there. *L. monocytogenes* can cause meningitis, encephalitis, abortions or septicemias in humans and a wide range of animal species [Gray & Killinguer, 1966]. However the link between listeriosis and silage remains unclear. Listeria are found in poor quality silages which have pH values greater than 5.5 allowing the survival and multiplication of the listeria microorganisms originally in the raw material [Gitter, 1986; Gronstol, 1979; Nicolas, 1983; Fenlon, 1985]. Vázquez-Boland *et al.* (1992), using a recently developed listeria selective medium in combination with serotyping and phage typing, reported that intake of such a poor quality silage (pH 7.8, *Listeria* count of up to 3.2×10^6 cfu/g silage) resulted in the death of 53 out of 450 sheep as a direct result of *L. monocytogenes* infection. In general, such a poor quality silage should not be fed to animals and since a pH of less than 4.2 is known to prevent the development of listeria in silages, [Vetter, 1978] this may not present a significant problem.

1.3.6 **Lactic acid bacteria**

The sources of lactic acid bacteria and the magnitude of the lactic acid bacteria population at the silage pit are to some extent uncertain due to the variability of the starting material, weather conditions and ensiling practices. Stirling and Whittenbury (1963) carried out a three year study and found that counts of lactic acid bacteria on grass were usually less than 10^2 cfu/g fresh matter, while release of plant juices (Seale *et al.*, 1982), wilting (Nilsson & Nilsson, 1956) and contact with farm machinery (Gibson *et al.*, 1961; Fenton, 1987) can result in an increase in numbers of lactic acid bacteria prior to ensiling. Pahlow and Dinter (1987) and Rooke (1990) reported that the majority of epiphytic lactic acid bacteria populations on crops at ensilage were between 10^4 and 10^5 cfu/g with the numbers peaking in July and August.

Studies which have examined the species of lactic acid bacteria on herbage have shown that these lactic acid bacteria consist of predominantly heterofermentative and relatively acid intolerant species [Fenton, 1987; Grazia & Suzzi, 1984]. The most commonly reported species isolated from silage include *Lactobacillus plantarum, Lactobacillus casei, Lactobacillus buchneri, Lactobacillus brevis, Pediococcus pentosaceus, Pediococcus acidilactici, Enterococcus faecium* and *Leuconostoc mesenteroides*. Table 1.4 lists the bacterial species reported in silages. *Lactobacillus* and *Pediococcus* species are generally more tolerant of low pH and many species will grow at pH 4.0 or less. In contrast *Lactococcus* and *Leuconostoc* species grow very slowly or not at all, below pH 4.5 and due to their heterofermentative activities should ideally be replaced with the homofermentative, acid tolerant species. Aside from producing a rapid reduction in pH, homofermentation of glucose and fructose results in no dry matter (DM) loss from the crop [Mc Donald, 1981] while heterofermentation results in a 24% DM loss.

Once the silo is sealed, anaerobic conditions develop and a major population increase in anaerobic and facultatively anaerobic microorganisms occurs. A dramatic increase in the populations of lactic acid bacteria occurs and they rapidly dominate the microflora [Seale, 1986]. It has been reported that maximum lactic acid bacteria numbers of about 10^9 cfu/g fresh matter are

reached after 2-4 days followed by a slow decline in numbers [Gibson *et al.*, 1958, 1961; Ohyama *et al.*, 1971; Ely *et al.*, 1981; Moon *et al.*, 1981]. The dominance of the lactic acid bacteria appears to be due to several factors. The lactic acid bacteria are tolerant of low pH and low water activities which affect enterobacteria and clostridia respectively. They are among the fastest growing natural anaerobic species and the end products of catabolism and the production of antimicrobial compounds, as described earlier, provide a competitive advantage. Within the lactic acid bacteria population, a change in predominant species occurs over the ensilage period with leuconostocs and lactococci present initially on the forage [Dellaglio & Torriani, 1985; Langston *et al.*, 1962; Mundt & Hammer, 1968; Pahlow, 1985; Seale, 1986; Stirling & Whittenbury, 1963], followed rapidly by homofermentative pediococci and lactobacilli. The heterofermentative lactobacilli predominate towards the end of ensiling and this has been attributed to their tolerance of low pH and the presence of acetic acid.

Homofermentative rods	**Homofermentative cocci**
Lactobacillus acidophilus	*Pediococcus acidilactici*
Lactobacillus alimentarius	*Pediococcus dextrinicus*
Lactobacillus casei	*Pediococcus halophilus*
Lactobacillus coryniformis	*Pediococcus parvulus*
Lactobacillus curvatus	*Pediococcus pentosaceus*
Lactobacillus delbrueckii	*Streptococcus bovis*
Lactobacillus farciminis	*Enterococcus faecalis*
Lactobacillus delbrueckii subsp.	*Enterococcus faecium*
leichmannii	*Lactococcus lactis*
Lactobacillus pentosus	
Lactobacillus plantarum	**Heterofermentative rods**
Lactobacillus sake	*Lactobacillus brevis*
Lactobacillus salivarius	*Lactobacillus buchneri*
Lactobacillus sharpeae	*Lactobacillus cellobiosis*
	Lactobacillus divergens
Heterofermentative cocci	*Lactobacillus fermentum*
Leuconostoc mesenteroides	*Lactobacillus reuteri*
Leuconostoc paramesenteroides	*Lactobacillus viridescens*
Leuconostoc cremoris	

Table 1.4. Lactic acid bacterial species found in silages

1.3.7 **Microbial inoculants**

As a fast reduction in pH is essential for crop preservation, the addition of a large number of homofermentative lactic acid bacteria to the forage prior to ensiling should guarantee a rapid, efficient fermentation. This would ensure that homofermentation dominated the initial stages of ensilage thus eliminating the spoilage species and reducing DM losses. Whittenbury (1961) defined the criteria required of an inoculant as follows;

1. It must be a vigorously growing organism able to compete with and
2. dominate other microorganisms.
3. It must be homofermentative.
   It must be acid tolerant and capable of producing a pH of at least 4.0 as
4. quickly as possible.
   It must be able to ferment glucose, fructose, sucrose and preferably
5. fructosans and pentoses.
   It should not produce dextran from sucrose or mannitol from fructose.
6. If mannitol appears, it should be able to ferment it rapidly.
7. It should have no action on organic acids.
8 It should have a growth temperature range extending to 50°C.
   It should be able to grow at high DM contents.

Wieringa and Beck (1964) considered lack of proteolytic activity to be essential, while Woolford and Sawczyc (1984a) considered a rapid establishment of low pH and domination of the epiphytic microflora as the most important criteria. Considering his criteria, Whittenbury suggested that the most suitable microorganisms for use as silage additives are *L. plantarum* and *P. acidilactici* and this was reinforced by Seale (1986).

Having selected the potential inoculant strain or strain mixture, reproducible and reliable testing should be carried out. This is an area of controversy due to the variability of crop composition, ensiling techniques, silo type and weather influences. The microflora of the grass is known to vary throughout the year [Dickenson *et al.*, 1975], the composition of the forage varies greatly depending on crop, region, season and year. Soil type, fertilizer application and grazing are also important. To compare a number of silage additives on a statistical basis, laboratory silo models which reflect farm scale silos should be used. Laboratory silos which can range from test tube silos to buckets or steel drums can be filled quickly and effectively sealed. Farm scale silos often take several days to fill, incurring losses due to delayed sealing. The principle differences between farm-scale silos and laboratory silos are the length of time it takes to ensile the crop, oxygen penetration and temperature

rise. It is preferable to trace the inoculant by effectively distinguishing it from the heterogenous indigenous population so that beneficial effects can be attributed to inoculation. If the inoculant is not effective in laboratory silos, it is unlikely to be of use in the adverse conditions of a farm-scale silo and so laboratory silos provide a useful indication of the effectiveness of potential inoculants [Seale, 1986]. Laboratory silos described by O'Kiely and Wilson (1991) have been shown to reflect conditions experienced in farm scale silos.

Pahlow and Dinter (1987) demonstrated that for an inoculant type additive to have an effect on the silage fermentation, the added lactic acid bacteria should be in a ten fold excess over the epiphytic lactic acid bacteria. Satter *et al.* (1987) have also shown that for responses in animal performance to be obtained from the use of microbial silage, a ten fold excess of lactic acid bacteria was required. As lactic acid bacteria on forage prior to ensilage are between 10^4 and 10^5 cfu/g, an inoculation rate of 10^6 cfu/g is clearly desirable to dominate the fermentation.

Many trials using inoculants have failed in the past due to the inoculation rate being too low to allow domination over the epiphytic population, the strain being unsuited to the silage microenvironment or the lack of adequate fermentable carbohydrate [Moon *et al,* 1981; Silley & Damoglou, 1985]. However, provided the conditions are correct, inoculation with *L. plantarum* at a rate of $1x10^6$ cfu/g grass has been shown to have beneficial effects when compared to control silos [Rooke *et al.*, 1985; Lindgren, 1988].

Improvements in animal performance have been obtained with some microbial silage inoculants. However this would seem to be dependent on the inoculant strain. Henderson *et al.* (1987) reported improved live weight gains in sheep as a result of both increased feed intake and feed conversion efficiency. Gordon (1989) observed increased milk yields (10%) as a result of increased (9%) silage intake but with no improvement in feed conversion efficiency. Martinsson (1992) reported an increase in milk yield (4-5%) mediated through increased intake of metabolizable energy in one of two experiments and Mayne (1990) reported that grass ensiled with $1x10^6$ cfu/g *L.*

plantarum produced large responses both in silage intake and milk yield despite a low WSC (10.9 g/kg). Steen *et al.* (1989) reported that silage DM intake and liveweight gain were significantly greater for *L. plantarum* ($1x10^6$ cfu/g) treated than for untreated silage. In contrast, Kennedy *et al.* (1989) reported no significant effects of *L. plantarum* ($1x10^6$ cfu/g) on DM intake (DMI), liveweight gain or carcass gain. Stokes (1992) reported no improvement of DMI or animal performance with microbial inoculants but this may have been as a result of the reduced aerobic stability of the silage.

Reports on effects of microbial inoculants on aerobic stability are limited and inconsistent. Pahlow and Zimmer (1985) and Jonsson and Pahlow (1984) reported that grass silages inoculated with lactobacilli were more stable than control silages when exposed to air. In legume silages, Kung *et al.* (1987) reported that microbial inoculation resulted in a lower silage pH and a delay in increase in pH as a result of aerobic spoilage. In contrast, microbial inoculation did not significantly affect aerobic stability of grass silages in the study of Lindgren *et al.* (1985). Kung (1991) explained these differences by noting that under certain conditions, some inoculants may cause such a rapid fermentation that unidentified fermentation products imparting aerobic stability in control silages, are never formed, or are in concentrations too low to be effective. This reflects Mc Donald's (1981) observation that the presence of higher volatile fatty acids in silage, resulting from poor fermentation, increased aerobic stability. Other inoculants may not cause such a rapid fermentation and so may not result in decreased aerobic stability. Another possible reason why some inoculated silages appear more stable than others may be due to the production of bacteriocins or other antagonistic agents by the additive strain which are active against spoilage species. ICI have included anti-fungal strains of *Bacillus subtilis* and *Serratia marcescens* for use in preserving feedstuff including silages and hay. The *Serratia* strain produces the anti-fungal antibiotic, prodigiosin (red pigment) and *Bacillus subtilis* displays activity against *Stachybotris alternans* and *Dendrodochrium toxicum* [European patent no. EP-408220, 1990]. Aerobic stability trials demonstrated that inocula containing *Serratia* alone or *Serratia* and *L. plantarum* (Ecosyl^{TM}),

showed good control of moulds and a reduction in temperature increase after five days air exposure, while the combined inoculum gave rise to a larger pH decrease in the fermentation.

1.3.8 **Improvements of future silage inocula**

Inoculants are desirable as silage additives because they are safe to handle, cheap to produce and act by aiding the natural fermentation [Seale, 1986]. However they are less effective on low DM crops with low WSC. This implies that inoculants may be of little use for lucerne or other low sugar, highly buffered crops. This can be overcome by the addition of other sources of fermentable carbohydrate such as molasses, whey or starch combined with α-amylase, cellulases, hemicellulases, endoglucanases and pectinases to release soluble sugars from the degradable fibre components of the ensiled crop. An alternative and more economical approach to the addition of extracellular enzymes, is the genetic manipulation of the inoculant strain so that enzyme activity is expressed by the organism itself. Such heterologous gene expression has been carried out by Scheirlinck *et al.*, (1989, 1990) who stably expressed α-amylase activity from *Bacillus stearothermophilus*, endoglucanase from *Clostridium thermocellum*, and xylanase from *Clostridium acetobutyricum* in *L. plantarum*. These enzymes were transcribed and translated from and secreted in *L. plantarum*, however, the expression levels were too low to be commercially useful. As both Bates (1989) and Scheirlinck (1990) suggested, increased expression could be obtained by the use of strong promoters or amplification of the number of gene copies in the chromosome. Genes allowing fermentation of these wide variety of substrates, in conjunction with increased lactic acid production, cloned in *L. plantarum* could provide improvements in crop preservation. Lactic acid bacteria do cause some deamination and decarboxylation (but not proteolysis) [Beck, 1978] and perhaps the genes involved should be inactivated. The inclusion of antifungal agents in the form of bacteriocins or anti-microbial compounds would lead to an inhibition of spoilage organisms.

The use of these genetically manipulated organisms would require

extensive study into the risks associated with the release of modified organisms in the environment. The genetic stability and competitiveness of genetically engineered microbes is also the subject of debate. However Sharp (1992) recently reported the proliferation and effective competition of two genetically manipulated forms of *L. plantarum* in test tube grass silages. Effective tracing methods are therefore required to accurately monitor the growth and survival of recombinant microbes in the environment.

1.4 Methods used to track microorganisms introduced into the environment

Traditionally, microbial identification was achieved by a mixture of biochemical assays and the use of selective media. However, some microbes are difficult to analyse by such methods and in some cases because of long incubation steps, results can be delayed. For experiments involving the release of organisms into the environment (whether natural or manipulated), target organisms typically comprise only a proportion of the total microbial community and so must often be cultured and the pure culture identified. This is time consuming and may not be applicable to a large number of samples.

1.4.1 Selective media

Specific groups of bacteria can be detected by plating on selective media and if the strain of interest has been genetically altered, it may be distinguishable from the indigenous microflora. Selective plating has been used in many soil experiments with introduced bacterial strains; often spontaneous mutants marked with resistances to rifampicin and nalidixic acid since resistance to these antibiotics was found to be low among soil isolates [Van Elsas & Waalwijk, 1991]. The use of colorimetric media has been widely reported. Drahos *et al.* (1986) used this method for monitoring a recombinant *Pseudomonas fluorescens* containing the *lac*ZY gene cassette from *E. coli*. The Z gene encodes for ß-galactosidase and *lac*Y encodes for lactose permease. Plating on agar containing X-gal, a chromogenic substrate resulted in blue-green colonies easily distinguishable from the non-recombinant non-lactose utilising *Pseudomonas*.

Another marker allowing the identification of colonies amongst backround colonies on (non)-selective plates is the *xyl*E gene product, 2,3-catechol dioxygenase which catalyses the conversion of catechol into 2-hydroxymuconic semialdehyde, permitting the detection of bright yellow *xyl*E positive colonies after spraying the plates with catechol. This has been used successfully by Winstanley *et al.* (1989) and Ingram *et al.* (1989). The *lux* genes from *Vibrio fischeri* encoding visible light emission have been used successfully to monitor microbes released into the environment by Rattray *et*

al. (1990), King *et al.* (1990) and Shaw and Kado (1986) among others. Ahmad *et al.*, (1991) cloned the *lux* A/B genes into *L. casei, L. lactis* and *L.lactis* subsp. *diacetylactis* in order to monitor antibiotics active against starter culture bacteria in milk. After plating, *lux* containing colonies may be recognised by exposure to X-ray film.

An unusual reporter gene has been described by Lindgren *et al.* (1989). The gene for ice-nucleation (*ina*Z) was isolated and incorporated into a transposon which was used to mutagenise plant pathogenic bacterial strains. Expression of the *ina*Z gene results in rapid freezing of cell suspension droplets at -9°C allowing a sensitivity that is reported to be far greater than the *lac*Z system.

A disadvantage of the use of marker genes, the detection of which depends on their expression, is the energetic load on the organism, which may affect its ecological competence. Non-expressed markers could therefore be useful as DNA probes for the identification of microbes.

1.4.2 **Nucleic acid probes**

Nucleic acid probes rely on the specificity provided by the hybridisation between two complementary (or nearly so) sequences of nucleic acids. Factors affecting the hybridisation or reassociation of two complementary DNA strands include temperature, salt concentration, the degree of mismatch between the base pairs and the length and concentration of the target and probe sequences. Probe sequences can be selected that are either very common among organisms for the identification of functional or phylogenetic groups, or very specific for the identification of only one strain of a species.

The main advantages of using gene probes include

(a) the ability to detect organisms that cannot be cultured because they are stressed or fastidious, which is a common limitation in the study of the total microbial population in nature,

(b) the ability to detect organisms without the necessity of having a specific selectable marker, such as antibiotic resistance or requiring the development of selective media,

(c) the ability to track the gene of interest regardless of whether it is expressed or transferred to another organism and
(d) the potential for higher sensitivity than is possible by any other methods.

The disadvantage of the gene probe method is that it is more complex, requiring the identification of a probe specific for the requirments of the researcher.

1.4.3 Extraction of DNA for the Detection of microbes in environmental samples

Three approaches have been used to recover DNA from environmental samples; (1) direct lysis of microbial cells in the environmental matrix followed by nucleic acid purification (2) the isolation of microbial cells followed by cell lysis and nucleic acid purification and (3) the isolation of microbial cells followed by cultivation and cell lysis.

Direct extraction of the DNA from environmental samples was first described by Ogram *et al.* (1987) where cells were lysed in the soil matrix by incubation with sodium dodecyl sulphate followed by physical disruption with a bead-beater. The extracted DNA was then purified by a variety of methods including cesium chloride - ethidium bromide ultracentrifugation, hydroxyapatite or affinity chromatography, phenol/chloroform extractions and ethanol precipitation. Significantly higher yields of DNA were recovered with the direct extraction method than with the cell recovery procedure indicating that DNA from non-culturable organisms was being recovered. It has also become apparent that a number of pathogens including *Legionella* species, *Vibrio cholerae*, *Campylobacter* and coliforms occur in the environment in a viable but non-culturable form [Cunningham & Harris, 1990].

Filtering of aquatic samples to recover microbes has been used extensively and lysis may be performed on the recovered cells directly or following cultivation on laboratory medium. [Sommerville *et al.*, 1989; Bej *et al.*, 1991a; Fuhrman *et al.*, 1988; Giovannoni *et al.*, 1990].

1.4.4 **Colony hybridisation**

Colony hybridisation is perhaps the simplest application of nucleic acid hybridisation and the easiest to integrate with conventional environmental microbiological sampling and analysis. Bacterial colonies or phage plaques can be transferred from primary environmental cultivation media to hybridisation filters (nitrocellulose, nylon etc.). The colonies or phage plaques are lysed by alkaline or enzymatic treatment and hybridised to a labelled single stranded nucleic acid probe. This method is dependent on the ability of the target microorganism to grow on the primary isolation medium and not be totally overgrown by non-target populations. The original protocol developed by Grunstein and Hogness (1975) was shown to be suitable for screening a large number of colonies. Sayler *et al.* (1985) detected one target *Pseudomonas putida* colony in a backround of one million *E. coli* colonies using the TOL plasmid as a probe. There are numerous reports of the application of colony hybridisations to environmental monitoring including the detection of *Salmonella* [Fits *et al.*, 1983], *Shigella* and enterotoxigenic *E. coli* [Sethabutr *et al.*, 1985] *Listeria* [Datta *et al.*, 1987, 1988; Flamm *et al.*, 1989; Noterman *et al.*, 1989], *Yersinia* [Hill *et al.*, 1983; Miliotis, 1989], *Rhizobium* [Hodgson & Roberts, 1983], *Erwinia amylovora* [Falkenstein *et al.*, 1988] *P. fluorescens* [Festl *et al.*, 1986] *L. plantarum* and *P. pentosaceus* [Cocconcelli, 1991].

Gene probes can be hybridised with primary isolates from environmental samples or from secondary cultivation and selection. The rationale for direct colony hybridisation on primary cultivation includes,

(a) avoiding a cultivation bias encountered by selective media that may underestimate total abundance of a given phenotype.

(b) ensuring that a given genotype is represented in the population sampled even if the genes are poorly expressed or are poorly selected,

(c) reducing the analysis time for cultivation, presumptive qualification and confirmation of a genotype/phenotype.

Colony hybridisation following selective cultivation of microbes is usually used to confirm a specific genotype or to test a unique DNA sequence for gene probe development.

1.4.5 **Labelling and detection of the probe**

Various substances have either been used or advocated as labels for DNA probes and have been reviewed in detail by Matthews and Kricka (1988). An ideal label for a nucleic acid probe would have the following properties;

(a) be easily attached to DNA,

(b) be detectable at very low concentrations using simple instrumentation,

(c) produce a signal which is modulated when the labelled DNA probe is hybridised to its complementary DNA sequence (thus facilitating the development of non-separation DNA probe assays),

(d) be stable at the elevated temperatures sometimes used in hybridisations.

Most attention has been focussed on alternatives to radioisotopic labels because of the associated problems of safety, stability and waste disposal. However, no single label has yet emerged as an ideal replacement for a radioisotope.

Two main types of labelling strategy have evolved; direct labelling, in which a label is attached directly via a covalent bond to DNA, and indirect labelling, in which either a hapten is attached to the DNA (and detected by using a labelled binding protein with specificity for the hapten) or a hybrid formation is detected by using a binding protein with specificity for double stranded DNA. An advantage of indirect procedures is the ability to build amplification steps into the assay. For example, if biotin is the primary label, it can be bound to one of the four binding sites on avidin and the remaining three sites can be filled with labelled biotin thus producing a three-fold amplification.

1.4.6 **Selection of probes**

There are three basic classes of genes which have been used successfully; (1) probes targeted for ribosomal RNA (rRNA), (2) for randomly cloned sequences of DNA unique to the strain of interest and (3) for engineered sequences.

1.4.6.1 Ribosomal RNA probes

For several reasons, rRNA is an appealing target for genus and species specific oligonucleotide probes. rRNA is ubiquitous and comprises variable and conserved regions ensuring that probes can be designed to identify virtually any organism or group of related organisms. Secondly, bacterial cells contain about 10,000 ribosomes each a potential target of an rRNA directed probe. Thus, probes to rRNA are more sensitive by several orders of magnitude than probes targeted to chromosomal DNA. Giovannoni *et al.* (1988) first demonstrated that because of the high copy number, individual cells can be identified by using singly radiolabelled rRNA targeted oligonucleotides.

In eubacteria, the RNA genes are a small (0.1%) but highly conserved part of the genome and are comprised of three distinct types; 16S, 23S and 5S rRNAs. The genes coding for rRNA exhibit an operon organisation that is essentially consistent from one bacterium to another. Briefly, the operon organisation consists of a promoter region followed by a sequence coding for the 16S rRNA (approx. 1,500 bases), a spacer or intergenic sequence (which in some instances may contain coding sequences for tRNA), the 23S rRNA coding sequence (approx. 2,500 bases), another short spacer sequence and the sequence coding for 5S rRNA (approx. 120 bases). This arrangement is present in the genome in multicopies and the number of operons present can vary from one group of microorganisms to another. *E. coli* for example, contains seven ribosomal operons, *Bacillus subtilis* contains 10 and *Mycoplasma hyopneumoniae* only one [Plohl & Gamulin, 1991].

In recent years, rRNA sequences have been used to infer quantitative evolutionary relationships among numerous, diverse organisms [Woese, 1987]. Due to their large size (1,500-2000 nucleotides), the 16S-like rRNAs have been particularly useful. Some segments in the 16S rRNA are invariant in all organisms and are therefore useful binding sites for oligonucleotide primers for sequencing protocols [Lane *et al.*, 1985, 1988]. Other portions of 16S rRNA are unique to particular organisms or related groups of organisms and hence offer targets for hybridisation probes with varying specificities.

Both 16S and 23S rRNAs have been used to construct genus and species specific probes for a wide variety of microorganisms. Rossau *et al.* (1989) carried out extensive hybridisations under varying conditions to test 18 oligonucleotide probes complementary to less conserved regions within the 16S and 23S rRNA of *Neisseria gonorrhoeae*. The criterium for specificity was the ability to differentiate between *N. gonorrhoeae* and *N. meningitidis* type strains both of which are genotypically extremely highly related. The DNA:DNA hybridisation homology values range between 64-93%. They observed that the specificity of the probes was highly dependent on the hybridisation temperature and wash conditions used. By simply altering the wash temperature, the detection range of the probes could be extended, so that the same probe could be used to detect *N. gonorrhoeae* specifically (at 80°C), or a larger group of organisms including *E. coli, Pseudomonas testosteroni* and *Chromobacterium violaceum* (at 50°C).

Regensburger *et al.* (1988) constructed probes specific for the *Micrococcus luteus-Micrococcus lylae*, the *Arthrobacter-Micrococcus* group, the eubacteria and a universal probe, from cloned 23S rRNA genes of *M. luteus*. The hybridisation conditions applied detected only organisms sharing at least 80% sequence homology, lower homologies did not hybridise.

Kraus *et al.* (1986) used cloned 23S rRNA from *Bacillus subtilis* as a probe to determine DNA homology values between species. Application of suboptimal (T_m-20°C) or stringent (T_m-10°C) hybridisation conditions yielded the best data for differentiating organisms related to *B. subtilis* from less or non-related bacteria. His experiments indicated a good correlation between oligonucleotide sequence analysis of 16S rRNA and DNA homology values.

Wilson *et al.* (1988) reported the construction of species specific oligonucleotide probes for *Clostridium difficile* and related species using unique segments of the 16S rRNA of three closely related clostridia; *C. bifermentans, Clostridium sordellii* and *C. difficile*. The rRNA of these 3 species showed 97-98% sequence similarity. The oligonucleotide probes were end-labelled with ^{32}P and hybridised to the immobilised rRNA of each clostridia. Each probe efficiently hybridised only to the rRNA of the species

to which it was directed. Complementary probes emmitted a signal that exceeded, by a factor of 100-1000, the signal of probes that mismatched the target rRNA by 2-5 bases. Even a 1 bp difference in rRNA sequence allowed a clear distinction between species (greater than 10 fold difference in efficiency of hybridisation).

Chen *et al.* (1989) described two oligomer probes that were broadly homologous to conserved eubacterial 16S rRNA sequences not present in human 18S rRNA or human mitochondrial 12S rRNA. One or both of the probes detected all 23 phylogenetically diverse eubacterial nucleic acids against which they were tested by dot blot hybridisation. A sensitivity of about one bacterium in ten eucaryotic cells was achieved. Possible uses for these probes would be in partial phylogenetic classification of detected organisms by direct sequence analysis of their PCR amplified product and their use in infection recognition in tissue culture or in animal and plant diseases of unknown cause.

Williams and Collins (1992) recently designed and evaluated a genus specific oligonucleotide probe derived from the V_3 region and species specific probes derived from the V_2 region of 16S rRNA for *Vagococcus fluivalis* (formerly motile *Streptococcus lactis*) and *V. salmoninarium* (a fish pathogen). Target rDNA fragments for hybridisation were obtained by PCR amplification of chromosomal DNA with two conserved primers located close to the termini of the 16S rDNA gene. Although vagococci are phylogenetically distinct, it is difficult to distinguish these organisms from other "lactic acid bacteria" by phenotypic criteria. These three probes were highly specific both for the designated genus and species, illustrating once again the usefulness of 16S rRNA directed probes.

These examples all used filter hybridisation methods to detect the target organisms but the hybridisation technique can also be applied to cells fixed to a microscope slide.

Giovannoni *et al.* (1988) synthesised oligodeoxynucleotide probes specific for each of the three primary lines of evolutionary descent; the eubacteria, the archaebacteria and the eukaryotes, based on 16S rRNA

sequence analysis. A probe complementary to a universally conserved sequence in 16S rRNA was used as a positive control. The probes were end-labelled with ^{32}P or ^{35}S and bound to whole fixed cells in colony blot assays and to single cells fixed to microscope slides (microautoradiography). The demonstration that fixed whole cells on microscope slides were permeable to short oligonucleotide probes, extended hybridisation studies to single cell indentification. De Long *et al.* (1989) identified certain disadvantages of using radioisotopes with microautoradiography including long exposure to photographic emulsions and inadequate resolution of less than 1μm because of the scatter of radioactive disintegrations - a problem also recognised by Giovannoni *et al.* (1988). De Long *et al.*, (1989) coupled oligonucleotide probes specific for 16S rRNA sequences to fluors (x-rhodamine or fluorescein) stained formaldehyde fixed intact cells and viewed the hybridised probes by fluorescent microscopy. The simultaneous use of multiple probes, labelled with different fluorescent dyes, allowed the identification of different cell types in the same microscopic field. All cell types examined, including Gram-positive bacteria, yeast spores, a variety of Gram-negative bacteria and eukaryotic tissues seemed freely permeable to the probes after fixation. Amman *et al.* (1990) used this technique to discriminate between *Fibrobacter* species and to demonstrate single mismatch discrimination between certain probe and non-target sequences.

Jurtshuk *et al.* (1992) extended the use of the rapid in-situ hybridisation (RIS) technique to specifically differentiate two closely related *Bacillus* spp.; *Bacillus polymyxa* and *Bacillus macerans*. The 16S rRNA probes were labelled with a rhodamine derivative (Texas red) and quantitative fluorescence measurements were made on individual bacterial cells microscopically. The RIS hybridisation technique offers several advantages over conventional identification protocols;

(1) the detection of a specific segment of the 16S RNA sequence, within intact individual cells allows the identification of a specific bacterial cell even within a mixed heterogenous population.

(2) a greater sensitivity for the detection of specific bacterial cells has been exhibited (Jurtshuk *et al.*, 1992)

(3) no extraction of nucleic acids from target cells is required,
(4) multiple probes with different labels can be used to allow the simultaneous detection of several target sites even within an individual cell,
(5) minimal sample sizes of bacteria can be used and only microquantities of reagents are required.

1.4.6.2 rRNA probes for the lactic acid bacteria

Identification of LAB with large numbers of tests is tedious, time consuming and determination of carbohydrate fermentations may be misleading due to the high frequency of mutants with altered carbohydrate fermentation. [Shaw & Harding, 1984; Ahrné, & Molin, 1991]. Therefore attention has turned to the design of genus and species specific rRNA probes for the LAB. Betzl *et al.* (1990) reported the construction of 23S rRNA based oligonucleotides which were able to distinguish and enumerate *Lactococcus lactis, Enterococcus faecalis, Enterococcus faecium* and *Enterococcus malodoratus / Enterococcus avium* in a mixed population of lactococci and enterococci and in spontaneously fermented milk, using successive colony hybridisation.

Klijn *et al.* (1991) described the construction of oligonucleotide probes designed from PCR amplified and sequenced V1 and V3 regions of 16S rRNA. The sequences of the V1 regions of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* displayed too many differences to allow the design of a species-specific *L. lactis* probe, however probes for each subspecies were constructed. The *L. lactis* subsp. *lactis* probe (pLl_1) did not give a signal with *L. lactis* subsp. *hordniae* containing a sequence in the V1 region that differed in only one nucleotide. The V3 region was used to discriminate between *Leuconostoc lactis* and *L. mesenteroides* despite only a 3 bp difference in sequence. Although the V3 probe was not tested against a complete range of LAB, the following species did not hybridise with it; *Leuconostoc lactis, Leuconostoc paramesenteroides, L. casei, Lactobacillus helveticus, Lactobacillus bulgaricus, Lactobacillus acidophilus, L. plantarum* and *Lactobacillus fermentum.*

Hertel *et al.* (1991) designed species specific probes based on a

variable region of the 5' terminus of the 23S rRNA for *Lactobacillus pentosus / L. plantarum, Lactobacillus curvatus* and *Lactobacillus sake*. The oligonucleotide probe for *L. pentosus* contained 6 and 5 base changes in comparison to those of *L. curvatus* and *L. sake* respectively whereas the latter two probes were distinguished by 2 bp changes. The specificity of the probes was evaluated by dot blot hybridisation to membrane bound crude nucleic acids. Oligonucleotides specific for *L. curvatus* and *L. sake* hybridised only to rRNA targets of strains belonging to the corresponding species, while the oligonucleotide for *L. pentosus* also hybridised to the closely related species, *L. plantarum*. Thirty different gram positive species were tested. These reports reflect the growing interest in rRNA for the construction of genus and species specific probes. There have been no reports of strain specific rRNA based probes and the GenBank does not as yet have sufficient entries to allow a comprehensive search of a variety of strains of a single species.

1.4.6.3 Strain specific plasmid DNA probes

The tracing of genetically manipulated microbes can usually be carried out using the manipulated gene as the probe sequence, however non-manipulated strains released into the environment are more difficult to distinguish from other strains of that species. Inoculation with "natural" strains occurs in the dairy industry in the production of cheese and yoghurt and in the agriculture industry in the preservation of silage. Evaluation of the efficacy of microbial inoculants usually entails microbial enumeration to estimate the levels of the inoculant and biochemical analysis on the fermentation end product. However, in a variable substrate with a mixed heterogenous population, these criteria may not indicate the successful establishment of the inoculant. To correlate the presence of the inoculant with the desired fermentation characteristics, a strain specific probe must be used.

Plasmid DNA sequences have been used as probes for the plant pathogen *Xanthomonas campestris* pv *phaseoli* [Gilbertson *et al.*, 1989], enteropathogenic *E. coli* [Vankatesan *et al.*, 1988], for epithelium associated strains of lactobacilli [Tannock, 1989] and for environmental isolates which

degrade 4-chlorobiphenyl [Pettigrew & Sayler, 1986]. Hendrick *et al.* (1991) in their evaluation of naturally occuring isolates of *Bacillus pumilis* for use as microbial hay preservatives used a plasmid probe to follow populations of these isolates in hay over time.

The presence of plasmids in a range of the LAB has been reported since they were first discovered in the *Lactobacillus* genus [Chassy *et al.*, 1976]. Phenotypes such as bacteriocin production and immunity [Muriana & Klaenhammer, 1987; Schillinger & Lücke, 1989], lactose metabolism [Chassy *et al.*, 1978] and drug resistance [Ishiwa & Iwata, 1980; Vescovo *et al.*, 1982; Morelli *et al.*, 1983] have been linked to extrachromosomal DNA in several species of LAB but most of the plasmids are still cryptic.

Hill and Hill (1986) first reported the use of plasmid profiling in monitoring *Lactobacillus plantarum* used to inoculate silage fermentations. They demonstrated that the plasmids of naturally occuring *L. plantarum* strains were stable during the ensiling process and so could be used to monitor the succession of the introduced organisms.

Plasmid profiling is tedious, time consuming and limited in the number of isolates which can be tested and so plasmid probes unique to two strains of *L. plantarum* and one strain of *Pediococcus pentosaceus* were developed by Cocconcelli *et al.*, (1991) in their evaluation of maize silage inoculants in farm silos. Their results indicated that the inoculants which were applied at $1x10^4$ cfu/g established well initially with a subsequent reduction in pH to about 4.0 (after 48 hours). However the difference between the total lactic acid bacterial population and the inoculant population increased with time. This indicated that the inoculants were not dominant over the indigenous population after three days ensilage. As previously mentioned, inoculants work most effectively when there is a tenfold excess over the indigenous population so perhaps a higher inoculation rate would have been more successful. An important result was the demonstration that the plasmids were stable and did not transfer to other silage strains. Plasmid probes are extremely useful for the monitoring of strains which contain a unique plasmid segment that may distinguish them from strains of the same species.

1.4.6.4 Engineered DNA probes

With the advent of genetic manipulation of strains and concern over the safety of releasing these microbes into the environment, nucleic acid probes specific for the manipulated sequences are invaluable in monitoring the spread of these microorganisms. Gene probe technology is ideally suited to tracking genetically manipulated microbes (GEMs) because the target gene is assayed directly. Several important factors to be considered before GEMs can be released into the environment include,

(a) the maintenance of the DNA of interest in the environment both within the original host and outside of the host,

(b) transfer of DNA between microbes and

(c) the effect of exogenous DNA sequences on the survival of GEMs.

Sharp *et al.* (1992) recently reported the tracing of two genetically engineered strains of *L. plantarum* inoculated into test tube silos. Although the GEMs were traced primarily by their resistance to rifampicin and erythromycin, confirmation was supplied by Southern hybridisation with the plasmid probe, pSA3, a shuttle vector for Gram-positive microorganisms that encodes erythromycin resistance. Sharp reported the maintenance of the DNA of interest in 100% of the integrant strains, no transfer of the genetically manipulated sequence between strains and that the presence of extra genetic material did not appear to disadvantage the bacterium in comparison to the parent strain.

1.5.1 Phylogenetics based on rRNA sequencing

The classification of organisms traditionally has been based on similarities in their morphological, developmental and nutritional characteristics. However, it is clear that with microorganisms, classification based on these criteria does not necessarily correlate well with evolutionary relationships as defined by macromolecular sequence comparisons.

Phylogenies derived from sequence analysis have to be accepted for what they maximally are; hypotheses to be tested and either strengthened or rejected on the basis of other types of data [Woese, 1987]. Sequence information is superior to phenotypic information because it is more readily, reliably and precisely interpreted and innately more informative of evolutionary relationships. The elements of a sequence (nucleotides) are restricted in number and well defined and are therefore less subjective. At the level of the genotype, change constantly occurs, however most of this is not reflected in a phenotypic change. It therefore becomes fixed randomly in time making its characterisation as "clocklike" in occurence appropriate. In other words, evolution has a tempo that is almost independent of the changes in phenotype. Woese (1987) explained this with an analogy to a car and its motor: a car does not go unless its motor is running but the motor can run without the car moving. A molecule whose sequence changes randomly in time can be considered a chronometer. The amount of sequence change it accumulates (distance) is the product of a rate x a time (over which the mutations have occurred).

All sequences are not of equal value in determining phylogenetic relationships. To be a useful chronometer, a molecule should exhibit

(1) clocklike behaviour - changes in its sequence have to occur as randomly as possible,

(2) range - rates of change have to correspond with the spectrum of evolutionary distances being measured and

(3) adequate size - the molecule has to be large enough to provide an adequate amount of information and to be a "smooth running" chronometer, ie. nonrandom changes affecting one part of the sequence should not appreciably

affect the others [Woese, 1987].

rRNA is at present the most useful of the molecular chronometers. It shows a high degree of functional constancy which assures relatively good clocklike behaviour. It occurs in all organisms and different positions in the sequences change at different rates allowing most phylogenetic measurements including the most distant to be measured. The sizes are large and consist of many "domains" (functional units) which makes their range all-encompassing. The rRNA genes seem to be free of the artifact of lateral transfer, an essential requirement if the molecular data are to be used to infer evolutionary relationships. The most compelling reason for its use is that it can be sequenced directly and rapidly by means of the enzyme reverse transcriptase.

The sequencing protocol is a variation of the base-specific dideoxynucleotide-terminated, chain elongation method. It has been modified for the use of reverse transcriptase and rRNA templates. A short oligonucleotide, complementary to a universally conserved site anneals specifically to its target site on the 16S rRNA, even in the presence of other cellular RNA and the chain elongation reaction proceeds. Three particularly useful priming sites, which provide access to the 3 major 16S rRNA structural domains, collectively yield 800-1200 nucleotides of sequence from each 16S rRNA [Lane *et al.*, 1985, 1988].

Each of the primary kingdoms; the eukaryotes, eubacteria and archaebacteria, has its particular form of rRNA. While there are general resemblences among them, there are also characteristic differences in structural detail. Strong rRNA sequence signatures (highly conserved kingdom specific sequences) define and distinguish the three kingdoms. Many of the traditional groupings of bacteria have been discarded. In the eubacteria, it has been agreed that Gram-positive is indeed a phylogenetically coherant grouping but Gram-negative is not [Woese, 1987]. The latter encompasses 10 distinct groups. The idea that the wall-less bacteria, mycoplasmas, are phylogenetically remote from other eubacteria is incorrect; the true mycoplasmas are merely "degenerate" clostridia [Woese, 1987]. Photosynthetic bacteria do not form a grouping genealogically distinct from

the nonphotosynthetic bacteria and neither do autotrophs and heterotrophs. In fact these "groups" are intermixed within the various eubacterial phyla. Thus the textbook views in which photosynthetic (or autotrophic) bacteria arise from nonphotosynthetic heterotrophic ancestors gain no support from the rRNA based phylogeny [Woese, 1987].

The eubacterial phyla and their subdivisions as they are now understood are listed in Table 1.5.

1.5.2 **Gram-positive eubacteria**

Cell wall type distinguishes the Gram-positive eubacteria from the other phylogenetic groups. The phylum is described in Table 1.6 and appears to consist of four subdivisions, only two of which are well characterised. These two are readily distinguished on the basis of DNA composition; high G+C content (>55%) or low G+C content (<50%). The phototroph, *Heliobacterium chlorum* is the only characterised representative of the third subdivision, while the genera *Megaspheara, Selenomonas* and *Sporomusa* constitute the fourth. Members of the third and fourth subdivisions do not have Gram-positive walls.

Species in the high G+C subdivision conform to a general actinomycete phenotype; they tend to be pleomorphic, form branched filaments and most are aerobic with the exception of the deeper branches (*Bifidobacterium*). The group as a whole is not particularly deep and would therefore not seem to be a particularly ancient one.

Species in the low G+C subdivision conform for the most part to a clostridial phenotype. They tend to be anaerobic, rod shaped and endosporeforming although a number have lost one or more of these characteristics. They form a phylogenetically deep and therefore ancient cluster [Woese, 1987].

Woese (1987) noted one subline in the low G+C subdivision which has given rise to four groups of particular interest; *Bacillus, Lactobacillus, Streptococcus* and the mycoplasmas. Their evolution parallels the development of aerobic conditions on the planet. *Bacillus* species are aerobic, though a few

also grow well anaerobically. *Lactobacillus*, *Streptococcus* and the mycoplasmas are basically anaerobic but tolerate, and in some cases utilise, a little oxygen, while their clostridial relatives are true anaerobes.

Table 1.5. Eubacterial phyla and their subdivisions

Purple bacteria
α subdivision
Purple non-sulphur bacteria, rhizobacteria, agrobacteria, rickettsiae, *Nitrobacter*

ß subdivision
Rhodocyclus, (some) *Thiobacillus*, *Alcaligenes*, *Spirillum*, *Nitrosovibrio*

γ subdivision
Enterics, fluorescent pseudomonads, purple sulphur bacteria, *Legionella*, (some) *Beggiatoa*

δ subdivision
Sulphur and sulphate reducers (*Desulfovibrio*), myxobacteria

Gram positive eubacteria
A. High G+C species
Actinomyces, *Streptomyces*, *Arthrobacter*, *Micrococcus*, *Bifidobacterium*
B. Low G+C species
Clostridium, *Peptococcus*, *Bacillus*, mycoplasmas
C. Photosynthetic species
Heliobacterium
D. Species with gram negative walls
Megasphaera, *Sporomusa*

Cyanobacteria and chloroplasts
Aphanocapsa, *Oscillatoria*, *Nostoc*, *Synechococcus*, *Gleobacter*, *Prochloron*

Spirochetes and relatives
A. Spirochetes
Spirochaeta, *Treponema*, *Borrelia*
B. Leptospiras
Leptospira, *Leptonema*

Green sulphur bacteria
Chlorobium, *Chloroherpeton*

Bacteroides, flavobacteria and relatives
A. Bacteroides
Bacteroides, *Fusobacterium*
B. Flavobacterium group
Flavobacterium, *Cytophaga*, *Saprospira*, *Flexibacter*

Planctomyces and relatives
A. Planctomyces group
Planctomyces, *Pasteuria*
B. Thermophiles
Isocystis pallida

Chlamydiae
Chlamydia psittaci, *Chlamydia trachomatis*

Radioresistent micrococci and relatives
A. Deinococcus group
Deinococcus radiodurans
B. Thermophiles
Thermus aquaticus

Green non-sulphur bacteria and relatives
A. Chloroflexus group
Chloroflexus, *Herpetosiphon*
B. Thermomicrobium group
Thermomicrobium roseum

Table 1.6 Gram positive eubacterial phylum[a]

High G+C subdivision

Bifidobacterium
Actinomyces
Arthrobacter
Micrococcus
Dermatophilus
Cellulomonas
Derskovia
Nocardia cellulans
Microbacterium
Corynebacterium (plant)
Brevibacterium linens
Streptomyces
Kitasatoa
Chainia
Microellobosporia
Streptoverticillium
Actinomadura
Streptosporangium
Mycobacterium
Nocardia
Brevibacterium ketoglutamicum
Corynebacterium
Geodermatophilus
Frankia
Dactylosporangium
Ampurariella
Actinoplanes
Micromonospora
Arthrobacter simplex

Photosynthetic subdivision

Heliobacterium

Species with gram negative walls

Megasphaera
Selenomonas
Sporomusa

Low G+C subdivision

Bacillus
Planococcus
Sporolactobacillus
Sporosarcina
Thermoactinomyces
Staphylococcus
Lactobacillus
Pediococcus
Leuconostoc
Streptococcus
Mycoplasma
Acholeplasma
Spiroplasma
Anaeroplasma
Clostridium innocuum
Erysipelothrix
Clostridium pasteurianum
Clostridium butyricum
Clostridium scatologenes
Sarcina ventriculae
Clostridium oroticum
Clostridium indolis
Clost. aminovalericum
Butyrivibrio fibrosolvens
Clostridium lituseburense
Eubacterium tenue
Peptostreptococcus
Clostridium aceticum
Clostridium acidiurici
Clostridium purinolyticum
Clostridium barkeri
Eubacterium limosum
Acetobacterium woodii
Clost. thermosaccharolyticum
Clostridium thermoaceticum
Acetogenium
Thermoanaerobium
Peptococcus
Ruminococccus

[a]Approximate phylogenetic clustering suggested by indentation. Table reproduced from Woese, (1987)

1.5.3 **Application of rRNA analysis in LAB classification**

The relationships between the lactic acid bacteria and the position of *Lactobacillus* relative to the genera *Aerococcus, Carnobacterium, Enterococcus, Leuconostoc, Pediococcus, Streptococcus* and *Tetragenococcus* has been thoroughly investigated using 16S rRNA sequencing [Collins *et al.*, 1991; Collins *et al.*, 1990; Martinez-Murcia & Collins, 1990].

The 16S rRNA sequences of fifty five species of the genus *Lactobacillus* [Collins *et al.*, 1991], eight *Leuconostoc* [Martinez-Murcia & Collins, 1990] and *Aerococcus viridans. P. acidilactici, Pediococcus damnosus, Pediococcus dextrinicus, Pediococcus halophilus, Pediococcus parvutus, P. pentosaceous* and *Pediococcus urinae-equi* [Collins *et al.*, 1990] were determined by reverse transcription. It was found that considerable genetic diversity exists amongst the *Lactobacillus* species examined. As shown in figures 1.1 and 1.2, the majority of *Lactobacillus* species form three phylogenetically distinct clusters; the *Lactobacillus delbrueckii* group, *Lactobacillus casei/ Pediococcus* group and the *Leuconostoc paramesenteroides* group.

The rRNA groups identified by Collins *et al.* (1991) did not support the classical division of *Lactobacillus* into the subgenera,"*Thermobacterium*", "*Streptobacterium*" and "*Betabacterium*" [Orla-Jensen, 1942], nor the three physiological groups, obligately homofermentative, facultatively heterofermentative and obligately heterofermentative as outlined in Bergey's Manual of Systematic Bacteriology [Kandler, 1986]. All of the species included in the *L. delbrueckii* rRNA cluster are obligately homofermentative (Orla-Jensen's thermobacteria). Several obligately homofermentative species were however interspersed in the *L. casei* rRNA cluster. The recovery of pediococci within the *L. casei* group and the clustering of several atypical heterofermentative species into cluster 3 demonstrates once again that morphology is a poor and unreliable indicator of relatedness for this group of microorganisms [Collins *et al.*, 1991]. rRNA sequencing has become a valuable tool in the construction of phylogenetic trees and in the future may lead to our correct understanding of bacterial evolution.

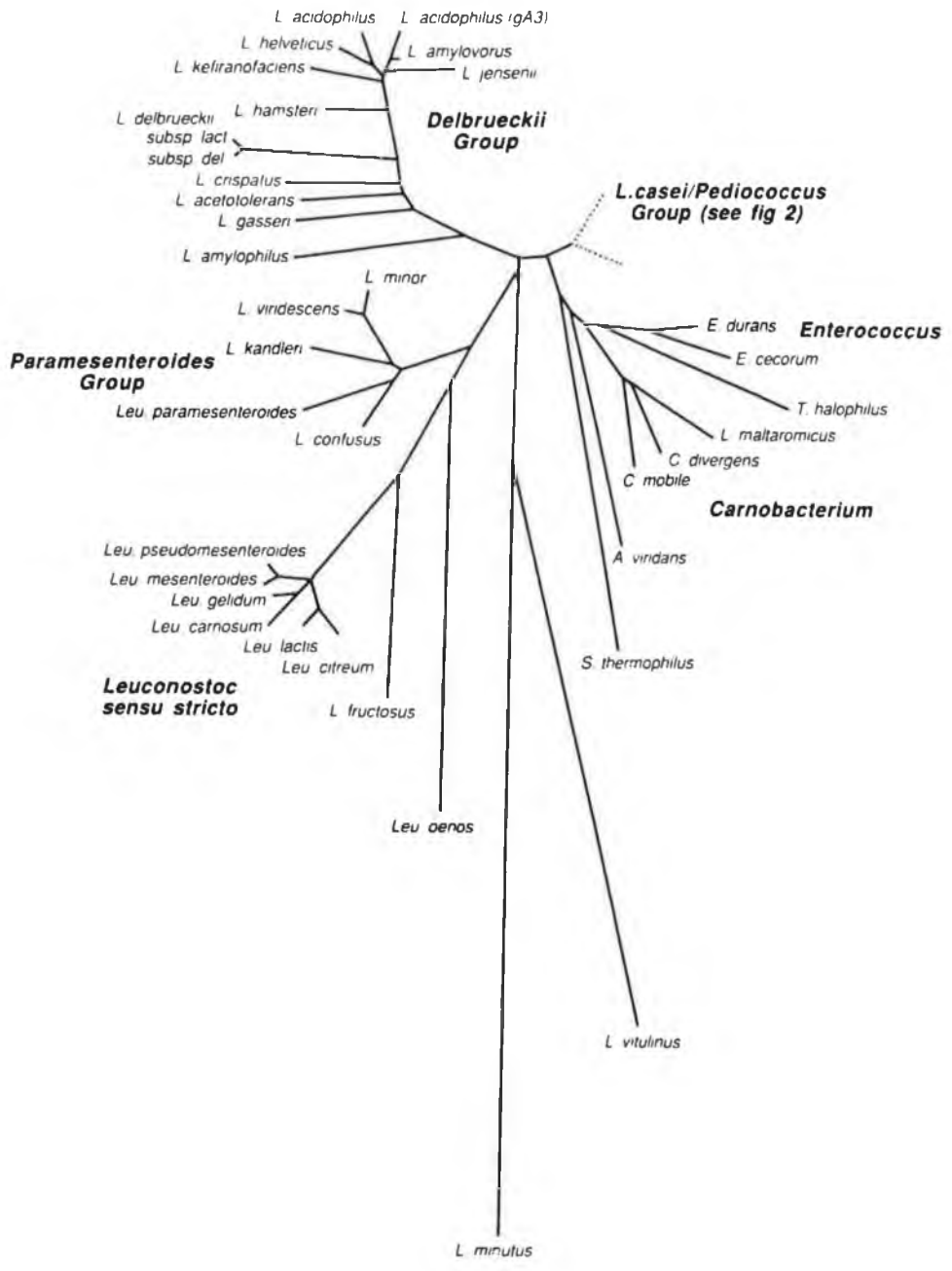


Figure 1.1 Reproduced from Collins *et al.*, (1991). Evolutionary distance tree showing the intra- and inter-generic relationships of the genus *Lactobacillus* and other lactic acid bacteria. The values were based on a comparison of 1340 nucleotides, ranging from positions 107 (G) to 1433 (A) of the *E. coli* numbering system. The evolutionary distance between two species is the sum of the total branch lengths.

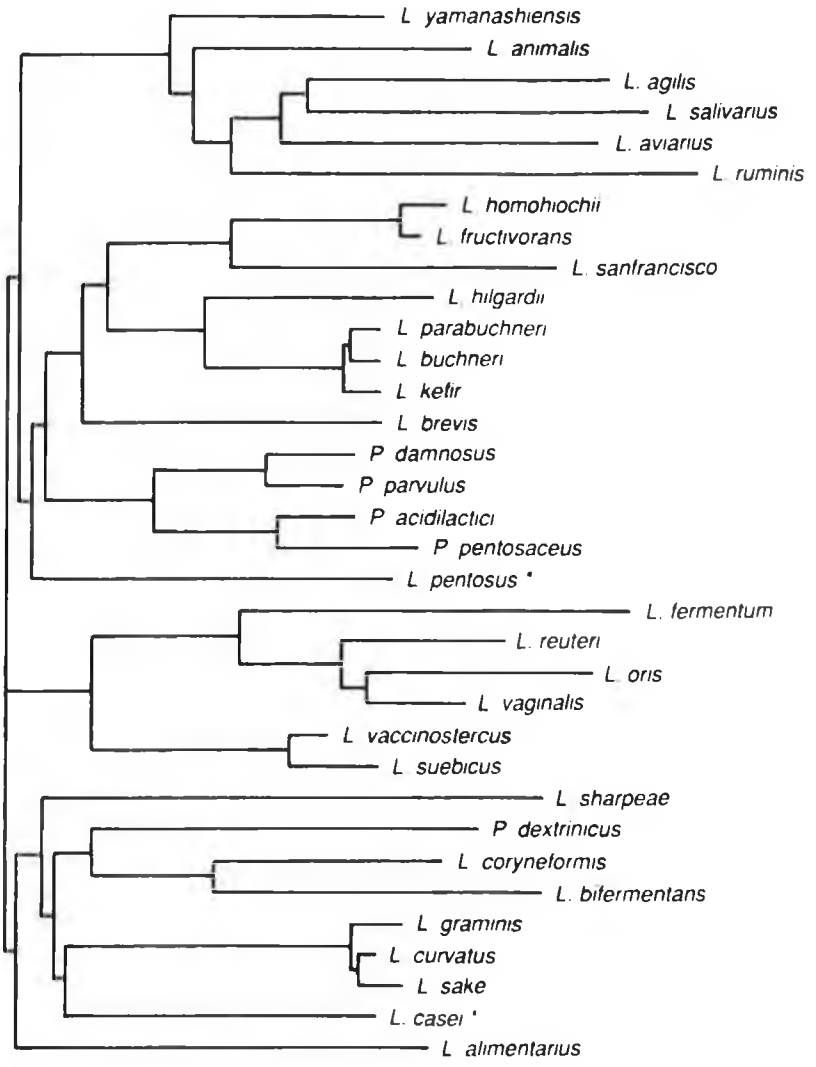


Figure 2. Reproduced from Collins *et al.* (1991). Evolutionary distance tree showing the interrelationships of species of rRNA group 2. The evolutionary distance between two species is the sum of the vertical lengths. * *L. paracasei* and *L. rhamnosus* exhibited >99% sequence homology with *L. casei*; *L. pentosus* exhibited >99% homology with *L. plantarum.*

1.5.4 **Rapid determination of rRNA sequences using PCR amplification**

The determination of rRNA sequences with reverse transcriptase is hampered by a few problems including the requirement for large quantities of good quality rRNA and the presence of sequencing "anomalies" which result from an inability of the enzyme reverse transcriptase to transcribe regions of strong secondary structure or termination due to reverse transcriptase being released from the template [Lane *et al.*, 1985, 1988]. An alternative strategy using the PCR reaction to amplify rRNA genes has been described by Böttger (1989) who directly sequenced an 880bp fragment of rDNA from *E. coli, Legionella pneumophila* and *Mycobacterium tuberculosis*. Similar procedures involving linear PCR reactions [Embley, 1991] and direct sequencing of amplified product [Both *et al.*, 1991] to sequence 16S rRNA genes from various bacterial species have been described. The PCR reaction is described in more detail in chapter 5.

1.6.1 Chromosomal DNA fingerprinting

One application of DNA probes is the construction of DNA fingerprints for strain or species indentification. Bacterial restriction endonuclease digest analysis (BRENDA) provides a sensitive means of detecting minor genomic differences between microorganisms. Restriction endonucleases specifically cleave DNA into different lengths, depending on the number and position of the individual recognition sequences. A DNA polymorphism refers to the change in the size of restriction fragments due to a modification in the enzyme recognition site. Sequence changes may arise as a result of mutations, insertions, deletions or inversions of DNA between sites. The significance of DNA polymorphisms is that they most often represent neutral mutations and do not cause any phenotypic change.

The restriction enzyme recognition sites consist in most cases of four or six nucleotides arranged in diad symmetry. Because the genomes of bacterial species vary widely in their base composition (26-72 mol% G+C), the distribution of fragment sizes produced by restriction endonuclease digestion can also be expected to vary considerably. The expected frequency, *a* of specific restriction sites can be predicted from

$$a=(\frac{g}{2})^{r1}(1-\frac{g}{2})^{r2}$$

where g is the fractional G+C content of the genome, *r1* the number of G+C base pairs and *r2*, the number of A+T base pairs in the enzyme recognition site [Nei & Li, 1979]. The expected number of restriction sites is given by frequency x genome size. The genome sizes for most prokaryote DNA falls between 800-8000 kb with a typical genome being about $6x10^6$ bases in length [Gillis & De Ley, 1975]. The probability of a G+C rich site in G+C rich DNA is about 1 in 500, but only 1 in 80,000 in G+C poor DNA [Owen, 1989]. CCG and CGG are the rarest trinucleotides in many genomes with <45% mol G+C content and so enzymes such as *Sma*I, *Rsr*II, *Nae*I and *Sac*II which have those sets of bases in their recognition sequences are rare cutters for low G+C content DNA. Table 1.7 lists the G+C mol% of the

DNA of lactic acid producing bacteria and related gram positive microorganisms of importance for the food industry [Stackenbrandt & Teuber, 1988].

Restriction endonuclease digest patterns have been used to distinguish various bacteria including a number of genera containing species of medical importance as well as strains of other microbial groups such as *Candida, Chlamydia, Giardia* and Cytomegalovirus [Owen, 1989].

The usefulness of such patterns as diagnostic tools is limited by their complexity because they may comprise approximately 1000 bands of various sizes depending on the cutting frequency of the enzyme used and the genome size of the organism. There is also the possibility, when strains contain plasmids, that significant differences in the chromosomal banding pattern are obscured by a similar plasmid pattern. The detection of strain differences in DNA fingerprints relies largely on direct visual comparison but computer methods have been used to make more objective comparisons between strain fingerprints [Bruce, 1988].

Table 1.7 G+C content (mol%) of the DNA of lactic acid producing bacteria and related Gram-positive microorganisms of importance in food technology.

Microorganism	G+C content
Clostridium perfringens	24-27%
Clostridium tyrobutyricum	28%
Staphylococcus aureus	32-36%
Enterococcus faecalis	34-39%
Bacillus cereus	36%
Listeria monocytogenes	37-39%
Lactobacillus helveticus	37-40%
Leuconostoc mesenteroides ssp. *cremoris*	38-40%
Pediococcus acidilactici	38-44%
Lactococcus lactis ssp. *lactis*	39%
Streptococcus thermophilus	40%
Lactobacillus brevis	44-47%
Lactobacillus delbrueckii spp. *bulgaricus*	49-51%
Bifidobacterium bifidum	58%
Brevibacterium linens	60-64%
Propionibacterium freundenriechii	64-67%
Microbacterium lacticum	69-75%
Micrococcus luteus	70-75%

1.6.2 Chromosomal probe hybridisation patterns

To reduce the number of bands in the fingerprint and to facilitate comparison between strains, a nucleic acid probe used in southern hybridisation to highlight specific DNA restriction site heterogeneities can be used to detect strain differences within and between species. The probes which have been used for this purpose include cloned random chromosomal sequences [Tompkins *et al.*, 1986], toxin production genes [Pappenheimer & Murphy, 1983] and most widely used of all, ribosomal RNA sequences. The rRNA based probes provide a widely applicable system to investigate the molecular epidemiology of diverse bacteria [Stull *et al.*, 1988], whereas other probes may be more limited in being species or strain specific. Due to the high sequence homology between eubacterial species, *E. coli* rRNA can hybridise with rRNA of a wide range of Gram-negative and Gram-positive bacteria.

A large number of microbial groups have been studied by means of chromosomal rRNA probe fingerprinting, including those listed in Table 1.8, with the purpose of distinguishing between species or between strains within species.

Table 1.8 Summary of microbial species investigated by chromosomal rDNA probe fingerprinting

Species	Probe		Reference
Mycoplasmas			
Acholeplasma laidlawii	pMC5 containing 16+23S and part 16S rRNA gene of *Myco. capricolum*	*Eco*RI, *Pst*I	Yogev & Razin (1986), Yogev *et al.*, (1988a)
Myco. genitalium	rRNA gene probe pMC5	*Hind*III	Yogev & Razin (1986)
Myco. hominis	pMYC4 containing insert of rRNA gene of *Mycoplasma* PG50	*Eco*RI	Christiansen *et al.*, (1987)
Myco. pneumoniae	pMC5 containing 5S+23S+part of 16S rRNA genes of *Myco.capricolum*	*Eco*RI, *Hind*III	Yogev *et al.*, (1988b)
Gram - eubacteria			
Aeromonas species	16S+23S rRNA from *E. coli*	*Sma*I, *Pst*I	Altwegg *et al.*, (1988)
Providencia stuartii, Prov. alcalfaciens, Prov. rustigianii, Proteus vulgaris	*Prov. stuartii* 16S + 23S rDNA	*Eco*RI, *Hind*III	Owen *et al.*, (1988)
Proteus vulgaris	Synthetic oligonucleotide probe PV25 of selected 16S rRNA sequences	*Eco*RI	Haun & Gobel, (1987)
Pseudomonas aeruginosa	*E. coli* 16S and 23S/5S rDNA	*Bam*HI / *Eco*RI, *Bam*HI / *Hind*III	Hartmann *et al.*, (1986)
Pseudomonas cepacia	*E. coli* and *P. cepacia* rRNA	*Eco*RI	Stull *et al.*, (1988)
Haemophilus influenzae	*E. coli* and *H. influenzae* rRNA	*Eco*RI	Stull *et al.*, (1988)
H. influenzae biovar aegyptius	*E. coli* rRNA	*Eco*RI, *Eco*RV	Irino *et al.*, (1988)
Salmonella typhi	pKK3535 containing *E. coli* cloned rDNA sequences	*Pst*I	Altwegg *et al.*, (1989)
Salmonella enteritidis	pKK3535	*Sma*I, *Sph*I	Martinetti & Altwegg, (1990)
Legionella pneumophila and other *Legionella* spp.	Cloned fragment containing 16S+23S rRNA genes of *L. pneumophila*	*Nci*I	Saunders *et al.*, (1988)
Campylobacter jejuni and other species	17-nucleotide synthetic probe complementary to a unique sequence in 16S rRNA of *Campylobacter*	*Eco*RV, *Rsa*I	Romaniuk & Trust, (1987)
C. jejuni and *C. coli*	cDNA from 16S+23S rRNA from *E. coli*	*Hae*III	Hernandez & Owen, (1991)
Gram + eubacteria			
Bacillus species	Various cloned rDNA probes	*Eco*RI	Gottlieb & Rudner, (1985)
Mycobacterium strains	16S rRNA, 16S+23S rRNA and 23S+5S rRNA probes of the *E. coli* rrnB operon	*Pst*I	Bercovier *et al.*, (1986)
Mycobacterium fortuitum-cheloni	rRNA from Mycobacteria	*Eco*RI	Kanaujia *et al.*, (1991)

Streptococcus uberis, S. parauberis	cDNA from 16S+23S rRNA from *S. parauberis*	*Hind*III	Williams & Collins, (1991)
Staphlococcus aureus, S. epidermidis, S. capitis and others	16S+23S rRNA from *E. coli*	*Hind*III or *Eco*RI	Thomsom-Carter *et al.*, (1989)
Lactococcus lactis	16S+23S rDNA PCR'ed 500bp fragment from *L. lactis* and 2 oligonucleotides	*Eco*RI, *Hind*III	Köhler *et al.*, (1991)
Other microbial groups			
Candida albicans and other species	plasmid containing *Saccharomyces cerevisae* rDNA (17S+25S rRNA genes)	*Eco*RI	Magee *et al.*, (1987)
Cyanonacteria including species of *Acacystis, Anabaena* and *Nostoc*	rRNA	*Hind*III	Nichols *et al.*, (1982)

Stull *et al.* (1988) compared the hybridisation banding patterns of DNA from three genetically diverse groups of bacteria, *E. coli, Pseudomonas cepacia* and *Haemophilus influenza*, probed with *E. coli* 16S and 23S rRNA. The restriction endonucleases used were *Eco*RI, *Bam*HI, *Xho*I, *Kpn*I and *Hind*III but only digestion with *Eco*RI yielded a homogenous distribution of DNA fragment lengths for all 3 species and so was chosen for further probe analysis. Nine isolates of *E. coli* were probed and 8-13 hybridisation bands were detected for each isolate. Although the banding pattern of each isolate was unique, bands of 1.6kb, 2.4kb and 7.3kb were detectable in each of these isolates. When extended to *P. cepacia* and *H. influenzae* similar results were obtained, with 7 out of 8 *P. cepacia* and 9 out of 10 *H. influenzae* isolates distinguishable from each other but common bands being detected within most of the isolates of each species.

The stability of the DNA banding patterns was investigated by the analysis of three isolates of *P. cepacia* and three *E. coli* after non-selective serial subculturing. No change in the banding pattern was observed after 16 serial subcultures indicating the stability and broad spectrum suitability of *E. coli* rRNA as a useful tool for molecular epidemiology or "ribotyping" of bacterial infections.

The rRNA gene restriction patterns of 92 isolates of *H. influenzae* biogroup aegyptius were studied by Irino *et al.* (1988) with *E. coli* 16S and

23S rRNA as a probe, using the restriction enzymes *Eco*RI and *Eco*RV. All strains were classified into one of 15 patterns and most strains showed 6-8 strong bands and 1-5 weak bands. One of the patterns was associated with most of the cases of Brazilian purpuric fever and was therefore a good indicator of the causative agent in the disease.

Moureau *et al.* (1989) used a 23bp oligonucleotide complementary to the 3' end of the 16S rRNA of *Campylobacter jejuni* in combination with *Xho*I-*Bgl*II digests to identify species specific patterns comprised of 1-3 bands. This simple pattern allowed easy discrimination among the commonly found species of *Campylobacter* however it did not allow individual strains to be distinguished.

Regarding ribotyping of the lactic acid bacteria, the only known report to date is by Köhler *et al.* (1991) who differentiated *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis, E. faecalis, L. delbrueckii* subsp. *bulgaricus, L. delbrueckii* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar. *diacetylactis* using three rRNA based probes to hybridise to *Eco*RI or *Hind*III digested chromosomal DNA. The probes were a 16S rRNA targeted oligonucleotide specific for eubacteria, a 500bp PCR amplified 16S rDNA gene fragment of *L. lactis* subsp. *cremoris* and a site specific oligonucleotide probe for the intergenic spacer between 23S and 5S rRNA genes of *L. lactis*. The restriction patterns of the lactococci had no bands in common with *E. faecalis* and *L. delbrueckii*. For all lactococcal strains the presence of six rRNA operons was confirmed. It was not possible to distinguish all strains in one experiment using a single restriction endonuclease only, however by comparing the results of either two different hybridisation probes or restriction enzymes, all strains could be separated from one another.

The substantial set of data now available on rDNA banding patterns of chromosomal digests demonstrates that they provide a valuable new method of distinguishing between related species and most significantly, of detecting variation among strains within species. The approach is facilitated by the use of broad-spectrum probes and because patterns are amenable to computer analysis, it should be possible to generate a database to aid microbial

identification [Owen, 1989].

It is important that the rDNA patterns should be interpreted in conjunction with the total chromosomal digest patterns because in some circumstances the latter may provide a more sensitive measure of variation between closely related strains at the subspecies level. The sensitivity of the method is dependent on the restriction enzymes used; Altwegg *et al.* (1989) attempted to type *Salmonella typhi* strains using rDNA patterns and found *Pst*I the most discriminatory restriction endonuclease while Martinetti and Altwegg (1990) found that of 14 different enzymes, only *Sma*I and *Sph*I yielded different rDNA patterns for *Salmonella* strains. Differences were detected in high molecular weight bands and by restriction enzymes which generated large DNA fragments. Despite this, they concluded that in their evaluation of DNA restriction patterns, rDNA patterns and plasmid analysis, rDNA patterns provided the most sensitive method for strain discrimination.

With the advent of DNA probe technology and especially rRNA sequencing, genus, species and strain specific probes enable the researcher to evaluate the efficacy of lactic acid bacterial inoculants in natural fermentations without the need to develop selective media or incorporate target sequences into the microbe of interest. Since the lactic acid bacteria are at present involved in a wide variety of fermentations with the future presenting exciting new possibilities using genetically modified strains, effective tracing methods are essential.

Genus specific probes based on rRNA sequences could be used to detect spoilage by the LAB in certain alcoholic beverages, meat products and refined sugar. Microbial identification of the LAB could become more rapid and reliable using species specific probes based on the more variable regions of the rRNA which in combination with a non-radioactive signal, could be commercially viable. Strain specific probes based on rRNA have not as yet been described and there is unlikely to be a large enough difference in rRNA sequences to allow such a probe to be developed, however the presence of stable natural plasmids in the LAB can be exploited towards the same end. Plasmid based probes can be used to evaluate the competitive ability and

dominating potential of a strain as part of a selection procedure to identify suitable inoculants.

Chapter 2
Materials and Methods

2.1 **Materials**

Except where otherwise stated, all chemicals were obtained from Sigma Corporation, BDH and Riedel de Haen and were of AnalaR grade. All enzymes were from BRL and were used according to manufacturers instructions.

2.2 **List of Strains**

Strain	Relevant phenotype/ genotype	Source or Reference
E. coli		
JA221	F' *rec*A1, leuB6, trpΔ5, *hsdMT, hsdMT, hsdR*⁻, *lac*Y, *xyl*	Clark & Carbon, (1981).
JM83	ara,Δlac-pro,Sm^r,thi, ϕ80d*lac*ZΔM15	Viera & Messing, (1982).
TG1	SupE,hstΔ5,thiΔ(lac-proAB) F'[tra D36 proAB$^+$ LacI^Q *Lac*ZΔϕM15]	
L. plantarum		
DCU101	contains p101a	Irish silage isolate
LP115		Commercial inoculant
A1-A33 incl.		Irish silage isolate
AF1-AF3 incl.		"
B1-B24 incl.		"
1-1-49		"
1-2-5		"
1-2-7		"
1-3-3		"
1-3-12		"
1-3-13		"
1-3-33		"

2-1-1		Irish silage isolate
2-1-3		"
2-1-8		"
2-1-9		"
4-1-1		"
4-1-2		"
4-1-3		"
4-1-4		"
OP1.18		"
OP2.5		"
OP2.17		"
OP2.23		"
OP2.24		"
LP80		Scheirlinck *et al.*, (1990).
LMG10755		Dellaglio, F., Italy.
NCDO type L		
Lc. lactis		
CH919	tn919, Tc^r	Fitzgerald, G., UCC.
P. acidilactici		
G24		Irish silage isolate
pll04	pediocin producer	Texel, France.
pll03		Texel, France.
pll08		Texel, France.
PAC1.0	pediocin producer	Dellaglio, F., Italy.
Pediococcus pentosaceus		
FBB61		Dellaglio, F., Italy.

Lactobacillus reuteri 20016	Dellaglio, F., Italy.
Lactobacillus helveticus	DCU stocks
Lactobacillus hilgardii	DCU stocks
Lactobacillus delbrueckii	DCU stocks
Lactobacillus amylovorus NRRLB 4540	Nakamura, (1981)
Lactobacillus amylophilus NCIB 11546	Nakamura & Crowell, (1979)
Bacillus cereus	DCU stocks
Cellulomonas flavigena ATCC 482	Corcoran, B., DCU.
Enterococcus faecalis	DCU stocks
Saccharomyces cerevisiae DBY746	Duffy, M., DCU.
Rhizobium leguminosarum biovar ***vicae***	Reigh, G., DCU.
Rhizobium meliloti	Reigh, G., DCU.

2.3 List of Plasmids

Plasmid	Marker	Source or Reference
pUC19	Amp^r,*lacZ*	Norrander, J. *et al.*, 1983.
p101a	-------	Brophy, G., 1990.
pGB100	Amp^r,Tc^r	Brophy, G., 1990.
pAT153	Amp^r,Tc^r	Twigg, A.J. & Sherrat, D., 1980.
pKK3535	Amp^r	Brosius, J. *et al.*, 1981.

2.4 **Microbiological Media**

All media components were obtained from Oxoid except where otherwise stated. Medium was solidified with 1.2% (w/w) Agar Technical No. 3 and autoclaved at 121°C for 20 minutes with the exception of LP and VRBA.

Luria Bertani (LB) medium (Maniatis *et al.*, 1982)
This medium was used for the routine culturing of *E. coli*.

Tryptone	10.0g
Yeast extract	5.0g
NaCl	10.0g
H_2O	1.0L

MRS medium (de Man *et al.*, 1960)
This medium was used for the routine culturing of lactic acid bacteria and prepared according to the manufacturers instructions (52g/L).

LP medium (Brophy, G., 1989)
LP medium was used for the selective growth of *Lactobacillus plantarum*.

Bacteriological peptone	10.0g
yeast extract	5.0g
KH_2PO_4	6.0g
sodium acetate	2.5g
diammonium citrate	2.0g
Tween 80	1.0g
$MgSO_4.7H_2$	0.2g
$MnSO_4.4H_2O$	0.1g
sorbitol	20.0g

Dissolved in 1 litre of distilled water, the pH was adjusted to 5.5 with glacial acetic acid, 1.2% Agar technical no. 3 was added and the medium was autoclaved at **110°C** for 20 minutes. When used for the identification of lactobacilli, the medium was prepared from its constitutive parts with the appropriate sugar replacing sorbitol.

When used for silage samples, 1% (v/v) of Amphotericin B (Fungizone, GIBCO) was added to inhibit fungal growth.

All 5 Carbon sugars were filter sterilised while the rest were autoclaved.

Sugar	Description	Subunits	Working Concentration
trehalose	disaccharide	2 glc. residues	10mg/ml
sucrose	disaccharide	glc. and fruct.	11mg/ml
sorbitol	6 carbon sugar		20mg/ml
lactose	disaccharide	gal. and glc.	10mg/ml
galactose	6 carbon sugar		20mg/ml
maltose	disaccharide	2 glc. residues	10mg/ml
ribose	5 carbon sugar		24mg/ml
rhamnose	6 carbon sugar		20mg/ml
raffinose	trisaccharide		20mg/ml
mannitol	6 carbon sugar		20mg/ml
xylose	5 carbon sugar		24mg/ml
cellobiose	disaccharide		10mg/ml
melibiose	galactopyranosyl -D-glc.	2 glc. residues	10mg/ml

Violet Red Bile Agar (VRBA) was used for the selection of coliforms from silage and was prepared by boiling 38.5g/L. Thin plates were poured as an overlay of VRBA is required prior to incubation at 37°C for 16 hours.

Plate Count Agar (PCA) and Nutrient agar were prepared according to the manufacturers instructions.

Modified GM17 medium

This medium was used for the growth of *Lactococcus lactis* and *Enterococcus faecalis* and was prepared as follows.

Phytone peptone	5g
Poly peptone	5g
yeast extract	2.5g
brain heart infusion	5g
ascorbic acid	0.5g
glucose	5g
ß-disodium glycerophosphate	19g
1M $MgSO_4$	1ml
H_2O	1 litre

M9 medium (Maniatis *et al.*, 1982)

This minimal medium was used for storage of the *E. coli* strains, JM83 and TG1 and was solidified using 1.5% Bacto agar (Difco).

Na_2HPO_4	6.0g
KH_2PO_4	3.0g
NaCl	0.1g
NH_4Cl	1.0g
H_2O to	1.0L

After autoclaving the following and the appropriate amino acids (depending on the strain) were added to 1 litre;

1M $MgSO_4$	2.0ml
20% Glucose	10.0ml
1M $CaCl_2$	0.1ml

SOC medium (for electroporation of *E. coli* TG1)

tryptone	2% (w/v)
yeast extract	0.5% (w/v)
NaCl	10mM
KCl	2.5mM
$MgCl_2$	10mM
$MgSO_4$	10mM
glucose	20mM

2.5 **Antibiotics**

All antibiotics used were purchased from Sigma and stock solutions were stored at -20°C. Stock solutions of ampicillin (25mg/ml) were used with *E. coli* at a working concentration of 50μg/ml for solid media and 35μg/ml for liquid media. Tetracycline stock solution (10mg/ml) was prepared in 50% ethanol, stored in the dark and used with *E. coli* at a concentration of 10μg/ml. Streptomycin sulphate, was prepared in sterile H_2O at a concentration of 20mg/ml and used at a working concentration of 20μg/ml with *E. coli*.

2.6 **Growth conditions and storage of strains**

Lactobacillus and *Pediococcus* strains were routinely grown static in MRS at 37°C for 24h unless otherwise specified and were stored for up to 2 weeks at 4°C on agar plates. Due to the lactic acid becoming toxic to the cells, storage for any longer period at this temperature is not recommended. Long term storage was in 50% glycerol in 4ml volumes at -20°C. Silage samples were incubated at 30°C for 48h on LP medium or MRS for the initial selection period to ensure accurate cell counts and adequate growth conditions for all types of lactic acid bacteria. *Lactococcus lactis* CH919 was grown on modified GM17 medium with 10μg/ml of tetracycline at 30°C for 48 hours.

E. coli strains were routinely grown shaking in LB at 37°C. Long term storage of *E. coli* strains was in 50% glycerol at -20°C. *E. coli* TG1 cells were kept on M9 minimal medium to prevent the loss of the *lacZ* phenotype.

Bacillus cereus was grown at 30°C on nutrient agar.

2.7 **Buffers and Solutions**

All the solutions used for DNA manipulation were prepared in distilled water, autoclaved at 121°C for 20 minutes and stored at room temperature except where stated.

TE buffer (10:1)

Tris-HCL	10mM
Na_2-EDTA	1mM
pH	8.0

TE buffer (50:1)

Tris-HCL	50mM
Na_2-EDTA	1mM
pH	8.0

0.5 M EDTA (pH 8.0)

Disodium ethylene diamine tetraacetate.$2H_2O$ (181.6g) was added to 800ml of H_2O and stirred vigorously. The pH was adjusted to 8.0 with NaOH and the volume made up to 1 litre with distilled water.

Solutions involved in electrophoresis

10x Tris Borate Electrophoresis buffer (TBE)

Tris-HCL	108.0g
Na_2-EDTA	9.3g
boric acid	55.0
H_2O to	1 litre
pH	8.3

50x Tris Acetate Electrophoresis buffer (TAE)

Tris-HCL	242.0g
glacial acetic acid	57.1ml
0.5M Na_2-EDTA (pH 8.0)	100.0ml
H_2O to	1 litre
pH	8.0

10x MOPS (stored in the dark)

To 800ml of DEPC treated H_2O, 41.8g of 3-[N-Morpholino] propanesulfonic acid was added and the pH adjusted to 7.0 with NaOH or acetic acid. 16.6ml of 3M DEPC treated sodium acetate and 20ml of 0.5M DEPC treated EDTA (pH 8.0) were then added and the solution was brought up to a volume of 1 litre with DEPC treated water and filter sterilised. The buffer yellows with age if autoclaved or exposed to the light. Straw coloured buffer works well but darker buffer does not.

6x Bromophenol blue (stored at 4°C)

bromophenol blue	0.25% (w/v)
sucrose in H_2O	40.% (w/v)

DNA samples were mixed in a 5:1 ratio with bromophenol blue prior to loading on agarose gels.

6x Formaldehyde loading buffer (stored at 4°C)

Na_2-EDTA pH 8.0	1.0mM
bromophenol blue	0.25% (w/v)
xylene cyanol	0.25% (w/v)
glycerol	50.0 % (v/v)

Solutions involved in blotting and hybridization

20x SSC

NaCl	175.3g
sodium citrate	88.2g

Dissolved in 800ml of distilled water, the pH was adjusted to 7.0 and the volume brought up to 1 litre.

20x SSPE

NaCl	174.0g
$NaH_2PO_4.H_2O$	27.6g
Na_2-EDTA	7.4g

Dissolved in 800ml of distilled water, the pH was adjusted to 7.4 with NaOH and the volume adjusted to 1 litre.

Denaturing solution (1.5M NaCl, O.5M NaOH)

NaCl	87.66g
NaOH	20.0 g
H_2O to	1.0 litre

Neutralising solution (1M Tris-HCl, 1.5M NaCl)

Tris-HCL	121.1g
NaCl	87.66g

Dissolved in 800ml of distilled water, the pH was adjusted to 8.0 and the volume adjusted to 1 litre.

50x Denhardts solution (stored at -20°C)

Ficoll	5.0g
Polyvinylpyrrolidone	5.0g
Bovine Serum Albumin	5.0g
H_2O	500ml

Prehybridisation solution (for 64°C hybridisation)

5x SSC

0.5% (w/v) Sodium dodecyl sulphate (SDS)

5x Denhardts solution

100μg/ml denatured salmon sperm or calf thymus DNA

Prehybridisation solution (for 68°C hybridisation)

6x SSC

0.5% (w/v) SDS

5x Denhardts solution

100μg/ml denatured calf thymus or salmon sperm DNA

Prewashing solution (for colony hybridisation)

50mM Tris-HCL (pH 8.0)

1M NaCl

1mM EDTA

0.1% (w/v) SDS

Prehybridisation solution (for 42°C colony hybridisation)

50% (v/v) formamide

5x Denhardts solution

5x SSPE

0.1% (w/v) SDS

100μg/ml denatured calf thymus or salmon sperm DNA

Prehybridisation solution (for northern hybridisation)

1M KPO_4, pH 7.4	12.5ml
20x SSC	125.0ml
50x Denhardts solution	50.0ml
5μg/ml denatured calf thymus or salmon sperm DNA	5.0ml
100% (v/v) formamide	250.0ml
H_2O	82.5ml
	500.0ml

This solution was stored at -20°C and prewarmed to 42°C before use.

Hybridisation solution

Hybridisation solution was as for prehybridisation solution with the addition of 0.01M EDTA, 10% (w/v) dextran sulphate and the denatured labelled probe.

Preparation of calf thymus/salmon sperm DNA (10mg/ml)

Salmon sperm or calf thymus DNA was used in hybridisations with radiolabelled probes and was prepared as follows; 500mg of calf thymus DNA (Sigma, type I) or salmon sperm DNA (Sigma, type IV) was dissolved in 50ml of distilled water for at least 4 hours. The DNA was sheared by passing it through a syringe with a microlance at the tip (21G, Becton Dickenson, Dublin), aliquoted and stored at -20°C. The DNA was denatured before use by placing it in a boiling water bath for 5min and then placing immediatedly on ice.

Solutions for the preparation of plasmid DNA from *E. coli.*

EIF solution (for use in the Eckhardt procedure)

8% (w/v) sucrose
2% (w/v) ficoll
50mM Tris
25mM Na_2EDTA
pH 8.4

This solution was boiled for 15-30 minutes in a water bath, aliquoted into 500μl quantities and stored at -20°C. Before use, 1mg of lysozyme and 10μl of RNase A (10mg/ml) were added to 500μl of EIF.

STET buffer (for lysis of *E. coli* transformants)

8% (w/v) sucrose

5% (v/v) Triton X 100

50mM Na_2EDTA

50mM Tris-HCL (pH 8.0)

Solution 1

0.5M glucose	1.0ml
0.1M Na_2EDTA	1.0ml
1.0M Tris-HCL (pH 8.0)	0.25ml
H_2O	7.75ml

Solution 2

1N NaOH	2.0ml
10% (w/v) SDS	1.0ml
H_2O	7.0ml

Solution 3

5M potassium acetate*	30.0ml
glacial acetic acid	5.75ml
H_2O	1.425ml
pH	4.8

***5M potassium acetate**

To 60ml of 5M potassium acetate, 11.5ml of glacial acetic acid and 28.5ml H_2O were added. This solution was 3 molar with respect to potassium and 5 molar with respect to acetate and was used in the preparation of Solution 3.

STE buffer (used in the mini-maxi procedure)

15% (w/v) sucrose

50mM Tris-HCl (pH 8.0)

50mM Na_2EDTA (pH 8.0)

TTE buffer

0.1% (v/v) Triton-X-100

50mM Tris-HCl (pH 8.0)

50mM Na_2EDTA (pH 8.0)

Triton mix (solution for large scale preparation of plasmid DNA from *E. coli.)*

20% (v/v) Triton-X-100	5.0ml
0.25M EDTA (pH 8.0)	12.5ml
1M Tris-HCL	2.5ml
H_2O to	50.0ml

Preparation of dialysis tubing

The dialysis tubing was boiled in 10mM Na_2EDTA for 10 minutes, washed in distilled water and boiled again for 10 minutes in distilled water. The boiled tubing can be stored at 4^0C and gloves must be worn when handling.

DEPC treatment

Diethylpyrocarbonate (toxic) was used to inhibit RNases in the preparation of solutions for RNA manipulation. DEPC (0.2% v/v) was added to the solution, left at room temperature for at least 20 minutes and then autoclaved to inactivate the DEPC. DEPC was not added directly to solutions containing Tris as it is highly unstable in these solutions decomposing rapidly into ethanol and carbon dioxide (Maniatis *et al.*, 1982). Gloves were worn at all times in the preparation of solutions for the manipulation of RNA to avoid the introduction of RNases.

Phenol/Chloroform (Kirby mix)

100g of AnalaR grade phenol was dissolved in 100ml chloroform. To this, 4ml of isoamylalcohol and 0.8g of 8-hydroxyquinoline were added and the mixture was stored under 100mM Tris-HCL, pH 7.5 in a dark bottle at 4°C.

Solutions used in ligations

10x STE/TNE (used in the CIP reaction)

100mM Tris-HCl (pH 8.0)

1M NaCl

10mM Na_2EDTA

10x Nick translation buffer/ Klenow reaction buffer

0.5M Tris-HCL (pH7.2)

0.1M $MgSO_4$

1mM dithiothreitol

500μg/ml BSA (fraction V, Sigma)

Aliquoted and stored at -20°C.

10x ligation buffer (for blunt ended ligations)

500mM Tris-HCL (pH 7.4)

100mM $MgCl_2$

10mM Spermidine

10mM ATP

150mM dithiothreitol

500μg/ml BSA

Aliquoted and stored at -20°C.

10x ligation buffer (for cohesive ended ligations)

200mM Tris-HCL (pH 7.6)

100mM $MgCl_2$

100mM dithiothreitol

6mM ATP

Aliquoted and stored at -20°C.

Dithiothreitol (1M DTT)

DTT (3.09g) was dissolved in 0.01M Sodium acetate (pH 5.2) and filter sterilised. Aliquoted and stored at -20°C.

3M Sodium acetate pH 5.2

Sodium acetate.$3H_2O$ (408.1g) was dissolved in 800ml H_2O, the pH adjusted to 5.2 with glacial acetic acid and the volume brought up to 1 litre.

ATP (0.1M)

ATP (60mg) was dissolved in 800μl of sterile H_2O and the pH adjusted to 7.0 with 0.1M NaOH. The volume was adjusted to 1ml with H_2O aliquoted and stored at -20°C.

10x PCR buffer

500mM KCl

100mM Tris-HCL (pH 8.3)

15mM $MgCl_2$

1% (w/v) gelatin

Aliquoted and stored at -20^{0}C.

2.8 Enzymes

DNAse free RNase

RNase A was dissolved at a concentration of 10mg/ml in Tris-HCl (pH 7.5) and 15mM NaCl, heated to 100°C for 15minutes, allowed to cool slowly to room temperature, dispensed into aliquots and stored at -20°C. RNase was used at a concentration of 500μg/ml.

Pronase

Pronase was prepared at a concentration of 20mg/ml in sterile H_2O, self digested at 37°C for 2 hours, aliquoted and stored at -20°C. Pronase was used

at a concentration of 1mg/ml in a reaction buffer of 0.01M Tris (pH 7.8), 0.01M EDTA, 0.5% (w/v) SDS at 37°C.

Proteinase K

Proteinase K was prepared at a concentration of 20mg/ml in sterile H_2O and stored at -20°C. No pre-treatment is required. Proteinase K was used at a concentration of 50μg/ml in a reaction buffer of 0.01M Tris (pH 7.8), 0.005M EDTA, 0.5% (w/v) SDS at 37°C.

2.9 **Electrophoresis and Visualisation of Nucleic Acids**

Electrophoresis of DNA

Agarose (0.7% w/v) in TAE was routinely used for the analysis of plasmid and chromosomal DNA. Mini gels were run at 100V in TAE buffer on a Horizon 58 or Pharmacia GNA-100 gel box system for approximately 1 hour, while total DNA digests were run at 40-50V on a Pharmacia GN-200 or BRL H4 gel box for 14-16 hours.

4% (w/v) Nusieve^R GTG (FMC) agarose in TAE or 2% (w/v) agarose in TAE were used to separate PCR products and primers, and were run at 75V in the Horizon 58 or Horizon 11.14 Midi gel systems for 2-4 hours. Nusieve^R GTG agarose is a low gelling (<30°C) and low melting (<65°C) temperature agarose. It finely resolves nucleic acid fragments less than 1000bp and can distinguish fragments as small as 8bp. At a given Nusieve GTG agarose concentration, DNA electrophoresed in gels in the presence of TBE buffer runs more slowly than DNA electrophoresed in TAE buffer. In 4% (w/v) gels using TAE buffer, bromophenol blue migrates similarly to a 70bp fragment, while in TBE buffer this dye migrates similarly to 30bp (FMS Bioproducts, Denmark).

Range of separation for agarose gels

Agarose % (w/v)	**Optimal range of separation of linear DNA (kb)**
0.3 (run at 4°C)	60 - 5.0
0.6 "	20 - 1.0
0.7	10 - 0.8
0.9	7.0 - 0.8
1.2	6.0 - 0.4
1.5	4.0 - 0.2
2.0	3.0 - 0.1

Electrophoresis of RNA

Commercially prepared *E. coli* 16S + 23S ribosomal RNA (Boehringer Mannheim) was electrophoresed using formaldehyde gels. All manipulations of agarose gels containing formaldehyde were carried out in the fume cupboard. Two minor advantages of using low formaldehyde agarose gels for RNA are that they take less time to run than glyoxal gels and their running buffers do not need to be recirculated. For a 1.2% (w/v) gel, 1.4g of agarose was boiled in 101.5ml DEPC treated H_2O and when cooled to 60°C, 11.7ml of 10x MOPS and 3.5ml of 37% formaldehyde were added. It is essential that the pH of the formaldehyde is greater than 4.0. The mini gel was poured and the gel box filled with 1x MOPS. The samples were prepared by the addition of the following to a microfuge tube;

10x MOPS	10μl
37% formaldehyde	17.5μl
formamide	50μl
rRNA (16S+23S)	22.5μl
	100μl

The sample was mixed well by vortexing, spun down and incubated at 55°C for 15 minutes. Twenty μl of formaldehyde loading buffer were added to the sample and mixed well. Five μl were loaded in each well, which corresponded to 4.5μg of rRNA. The gel was run at 100V for about 3 hours or until the tracker dye had migrated halfway down the gel. One of the lanes containing the rRNA was cut, stained in ethidium bromide and viewed on the UV transilluminator. Using this procedure, the RNA is fully denatured and its rate of migration through the gel is in linear proportion to the $\log_{10}$ of its molecular weight (Maniatis *et al.*, 1982). The gel to be transferred to nitrocellulose was washed several times in water to remove the formaldehyde and the remainder of the procedure was carried out on the bench. Gels containing formaldehyde are less rigid than nondenaturing agarose gels and care must be exercised when handling them (Maniatis *et al.*, 1982). **It is recommended not to stain the gel before transferring to nitrocellulose.** The electrophoresis buffer was discarded after each use as the gel overheated and did not remain denaturing when the buffer was used twice. There was no special cleaning procedure carried out on the electrophoresis tanks before use although it is recommended that they should be cleaned with a detergent solution, rinsed in water, dried with ethanol and then filled with a solution of 3% (v/v) H_2O_2. After 10 minutes at room temperature, the tank should be thoroughly rinsed with water treated with 0.1% (v/v) DEPC. This procedure was found to be unnecessary, perhaps due to the high quality of the commercial rRNA.

Visualisation of nucleic acids

All nucleic acid gels were stained in 1μg/ml ethidium bromide and visualised on a UV transillumunator. Photographs were taken on an Olympus OM-20 camera using T-max 100 film (Kodak) and an A003 red filter (Cokin, France) which blocks UV light. Vivitar close-up lenses were used when required.

Ethidium Bromide (10mg/ml)

One gram of ethidium bromide (warning, a mutagen) was added to 100ml H_2O and stirred for several hours to ensure that the dye was dissolved. The container was wrapped in tin foil and stored at 4°C. Gloves were worn at all times when handling EtBr containing solutions. EtBr waste was collected, treated with activated charcoal and filtered through 3MM Whatman filter paper. The clear liquid was disposed normally and the solids contained on the filter paper were incinerated.

2.10 Development of black and white photographs

All the materials were supplied by Kodak. In complete darkness, the film was loaded onto the spool of a small tank developer and 250ml of D76 developer were added. Air bubbles were removed by tapping a few times off the bench. After the required development time (temperature dependent), the developer was poured out, the tank rinsed once with tap water and 250ml Unifix (1/3 in water) were added. When completely fixed, usually after 10 minutes, the negatives were rinsed for at least 1 hour in tap water and then air dried.

The photographs were printed onto Kodak Kodabrome II RC F4 high contrast paper, developed in Dektol (1/10 in water), rinsed briefly in a weakly fixing water bath and fixed in Unifix (1/3) for 5 minutes. After rinsing thoroughly in tap water, the prints were dried.

2.11 Total DNA isolation from *Lactobacillus plantarum* and other species

One ml of a fresh overnight MRS culture was centrifuged at 13,000 rpm for 5 minutes, the supernatant removed and the pellet resuspended in 0.5ml of 6.7% (w/v) sucrose/TE (pH 8.0). To this 100μl of freshly prepared lysozyme (10-50 mg/ml in TE) were added and the suspension mixed briefly and incubated at 37°C for at least 10 minutes until lysis was apparent. Fifty μl of 20% (w/v) SDS were added, mixed and incubated for a further 10 minutes at 37°C until clearing was observed. The cell lysate was extracted twice with 0.5ml phenol/chloroform, centrifuging at 13,000 rpm for 10 minutes to remove proteins and each time the supernatant containing the DNA was

carefully removed from the protein layer and placed in a clean sterile microfuge tube. One chloroform/isoamylalcohol (24:1 v/v) extraction was performed to remove any residual phenol, the DNA was precipitated at room temperature for 30 minutes by the addition of an equal volume of isopropanol and then pelleted by centrifugation at 13,000rpm for 15 minutes. The pellet was washed twice with 70% (v/v) ethanol to remove salts, dessicated and resuspended in 100μl of sterile TE. This procedure regularly yielded clean DNA of which 17μl was used for restriction.

2.12 **Plasmid preparation from *Lactobacillus plantarum***

This method is essentially that of Anderson and M^c Kay (1983) with a few important points emphasised. **All the solutions were prewarmed to 37°C before use.**

One ml of a **fresh log phase** (after 5 hours growth) MRS broth culture was centrifuged at 13,000 rpm for 5 minutes, the supernatant removed and the pellet resuspended in 379μl of 6.7% (w/v) sucrose/TE (pH 8.0). To this, 96.5μl of **freshly prepared lysozyme** (10mg/ml in 25mM Tris, pH 8.0) were added, the microfuge vortexed briefly and incubated at 37°C for 5 minutes. This was followed by the addition of 48.2μl of 0.25M EDTA/50mM Tris (pH 8.0). To this, 27.6μl of 20% (w/v) SDS in 50mM Tris/20mM EDTA (pH 8.0) were added to each tube individually, vortexed for a few seconds **immediately after addition** and incubated at 37°C for 10 minutes to complete lysis. After 10 minutes, the suspension was clear or a little more SDS was added if this were not the case. The microfuge tubes were then vortexed at the highest setting for **exactly 30 seconds**, as any longer causes the chromosome and larger plasmids to break. To this, 27.6μl of **freshly prepared** (no older than 1 hour) 3N NaOH were added to each tube and mixed gently by inversion for 10 minutes. Following this, 49.6μl of 2M Tris-HCL (pH 7.0) were added and the inversion continued for a further 3 minutes. 71.7μl of 5M NaCl were then added and the tubes were incubated at 4°C for 30 minutes to aid protein precipitation. The protein was pelleted by centrifugation at 13,000rpm for 5 minutes and the supernatant was removed to a clean microfuge tube. Seven

hundred μl of **phenol saturated with 3% NaCl** was added, mixed thoroughly and centrifuged at 13,000 rpm for 10 minutes. The clear supernatant was removed and extracted with 700μl of chloroform/isoamylalcohol (24:1) to remove any residual phenol and centrifuged at 13,000 rpm for 5 minutes. The upper phase was removed and the plasmid DNA was precipitated with an equal volume of isopropanol and chilled at -20°C overnight.

The DNA was pelleted at 13,000 rpm for 20 minutes, washed twice with 70% (v/v) ethanol, dried and resupended in **20μl** of sterile water. All of this was used to load either mini or maxi horizontal agarose gels.

2.13 Manipulation and ligation of vector and insert DNA

Klenow reaction

The Klenow fragment of the enzyme DNA polymerase I catalyses the addition of nucleotide residues to the 3'-OH terminus of nicked or restricted double stranded DNA where there is a single stranded template. This property was used to repair the ends of PCR products as is described in section 2.23.5 and in the random priming labelling reaction, section 2.20.1.

CIP treatment

The enzyme calf intestine alkaline phosphatase catalyses the removal of 5'-phosphate residues from single or double stranded DNA and RNA and was used to treat vector DNA to prevent self re-ligation and to dephosphorylate the rRNA prior to end labelling with [γ ^{32}P] ATP. To treat vector DNA, 0.5μl of CIP enzyme (0.5 units) was added to 12μl of vector DNA and incubated at 37°C for 20 minutes. To stop the reaction the following were added and incubated at 65°C for 10 minutes;

10x STE (TNE)	10μl
10mM Na_2EDTA	10μl
20% SDS	2.5μl
TE	100μl
H_2O	60μl

To dephosphorylate the rRNA before labelling, the procedure outlined in section 2.20.2 was used.

T4 polynucleotide kinase reaction

The T4 PNK enzyme catalyses the transfer of the γ-phosphate of ATP to a 5'-OH terminus in single or double stranded DNA or RNA. This enzyme was used in the radiolabelling of rRNA as described in section 2.20.2 and in the repair of the PCR generated fragments prior to ligation.

Ligation reaction

The enzyme T4 DNA ligase catalyses the formation of a phosphodiester bond between adjacent 3'-OH and 5'-phosphate termini in double stranded DNA. This enzyme was used routinely in the ligation of cohesive and blunt ended fragments to vector DNA. The buffers for cohesive and blunt ended ligations differ from each other and are described in section 2.7. Ligation reactions were carried out overnight at 16°C and were set up as follows;

insert DNA in H_2O	15.4μl
vector DNA	1μl
10x ligation buffer	2μl
0.5m DTT	0.6μl
T4 DNA ligase	1μl
	20μl

Ligation of the PCR amplified fragments to pUC19 used a combination of the T4 PNK, Klenow and ligation reaction and is described in section 2.27.

2.14 Transformation of *E. coli*

2.14.1 Preparation of Competent *E. coli* JM83 cells for transformation.

Using an overnight broth culture, 100mls of LB were inoculated at 1% (v/v) and incubated in a shaking water bath at 37°C until the A_{600nm} was between 0.3-0.4. The cells were then placed on ice for one hour. Twelve mls of culture were spun in a cold centrifuge at 4°C, 5000 rpm for 5 minutes. The cells, placed immediately on ice, were resuspended in 6ml of cold 0.1M $MgCl_2$ and centrifuged again at 5000 rpm for 5 minutes. The pellet was resupended in 6 ml of ice-cold 50mM $CaCl_2$ and left on ice for 30 minutes. This was followed by another centrifugation step at 5000 rpm for 5 minutes. The final pellet was resuspended in 1.2ml of 50mM $CaCl_2$. The cells were stored on ice and were used for transformations up to 16 hours later.

2.14.2 Transformation of Competent *E. coli* JM83 cells.

Two hundred μl of competent cells were added to the ligation/plasmid and left on ice for at least 1 hour. The cells were then heat shocked at 42°C for exactly 2 minutes and immediately returned to ice. 0.7ml of LB without the selection antibiotic were added and the microfuge tubes incubated at 37°C for 45 minutes to allow the cells to recover. 200μl (per plate) of the cells were spread onto the selective media and incubated at 37°C for 24 hours. The transformants were picked off, purified and plasmid DNA was isolated using one of the methods described in section 2.15. pUC19 based transformations were carried out in JM83 and TG1 to exploit the *lac*Z phenotype. Selection of the transformants included inoculation onto ampicillin plates containing 5-bromo-4-chloro-3-indolyl ß-D-galactopyranoside prepared in dimethylformamide (20μl per plate). Forty μl of 0.1M IPTG (isopropyl-ß-D-thiogalactopyranoside) was also spread on selection plates when used with TG1. 1M IPTG was prepared by dissolving 2.38g in 100ml sterile water. Colonies with inserts in the plasmid turned white while those with inserts remained blue.

2.14.3 **Preparation of electrocompetent *E. coli* TG1 cells.**

Electroporation became the routine method of introducing DNA into competent *E. coli* TG1 cells towards the end of the project, due to superior transformation efficiencies and these were prepared as follows; One litre of LB was inoculated with 1% (v/v) of overnight *E. coli* culture and shaken vigorously at 37°C to an A_{600nm} of 0.5-0.8. The flask was chilled on ice for 15-30 minutes and the cells were centrifuged at 4000x g_{max}, 4°C for 15 minutes. The cell pellets were resuspended slowly, in a shaking ice water bath, in 1 litre of ice cold distilled water. The suspension was centrifuged as before and the pellets were resuspended as before in 0.5 litres of ice cold distilled water. Following resupension, the cells were centrifuged as before and resuspended in 20ml of 10% (v/v) ice cold glycerol. Once again, the cells were centrifuged and resuspended finally in 2-3mls of 10% (v/v) glycerol. The competent cells were dispensed into 100μl aliquots and stored at -80°C for up to 6 months.

2.14.4 **Electroporation of *E. coli* TG1 cells**

Electroporation of electrocompetent cells was carried out as follows; The apparatus used was a BioRad Gene pulser™, set at 25μF capacitance, 2.5kV and 200 Ohms to deliver a single exponentially decaying pulse. The competent cells were thawed on ice and 1-5μl of plasmid DNA was added. The DNA must be cleaned of excess protein and salts to minimize arcing. The mixture of cells and DNA was transferred to an ice cold 0.2mm electroporation cuvette, dried and tapped sharply off the bench to dislodge bubbles which can cause arcing. The cuvette was placed into the sliding holder and the pulse applied by pressing the two red buttons simultaneously. Immediatedly following the pulse, 1ml of SOC medium (at room temperature) was added to the cuvette and the cell suspension placed at 37°C in a microfuge tube. Following the appropriate recovery time (antibiotic dependent), dilutions of the cells were plated onto selective medium and incubated at 37°C for 24 hours. Transformation efficiencies of up to 10^9 cfu/μg DNA were obtained using this method.

2.15 Small scale plasmid preparation from *E. coli*

2.15.1 **STET prep.** (described by Holmes and Quigley, 1981)

An overnight broth culture or a toothpick colony was resuspended in 300μl of STET buffer. To this, 20μl of 10mg/ml lysozyme in STET buffer was added and the tubes were left at room temperature for 10 minutes. The samples were then denatured in a boiling water bath for exactly 60 seconds and were spun immediately at 13,000 rpm for 10 minutes. The supernatant was removed and precipitated with an equal volume of isopropanol. After incubation at -20°C for 20 minutes, the samples were spun at 13,000 rpm for 5 minutes. The resulting pellet of plasmid DNA was washed in ether, dessicated and resupended in 50μl of TE. The addition of RNase (500μg/ml) was included in the restriction of the plasmid.

2.15.2 **Solution 1,2,3 prep.** (described by Birnboim and Doly, 1979)

One to 1.5 ml of an overnight broth culture was pelleted at 13,000 rpm and resuspended by vortexing in 100μl of Solution 1 (section 2.7) and incubated at room temperature for 5 minutes. Two hundred μl of Solution 2 were added and the microfuge tubes were left on ice for a further 5 minutes. To this, 150μl of Solution 3 were added, the samples were left on ice for 10 minutes and then centrifuged at 13,000 rpm for 5 minutes. The supernatant was removed and 400μl of Kirby mix added. After thorough mixing, the samples were spun at 13,000 rpm, the aqueous layer removed and precipitated with 0.8ml ice cold ethanol. Following 10 minutes at room temperature, the plasmid DNA was pelleted by centrifugation at 13,000 rpm. The pellet was washed twice in 70% (v/v) ethanol, dessicated and resuspended in 50μl of TE.

2.15.3 **Mini-maxi prep.** (described by Noguchi, 1991)

This plasmid isolation procedure yields very clean DNA which is suitable for sequencing.

Fifty ml of an overnight culture was centrifuged at 3,000 rpm, 4°C for 10 minutes and the pellet was resuspended in 2 ml of ice cold TE (50:1). The

microfuge tubes were spun for 1 minute at 13,000 rpm and the pellet resuspended in 300μl of ice cold STE buffer. One hundred μl of 5mg/ml lysozyme/STE were added and the tubes left on ice for 10 minutes after which 370μl of ice cold TTE buffer were added. After a further 10 minutes on ice, 2μl of DEPC were added to the tubes which were then boiled for 45 seconds. Following boiling, the tubes were centrifuged for 15 minutes and the pellets were removed with a toothpick. One ml of ethanol was added and the tubes were spun for 3 minutes. The supernatant was removed and the pellet was air dried for 10 minutes. The pellets were pooled by resuspension in a total of 700μl sterile water. One μl of RNase A (50mg/ml) was added to the resuspended DNA and incubated at 37°C for 30 minutes, followed by 1μl of proteinase K (10mg/ml) which was incubated at 50°C for 15 minutes. NaCl was added to a final concentration of 0.2M and the plasmid was extracted with phenol/chloroform twice and chloroform/isoamylalcohol (24:1 v/v) once. The supernatant was precipitated with 800μl of cold ethanol, centrifuged at 13,000 rpm for 5 minutes, washed twice in 70% (v/v) ethanol, dessicated and resuspended in 50μl of TE (10:1).

2.16 **Large scale plasmid preparation from *E. coli***

Two hundred ml of an overnight LB culture was centrifuged at 5000 rpm, 10 min and the pellet resusupended in 2ml of 25% (w/v) sucrose/50mM Tris-HCl (pH 8.0). The suspension was transferred to 50 Ti plastic screw capped tubes on ice and 0.4ml of lysozyme (20mg/ml in 0.25M Tris) was added and incubated at 37°C for 5 minutes. 0.8ml of 0.25M EDTA (pH 8.0) was added and incubated for a further 10 minutes. To this, 3.2ml of Triton-mix was added and incubated for 15 minutes. The cell lysates were centrifuged in the 50 Ti rotor at 40,000 rpm at 4°C for 40 minutes. 6.9g of Cesium chloride were weighed into a sterile universal, the cleared lysate added and mixed gently. The solution was added to an quick seal polyallomer ultracentrifuge tube (Beckman) and 0.18ml ethidium bromide (10mg/ml) was added. The total solution weight was increased to 14.1g using 10mM EDTA (pH 8.0). Air was removed from the tubes which were filled with mineral oil and sealed with a

Beckman heat sealer. The density gradient was formed by centrifugation at 50,000 rpm, 18°C in the 70.1 Ti rotor (Beckman Ultracentrifuge model LH-M). The plasmid band was visualised by UV light and extracted after the 22-24 hour centrifugation. Extraction involved piercing the tube at the top and under the plasmid band with a lance and withdrawing the band into a syringe. The plasmid DNA was extracted a few times with isopropanol saturated with 20x SSC to remove the ethidium bromide. This was followed by dialysis against several changes of TE buffer to remove the CsCl and other contaminating salts. The DNA was ethanol precipitated where necessary by the addition of 4 volumes of TE, 2.5x final volume of ethanol and incubation at -20°C overnight. The DNA was pelleted at 15,000 rpm, 4°C for 20 minutes, washed in 70% (v/v) ethanol, dessicated and resuspended in 400μl TE.

2.17 **Non preparative plasmid isolation procedure for *E. coli* (Eckhardt).** This is an "in gel" lysis procedure resulting in the separation of plasmids or cosmids via electrophoresis from the chromosomal DNA, which are then viewed directly following staining with ethidium bromide. This procedure does not result in the isolation of plasmids or cosmids, however the Eckhardt gels may be blotted and hybridised successfully using the usual procedures. This procedure was carried out using the maxi gel apparatus as there was very little success with mini gels.

An agarose gel (0.8% w/v) was prepared in 1x TAE and to each 120ml, 1ml of 10% (w/v) SDS was added after boiling. A toothpick colony from a fresh overnight culture was resuspended in 10μl of TE on the surface of an empty petri dish. Fifteen μl of EIF solution (with lysozyme and RNase A) were added to the suspension, mixed quickly and immediately loaded onto the 0.8% agarose/TAE/SDS gel. The gel was run at 15V for 15 minutes (or until the wells were clear) and then the voltage was increased to 100V. Five μl of bromophenol blue were loaded in an empty lane as a marker and the gel was allowed to run until the bromophenol blue reached 3/4 of the length of the gel.

2.18.1 **Southern Blot**

This procedure described by Southern (1975) results in the transfer of electrophoresed DNA from an agarose gel to a nitrocellulose filter thereby allowing the long term storage of the DNA and its subsequent analysis using hybridisation to a labelled probe. After electrophoresis, the gel was visualised and photographed. When the fragments of interest were larger than 10kb, the gel was placed in 0.2N HCL and gently shaken for 10 minutes or until the bromophenol blue turned yellow. The gel was then washed 3 times in distilled water and Denaturing solution (section 2.7) added. The gel was gently rocked for 1 hour and then the Denaturing solution was replaced with Neutralising solution and rocked for another hour. At this stage the pH of the gel was below 8.5. The transfer took place overnight using either a straightforward Southern or the bidirectional procedure, depending on the concentration of DNA on the gel.

For the standard Southern blot, a piece of nitrocellulose slightly larger than the gel was floated on the surface of 2x SSC for 5 minutes and then submerged for a further five minutes. This ensures even wetting of the nitrocellulose, eliminating air bubbles. The gel was placed on a 3MM Whatman filter paper wick, the ends of which were in contact with 20x SSC. The wet nitrocellulose was laid carefully on the gel and the air bubbles removed by rolling a glass test tube over the surface. Two pieces of filter paper soaked in 2x SSC were placed on top of the nitrocellulose and the stack built up with paper towels. A weight was placed on top to aid the transfer. Parafilm was used to prevent the towels coming in contact with the gel or 20x SSC, thereby preventing the liquid bypassing the nitrocellulose.

The bidirectional blotting procedure was essentially the same except the gel was sandwiched between two nitrocellulose filters and four 3MM Whatman filter papers soaked in 20x SSC with towels on either side. There was no need for a wick or reservoir of 20x SSC in this method. The structure was inverted a few times to ensure even transfer to each nitrocellulose filter. Hybridisation to each of these filters yielded a positive result with no apparent difference in signal strength.

After the overnight transfer of DNA to the nitrocellulose with either procedure, the towels and filter paper were removed and the position of the wells were marked on the nitrocellulose. They were then washed in 6x SSC to remove any fragments of agarose, air dried for one hour and baked between two pieces of filter paper for 2 hours at 80°C.

2.18.2 **Northern Blot**

This procedure was used for the transfer of rRNA to nitrocellulose and differed slightly to the Southern Blot procedure for DNA transfer. Electrophoresis was carried out as described in section 2.9. Electrophoresis and transfer to nitrocellulose were carried out sequentially as there is no point at which the procedure can be left. Neither DBM or Nylon filters were recommended for this protocol. Both denatured and native RNA molecules are capable of binding to nitrocellulose filters, however, in order to transfer efficiently from agarose, the RNA must be denatured. Ribosomal RNA bands should be sharp. If the RNA is known to be of high quality but the stained section of the gel doesn't show sharp ribosomal bands, the most likely explanations are that either the gel solutions were not mixed adequately or that the pH of the formaldehyde was too low.

Following electrophoresis, the gel was washed several times in water and then washed in 10x SSC with agitation for 45 minutes. If the gel were $>1\%$ (w/v) agarose or more than 0.5cm thick or if the RNA to be analysed were >2.5kb in length, the gel was soaked in 0.05N NaOH for 20 minutes. This partially hydrolyses the RNA and improves transfer efficiency. The nitrocellulose was placed in water for 1 minute and then in 20x SSC for 5-10 minutes. The gel was blotted either one way or bidirectionally as described for Southern blotting. The following day, the wells were marked and the filters were baked at 80°C for 2 hours. The blots were stored dry at room temperature.

2.18.3 **Colony Blot of *E. coli*** (Maniatis *et al.*, 1982).

Transformants can be screened for correct inserts without the need to isolate plasmid DNA if the colonies are transferred to nitrocellulose and hybridised with a labelled probe specific for the insert.

Nitrocellulose was placed on the medium and the colonies were transferred to the nitrocellulose with sterile toothpicks. Lysis was carried out by placing the nitrocellulose sequentially onto 3MM Whatman filter paper soaked with the following solutions; 10% (w/v) SDS for 3 minutes; Denaturing solution for 5 minutes; Neutralizing solution for 5 minutes and 2x SSPE for 5 minutes. The filters were then allowed to air dry for 1 hour and were baked between 2 pieces of filter paper at 80°C for 2 hours.

2.18.4 **Colony Blot of *Lactobacillus plantarum*.**

The colonies were placed on the nitrocellulose and incubated at 30°C for 48 hours. Lysis was effected by placing the nitrocellulose sequentially onto 3MM Whatman filter paper soaked with the following solutions; 50mM Tris (pH 8.0) for 5 minutes; 10mg/ml lysozyme in 6.7% (w/v) sucrose/TE (pH 8.0) for 3 hours at 37°C; 10% (w/v) SDS for 10 minutes; 0.5M NaOH, 1.5M NaCl for 5 minutes; 0.5M Tris (pH 8.0), 1.5M NaCl for 5 minutes and then 2x SSPE for 5 minutes. The filters were then allowed to air dry for 1 hour and baked between two pieces of filter paper at 80°C for 2 hours.

2.18.5 **Dot Blot**

2μg of DNA, heat denatured by boiling for 10 minutes and rapidly cooled on ice was placed using a finntip onto nitrocellulose filters and allowed to dry. The filters were washed in 6x SSC and dried at room temperature for one hour. The filters were baked at 80°C for 2 hours to fix the DNA prior to storage or hybridisation.

2.19 **Spin columns**

Column cleaning was used in the preparation of DNA for sequencing reactions, in the preparation of DNA probes and cleaning ligations prior to electroporation.

Fifty μl of glass beads (40 mesh for GLC, BDH) were placed at the bottom of a 0.5ml microfuge tube which had been pierced with a hot lance. To this, 0.5ml of 70% (v/v) sepharose CL-6B were added and the column was equilibrated 3 times with sterile distilled water. To do this, a volume of water equal to the sample volume was added to the column and centrifuged at 1,500 rpm for exactly 2 minutes into an empty microfuge tube. Once the column was equilibrated and eluting the exact sample volume, the sample was added and the column spun at 1,500 for 2 minutes into a clean sterile microfuge tube.

2.20 **Preparation of radiolabelled probes and hybridisation conditions.**

DNA and rRNA probes were prepared using one of the following procedures;

2.20.1 Random Primer Labelling (Promega Kit)

The DNA to be labelled (at least 25ng) was linearised and denatured at 95-100°C for 5 minutes. The tube was placed immediately on ice. In a separate microfuge tube the following were added:

5x Buffer	10μl
mixed dNTP's (1.5mM each)	2μl
25ng denatured template	31μl
1mg/ml BSA	2μl
[α-^{32}P] dATP (3000Ci/mMol)	4μl
Klenow enzyme (5u/μl)	1μl
	50μl

This was incubated at room temperature for at least 1 hour. Incubation for longer periods resulted in increased specific activity of the probe.

Random Primer labelling (BRL Kit)

The DNA (at least 25ng) was linearised and denatured for 5 minutes in a boiling water bath and immediately cooled on ice. In a separate microfuge tube, the following were added on ice;

dCTP (0.5mM)	2μl
dGTP (0.5mM)	2μl
dTTP (0.5mM)	2μl
denatured template (25ng)	24μl
Random Primers Buffer	15μl
[α-^{32}P] dATP (3000Ci/mMol)	4μl
Klenow enzyme (3u/μl)	1μl
	50μl

This was incubated at 25°C for at least 1 hour and once again, incubation for a longer period resulted in a higher specific activity of the probe.

2.20.2 5'End Labelling of rRNA (Boehringer Mannheim kit)

(1) Dephosphorylation:

The RNA used was 16S+23S ribosomal RNA from *E. coli* MRE 600 supplied by Boehringer Mannheim. The procedure was carried out following the manufacturer's instructions. The alkaline phosphatase enzyme was diluted 1:1 in buffer A and the following were added to a microfuge tube;

rRNA (4μg/μl)	1-10μl
buffer A	10μl
H_2O	60-69μl
diluted enzyme	1μl

After incubation at 37°C for 30 minutes, 20μl of DEPC treated 50mM EGTA (pH 8.0) were added and the tubes incubated at 65°C for 30 minutes. The tubes were cooled and 100μl of phenol saturated in buffer B (500mM Tris-HCl; 5mM EDTA pH 8.2 at 25°C) were added, the emulsion mixed and the

tubes were centrifuged for 1 minute at 13,000 rpm. The aqueous layer was removed and placed into a clean microfuge tube while 1μl of buffer B and 40μl of DEPC treated H_2O were added to the phenol layer and once again extracted. The aqueous layers were combined and extracted with 150μl of phenol and the upper phase removed. Once again, the remaining phenol layer was extracted with 1μl of buffer B and 40μl of DEPC treated H_2O and the aqueous layers combined. 500μl of chloroform were added to the aqueous layer and extracted twice. To the aqueous layer, 15μl of ice cold sodium acetate and 600μl of ice cold ethanol were added, mixed well and chilled at -70°C for 15 minutes. The RNA was pelleted at 13,000 rpm for 15min at 4°C. The supernatant was removed carefully and the pellet was washed with 1ml of ice cold 80% ethanol, chilled at -70°C for 15 minutes and centrifuged at 13,000 rpm, 4°C for 5 minutes. The ethanol was removed, the pellet was dessicated for 15 minutes and resuspended in 10μl of 1:50 buffer B.

(2) End Labelling:

On ice, the following were added to the dissolved RNA;

Buffer C	2μl
[γ^{32}P] ATP, (3000Ci/mMol)	2μl
H_2O	6μl

1μl (8units) of T4 polynucleotide kinase (PNK) was added and the mixture incubated at 37°C for 30 minutes. The reaction was stopped by cooling on ice.

(3) Ethanol Precipitation of Polynucleotides:

The following were added to the reaction tube;

2.5M ammonium acetate (pH 7.0)	100μl
1mg/ml tRNA	1μl
ethanol	400μl

The tube was mixed, chilled at -70°C for 15 minutes and pelleted at

13,000rpm for 15 minutes at 4°C. The supernatant was removed, and the pellet resuspended in 100μl of 0.3M DEPC treated sodium acetate (pH 7.5) and 300μl of ice cold ethanol. Once again, following chilling at -70°C for 15 minutes, the labelled polynucleotide was centrifuged and washed with 1ml 80% (v/v) ethanol. After one last precipitation at -70°C for 15 minutes, centrifugation at 13,000rpm for 15 minutes and dessication, the pellet was resuspended in 20μl of 1:10 buffer B. The labelled probe was stored at 4°C for up to 24h before use in hybridizations.

2.20.3 **Hybridisation with radioactive probes**

Prehybridisation involved floating the baked blot in 6x SSC for 5 minutes, placing in a heat sealable plastic bag with prehybridisation solution and incubation at the appropriate temperature for at least 4 hours. The volumes of prehybridisation and hybridisation solution were dependent on the size of the filters; 0.2ml/cm^2 prehybridisation solution and 0.05ml/cm^2 hybridisation solution were used in all cases unless otherwise specified. Hybridisations were carried out overnight at temperatures including 42°C, 64°C and 68°C and different hybridisation solutions were used for each temperature as described in section 2.7.

Blot type	Hybridisation temperature
Southern:	
homologous DNA	68°C
heterologous DNA	64°C
Northern blot	37-42°C
Colony blot	42°C

Following hybridisation, the radiolabelled probe was poured into a radioactive waste container, the bag cut open, the blot removed and washed according to the protocol required. The blots were then air dried and placed into heat sealable bags. Exposure to autoradiography film was carried out in light-proof film cassettes for between 24 hours and one month depending on the strength

of the signal, after which the film was developed as described in section 2.22.

Blot type	Washing conditions
Southern blot:	
homologous DNA	2xSSC/0.5% SDS at r.t. for 5min, 2xSSC/0.1% SDS at r.t. for 15min, 0.1xSSC/0.5% SDS at 68°C for 2h, 0.1xSSC/0.5% SDS at 68°C for 30min.
heterologous DNA	2xSSC/0.1% SDS twice at 64°C for 15min each, 0.2xSSC/0.1% SDS twice at 64°C for 15min each.
Northern blot	1xSSC/0.1% SDS twice at r.t. for 15min each, 0.25xSSC/0.1% SDS twice at r.t. for 15min each.
Colony blot	2xSSC/0.1% SDS twice at r.t. for 20min each, 1xSSC/0.1% SDS twice at 68°C for 1h each.

2.21 **Preparation of Non-Radioactive Labelled DNA probes and Hybridisation.**

2.21.1 DIG DNA labelling and Detection (Boehringer Mannheim Biochemica)

This procedure involved the labelling of DNA by random primed incorporation of digoxigenin labelled deoxyuridine triphosphate. The dUTP is linked via a spacer arm to the steroid hapten digoxygenin (DIG-dUTP). Following hybridisation, the target DNA hybrids were detected by an enzyme linked immunoassay involving an antibody conjugate and a subsequent colour reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium

salt. This procedure allows the efficient labelling of small (10ng) and large (up to 3μg) amounts of DNA. Linearised DNA labels more efficiently than circular and must be thoroughly heat denatured prior to labelling. The colour formation is between 1-24 hours and rehybridisation is possible after removing the colour with dimethylformamide. The kit claims to allow detection of 0.1pg of **homologous** DNA, however according to a trial dot blot, 1.0fg (10^{-15}g) of pBR328 could be detected when probed with pBR328.

DNA Labelling

The linearised DNA was denatured in a boiling water bath for 10 minutes and chilled quickly on ice. The following were added to a sterile microfuge tube;

denatured DNA	15μl
hexanucleotide mixture	2μl
dNTP	2μl
	19μl

1μl of Klenow enzyme (2 units) was added and the reaction carried out at 37°C for at least 1 hour. The reaction was terminated by the addition of 2μl of 0.2M EDTA pH 8.0 and the DNA was precipitated with 2.5μl of 4M LiCl and 75μl cold ethanol. The pellet was recovered by centrifugation after 2 hours at -20°C or 15 minutes at -80°C, washed with cold 70% ethanol, dessicated and resupended in 50μl TE.

2.21.2 Hybridisation

The blots were prehybridised with at least 20ml solution/100cm^2 filter at 68°C for at least 1 hour. This solution was replaced with 2.5ml/100cm^2 filter of hybridisation solution containing the labelled denatured probe and incubated at 68°C overnight.

Hybridisation solution:

5x SSC

1% (w/v) Blocking reagant (supplied)

0.1% (w/v) N-lauroyl sarcosine Na salt

0.02% (w/v) SDS

dissolved at 50-70°C for 1 hour and stored at -20°C.

The filters were washed twice for 5 minutes with 2x SSC/0.1% SDS at room temperature and twice at 68°C with 0.1x SSC/0.1% SDS.

2.21.3 Detection

The filters were washed briefly (1 minute) in buffer 1 and incubated with 100ml buffer 2 for 30 minutes. The antibody (Ab) conjugate was diluted to 150mU/ml (1:5000) in buffer 2 and the filters were incubated with about 20ml of the Ab conjugate for 30 minutes. The unbound Ab conjugate was removed by two 15 minute washes in 100ml buffer 1 and the filters equilibrated with 20ml buffer 3 for 2 minutes. The filters were then incubated with ca. 10ml colour solution in the dark until band formation one hour to 3 days later. The reaction was terminated by washing the filter for 5 minutes in 50ml of buffer 4. The buffers were prepared according to the manufacturers instructions.

2.22 X-ray film development

All of the film types used in conjunction with radioactive and non-radioactive probing systems were developed in the same way. In the darkroom with a red filter (GBX-2 safelight, Kodak), the film was marked by inserting a few holes in the blots and film to aid orientation. The film was placed in Kodak developer LX24 (1:4) for 5 minutes, washed in water briefly and fixed in Kodak Fixer LX20 (1:4) for 5 minutes until clearing occured. The film was washed in tap water and dried. Kodak Min-RE film (blue, 18x24cm) was used routinely for the radioactive blots. Amersham Hyperfilm was used if higher sensitivities were required. Kodak X-OMAT AR (35x43cm) was used for the sequencing gels.

2.23 Polymerase Chain Reaction

2.23.1 Extraction of oligonucleotides from the synthesis columns.

In general, all the primers and oligonucleotides were delivered on the synthesis columns and the following procedure was used in their purification. In the fume cupboard 1ml of ammonium solution [$(NH_4)_2OH$)] in a 1ml

syringe was passed through the column into another 1ml syringe attached to the other side of the column. This was repeated 3-4 times and then the solution was left in the centre of the column for at least 15 minutes. The 1ml was eluted into a sterile 4ml Bijou bottle. The 1ml elution step was repeated 2 more times each after 15 minutes in the column. The bottle was sealed tightly with parafilm and incubated at 55°C for at least 8 hours or overnight. The ammonia solution containing the oligonucleotide was freeze dried and resuspended in sterile TE. The oligonucleotide was ethanol precipitated overnight by the addition of 1/10 the volume of 3M sodium acetate and twice the volume of cold ethanol at -20°C. Following centrifugation and washing twice with 70% (v/v) ethanol, the pellet was resuspended in 300μl of sterile H_2O and stored in aliquots at -20°C. The primers were quantified using electrophoresis on 4% (w/v) Nusieve agarose with a sequencing primer (T7 Promega kit) as a marker.

2.23.2 Setting up the PCR reaction

All the PCR reactions were set up in the laminar air flow under sterile conditions and the target DNA was added last to each microfuge tube to prevent cross contamination. The oligonucleotides and dNTPs were kept on ice at all times. A typical PCR reaction was set up as follows;

primers (see chp5)	2x2μl
target DNA (see chp5)	5μl
dNTPs (10mM)	10μl
10x buffer (Promega)	10μl
H_2O	70.5μl
Taq polymerase (5u/μl)	0.5μl
	100μl

The tubes were mixed, centrifuged briefly and overlaid with 60μl of sterile mineral oil. Following the PCR reaction, a 10μl sample was examined on a 4% (w/v) Nusieve gel and the tubes stored at 4°C.

2.23.3 "Magic" PCR Preps™ DNA purification system for rapid purification of DNA fragments (Promega)

The magic PCR preps system provided a simple, reliable and effective way to purify PCR amplified DNA for sequencing, labelling and cloning. The PCR sample was purified "directly" from the microfuge tube following electrophoresis. The samples were pooled and extracted twice with chloroform/isoamylalcohol (24:1 v/v) to remove the mineral oil before purification with the columns.

Direct purification from PCR reactions

One hundred μl of direct purification buffer was added to 30-300μl of PCR reaction followed by the addition of 1ml of Magic PCR Preps resin. The tube was vortexed briefly 3 times over a 1 minute period, then pipetted into a 1ml syringe and the plunger depressed slowly to push the slurry into the mini-column. The column was washed twice with 1ml of "Magic" PCR Preps™ wash solution using the syringe to slowly push the solution through the column. The column was centrifuged at 14,000g for 20 seconds to dry the resin and the column allowed to air dry for 10 minutes to remove the last traces of isopropanol. The fragment was eluted from the column by the application of 50μl of sterile water to the column for 1 minute and centrifugation at 14,000g for 20 seconds into a sterile microfuge tube. The column was discarded and the DNA stored at 4°C or -20°C.

2.23.4 Qiagen >PCR purification spin kit<

One volume of buffer QP was added to the PCR sample and the Qiagen-spin column equilibrated with 0.8ml of buffer QP by centrifuging at 1000 x g into an empty microfuge tube. The sample was centrifuged through the Qiagen column and washed twice with 0.8ml isopropanol to remove mineral oil at 1000 x g. The column was washed twice with 0.8ml of buffer QB and the DNA was eluted and desalted as follows.

Desalting and concentration with QIAEX

This was recommended when the concentration of the DNA is below 1μg and high recovery in a small volume is important.

The DNA was eluted from the column with 0.8ml of buffer QXE and centrifuged at 1000 x g into a clean sterile microfuge tube. The QIAEX beads were vortexed until a homogeneous suspension obtained and 10μl were added immediately following resuspension to the DNA. The mixture was vortexed every 2 minutes for 10 minutes at room temperature to keep the beads in suspension and the sample was centrifuged for 30 seconds at 8000 x g. The pellet was washed twice with 0.5ml of buffer QX3 each time centrifuging for 30 seconds at 1000 x g and discarding the supernatant. The pellet was air dried for 10 minutes and resuspended in 20μl of TE buffer by vortexing and incubation at room temperature for 5 minutes. Following centrifugation the aqueous supernatant was removed carefully without removing any of the QIAEX beads.

Buffer QP : 400mM NaCl, 50mM MOPS; pH 7.0

Buffer QB : 750mM NaCl, 50mM MOPS, 15% ethanol; pH 7.0

Buffer QXE : 7M $NaClO_4$, 10mM Tris, 5% ethanol; pH 7.0

Buffer QX3 : 100mM NaCl, 10mM Tris/HCl, 70% ethanol; pH 7.5

2.23.5 Cloning of PCR fragments

Cloning the PCR fragments involved repairing the ends using a combination of T4 PNK, Klenow extension and T4 DNA ligase reactions. The template DNA was cleaned using the "Magic" PCR Preps^{tm} DNA purification system.

template DNA	18μl
10x NTB buffer	3μl
dNTP's (10mM)	2μl
H_2O	4μl
T4 PNK (8u/μl)	1μl
Klenow fragment (3u/μl)	2μl
	30μl

This reaction was incubated at 37°C for 30 minutes to 1 hour and then heat inactivated at 70°C for 10 minutes prior to ligation. The ligation reaction was set up as follows and incubated at 16°C overnight.

repaired template DNA	29μl
10x ligation buffer (blunt)	4μl
pUC19/*Sma*I (10ng/μl)	1μl
DTT (10mM)	4μl
T4 DNA ligase (1u/μl)	2μl
	40μl

E. coli TG1 electrocompetent cells were transformed with 5μl of the column cleaned ligation and transformants were selected on ampicillin and X-gal.

2.24 **DNA Sequencing**

2.24.1 Preparation of glass plates

The plates, spacers and combs were washed in tap water containing 2% RBS and rinsed in distilled water. The plates were placed on polystyrene slabs, air dried and polished with ethanol. After drying, the plates were treated with Repel Silane (Dimethyldichlorosilane solution 2% (w/v) in 1,1,1-trichloroethane, LKB, Sweden) and air dried. The plates were given a final ethanol polish before laying the spacers and taping up the sides.

2.24.2 Preparation of sequencing gel

The gel was prepared in a clean plastic beaker by the addition of 75μl TEMED and 196μl 10% Ammonium persulphate (in that order) to 60ml stock acrylamide (toxic, gloves worn at all times). The solution was gently mixed using a 20ml syringe to reduce the introduction of air and "poured" using the 20ml syringe. The plates were held at a shallow angle to prevent the formation of air bubbles and to reduce leakage. The combs were placed upside down to set in the acrylamide and excess acrylamide was removed. The gel usually required one hour to set after which it was stored in the sequencing

apparatus covered in 1x TBE or on the bench with tissue soaked in 1x TBE covering the wells and sealed with parafilm to prevent drying.

40% stock acrylamide solution for sequencing:

Acrylamide	380g
N,N'-methylenebisacrylamide	20g
H_20 to	600ml

The solution was heated to 37°C to dissolve and the volume adjusted to 1 litre with water. The acrylamide solution was then filtered through a Gelman filter (0.45μm) under vaccuum and stored in a dark bottle at room temperature.

6% Acrylamide/urea for sequencing:

10x TBE	100ml
Urea (ultrapure)	460g
40% (w/v) Acrylamide	75ml
H_2O	to 1 litre

The above were dissolved at room temperature on a magnetic stirrer and filtered through a Gelman filter (0.45μm) under vaccuum. The 6% solution was stored at 4°C in a dark bottle.

2.24.3 Preparation of DNA for sequencing

The DNA used in the sequencing reactions was prepared by pooling 4 mini (STET) preps of the plasmids and resuspending in 400μl of H_2O. To this 20μl of RNase A (1mg/ml) was added and the DNA incubated at 37°C for 30 minutes. Following this, 2-3 phenol/chloroform extractions and one chloroform/isoamylalcohol (24:1 v/v) extraction were performed to remove proteins and the residual phenol, which would inhibit the sequencing reaction. The DNA was freeze dried and the resulting pellet of DNA was resuspended in 16μl of H_2O. The DNA was stored long term at -20°C and short term at 4°C.

2.24.4 Annealing reaction

The DNA was denatured by the addition of 4μl 1M NaOH to 16μl DNA and incubation at room temperature for 10min. Ten μl of the denatured DNA was column cleaned into the microfuge tubes containing 2μl of annealing buffer and 2μl of primer. Following incubation at 37°C for 20 minutes and at least 10min at room temperature, the sequencing reactions were carried out immediately or the samples were stored at -20°C.

2.24.5 Sequencing reaction (T7 polymerase, Pharmacia)

The enzyme T7 polymerase was diluted to 1.5u/μl and stored on ice until required. The dNTP's were aliquoted (2.5μl) into microfuge tubes labelled A,C,G,T and kept on ice. The Labelling mix was prepared according to the manufacturers instructions with the exception of the quantity of [α-^{35}S] dATP used. Volumes as low as 0.4μl were substituted for the recommended 1μl without any significant deterioration in signal. To the annealed template, 6μl of enzyme premix was added and the reaction incubated at room temperature for 5 minutes. The dNTP's were warmed at 37°C for 1 minute and 4.5μl of the template/enzyme mix was added to each of the dNTP's, discarding the tip each time. This reaction was further incubated at 37°C for 5 minutes until the addition of 5μl of Stop solution (contains bromophenol blue). The sequencing reactions were stored at -20°C for up to one week.

2.24.6 *fMol*™ sequencing

The PCR amplified DNA to be sequenced was cleaned using either the Magic (Promega) or the Qiagen (2.23.4) protocols and freeze dried. The control reaction was carried out according to the manufacturers instructions. For each set of sequencing reactions, four tubes were labelled (G,A,T,C). Each of the four d/ddNTP mixes were prepared by adding 1 volume of nucleotides to 1 volume of sterile water. One μl of the appropriate diluted d/ddNTP mix was added to each tube and stored on ice or at 4°C until needed. For each set of four sequencing reactions, the following were added to a 0.5 ml microfuge tube.

Control DNA (200μg/ml)	5μl
Primer pUC forward (24mer)	2.5μl
[α-^{35}S]dATP (1500 Ci/mMol)	0.5μl
fmol™ Sequencing 5x buffer	4.25μl
sterile H_2O	3.75μl
	16.0μl

One μl of sequencing grade *Taq* DNA polymerase (5u/μl) was added and briefly mixed. Four μl of this enzyme/primer/template mix were added to the inside wall of each tube containing the d/ddNTP mix. A drop of mineral oil was added to prevent evaporation, centrifuged briefly and the tubes were placed in the PCR thermal cycler preheated to 95°C (this is important to prevent non-specifically annealed primers from extension). The following program was run;

1. 95°C for 2 mins
2. 95°C for 30 secs
3. 42°C for 30 secs
4. 70°C for 1 min

30 cycles from step 2,
refrigeration

Three μl of Stop solution was added to each microfuge tube and the samples were stored at -20°C. The samples were denatured at 75°C for 5 minutes before loading onto the sequencing gel.

2.24.7 Running the sequencing gel and exposing to autoradiography film

The gel was pre-run at 1,500V for at least 30 minutes in 1x TBE and the wells were cleaned of unpolymerised acrylamide with a syringe. 2-4μl of denatured sample were loaded onto the gel and the gel was run at 1,500 volts for the appropriate length of time. The first loading was regarded as the length of time taken for the bromophenol blue to reach the end of the glass plates.

This would allow the insert directly adjacent to the sequencing primer to be read.

After draining the buffer from the apparatus the plates were removed and prised apart with a thin spatula. The plate containing the gel was laid carefully in a bath of 10% (v/v) acetic acid and 10% (v/v) methanol for 20 minutes. Following this, two sheets of 1MM Whatman filter paper were placed over the gel and the gel was lifted from the glass plate. Clingfilm was used to cover the gel to prevent it cracking in the gel drier and the sandwich (filter paper-gel-clingfilm) was placed gel side up in the drier, sealing the edges of the rubber sheet with water and dried for 2 hours at 80°C. The clingfilm was peeled from the gel and the filter paper containing the gel was exposed (DNA side up) to Kodak X-OMAT AR film in a light proof cassette for 24-48 hours. Development was as described in section 2.22.

2.24.8 Computer analysis of sequences

All the cloned sequences were entered into the SEQAID™ programme (Rhoads, D.D. and Roufa, D.J., Molecular Genetics Laboratory, Kansas State University). This package was used for the orientation of sequences and restriction site searches. Extensive homology searches and analysis involved mailing the sequences via the Dublin City University and Trinity College Dublin (TCD) VAX mainframe computers to the BLAST programme (Altschul *et al.*, 1990) in the US. This was accessed by remote login to the Genetics Department of TCD.

Sequences were extracted from the GenBank using the QUERY command on the TCD vax and using the following format; sp = *Lactobacillus plantarum* et K=rRNA.

Alignments of sequences were facilitated by the Multiple Alignment with Hierarchical Clustering programme, version 3.0 written by F. Corpet, INRA, France (June '89) and published in *Nucleic Acids Research* **16:**10881-10890. Two files are required for alignment, the file containing the sequences and a symbol comparison table file. The sequences file contains all the sequences to be aligned each typed as follows: >seqname (7 characters long

with no blanks) followed by the sequence with a maximum of 80 bases per line. Editing to suit this format was carried out using Wordperfect 5.1 saving the results as a DOS file (ctrl F5, 1) suitable for the Multalin programme. The symbol comparison file designed for nucleic acid alignments (nucl.dat) was used. The alignment results were imported back into Wordperfect 5.1 and edited to clarify symbols and to facilitate visual comparisons.

The oligonucleotides selected, complementary to regions exhibiting the maximum diversity between genera and species, were mailed to the BLAST programme to analyse their usefulness as specific nucleic acid probes.

2.25 Silo trials

2.25.1 Preparation of inocula

The inocula were preprared from fresh overnight MRS cultures grown at 37°C. Generally 1-3ml was required to give a microbial count of $7x10^9$ cfu and this was increased to a volume of 10ml with sterile quarter strength Ringers diluent. The inocula were stored at room temperature until inoculation time later that same day. Cell counts were performed at the inoculum preparation stage to gain an accurate inoculation rate. It was assumed that as the cultures were in stationary phase, medium nutrients would be scarce and the dilution in Ringers would inhibit cell growth so that even if inoculation took place up to 8 hours later the cell count would be the same.

2.25.2 Setting up the silos

The first stage in setting up the silos was to weigh the freshly cut grass into 7kg amounts. The inoculation was performed on a table covered with a polythene sheet sterilized by swabbing with methylated spirits. The inocula were added dropwise with a syringe to the grass and mixed well. The silos were packed with 6kg of grass, a 10kg weight placed on top and the silos sealed. A gas release valve was fitted to the top of the silos. For a description of the silo, see chapter 5. Each treatment was performed in triplicate and separate silos were set up for each day of sampling. The control silos

inoculated with 10ml sterile Ringers were set up first and gloves were changed between treatments to avoid any contamination with the inocula.

2.25.3 Sampling

The silos were emptied, the contents well mixed and a 100g sample removed for further analysis.

2.25.4 Biochemical and microbiological tests on the forage

The pH was measured directly on the pressed silage samples. For microbial analysis, 10g of forage was added to 90ml Ringers diluent and mixed by stomaching for 10 minutes. Serial ten fold dilutions were carried out in 4.5ml of sterile Ringers, vortexing well between dilutions and 100μl samples were spread onto the relevant selective media. LP medium with sorbitol as a sugar source was used to estimate the *L. plantarum* numbers, MRS medium was used for total lactic acid bacterial enumeration and VRBA for the coliform - aerogenes population. Following growth at 30°C for 48 hours, the colonies were enumerated and stored at 4°C until further use.

2.26 Assays for enzyme activities in *L. plantarum*

2.26.1 Bacteriocin assay

Fresh overnight cultures of the test strains and control strain (*P. acidilactici* pLL04) were incubated on MRS medium at 37°C for 24 hours. The colonies were killed by chloroform vapours for 30 minutes in the fume cupboard by inverting the petri dishes over filter paper soaked in chloroform. A fresh overnight broth culture of the indicator strain was diluted in soft MRS agar (0.75% v/v) to give a cell concentration of $1x10^6$ cfu/ml and poured over the producer plate. Following incubation at 37°C for 24 hours, zones of inhibition could be noted.

2.26.2 Amylase activity

The starch agar plates were prepared by boiling 0.1g starch in 50ml distilled

water for 10 minutes. To this, 5.2g of MRS and 1.2g agar technical no.3 was added and the medium autoclaved. Following 48 hour growth at 37°C growth or absence of growth was noted and the plates were exposed to iodine crystals in the fume cupboard. Amylase activity was noted if there was clearing around growth. The starch degrading species *Lactobacillus amylovorus* was included as a positive control.

2.26.3 Protease activity

Skimmed milk agar plates were prepared by the addition of 20% (w/v) skimmed milk solution (2x) to MRS (2x) medium to give a final concentration of 10% (w/v) skimmed milk. Both solutions were autoclaved separately and added together aseptically. Following growth at 37°C for 48h, the plates were flooded with 10% mercuric chloride in the fume cupboard and protease activity was noted as clearing around growth. The proteolytic bacterium *Bacillus cereus* was included as a positive control. 10% (w/v) mercuric chloride was prepared by the addition of 10g to 54mls of HCl and 46ml water (20% HCl solution).

2.26.4 ß-glucanase activity

The lichenen agar plates were prepared by boiling 0.1g of lichenen (from *Usnea barbata*) in 50ml of distilled water. To this, 5.2g of MRS (enough for 100ml), 1.2g agar, 1g succininc acid and 0.6g NaOH were added and the volume adjusted to 100ml. The medium was buffered with succinic acid to optimize enzyme activity (pH of 6.5-7.0). Following autoclaving, the test strains were plated with the control *Saccharomyces cerevisiae* strain DBY746 containing the recombinant plasmid, pGL21 (Duffy, M., 1993) and incubated at 30°C for 48h. The plates were flooded with 0.1% (w/v) Congo red (in H_2O) and swirled for 5 minutes. This was followed by three rinses in tap water and 1M NaCl. Congo red binds to (1-3)(1-4)-ß-D-glucans, substituted celluloses, O-(Carboxymethyl) pachyamn and larchwood xylan and is therefore useful in detecting (1-3)(1-4)-ß-D glucanase and xylanase activities. Oligosaccharide reaction products resulting from enzyme activity on these polysaccharides do

not bind to congo red and so enzyme activity is detected by the presence of a clear halo.

Chapter 3

Analysis of strain heterogeneity among *Lactobacillus plantarum* silage isolates

Analysis of strain heterogeneity among *Lactobacillus plantarum* silage isolates.

3. Introduction

To investigate strain heterogeneity among *Lactobacillus plantarum* strains in silage, a method to differentiate between strains must be adopted. Initially chromosomal restriction endonuclease patterns were examined and while facilitating strain differentiation, this method is difficult to apply to a large number of isolates. Chromosomal DNA restriction patterns are difficult to compare under varying electrophoresis conditions and strain differences can be masked by similar plasmid patterns. However for rapid confirmation of strain identity and grouping of identical strains, this technique was applied routinely (Plate 3.1).

Analysis of ribosomal RNA fingerprints - "ribotyping" facilitates strain analysis as there are fewer bands to compare. The rDNA probe highlights the rRNA genes which are grouped into operons and are present in multiple copies. Restriction site polymorphisms result in variable banding patterns following hybridisation, which can be species or strain specific (section 1.6.2).

Finally, plasmid profiles were examined to assess the variability of extrachromosomal DNA among the isolates and to investigate the effect of plasmid content on comparison of chromosomal restriction patterns. To ensure that the indigenous plasmids did not affect the rDNA patterns, plasmid preparations were hybridised with the rDNA probe.

The investigation of strain heterogeneity in silage would yield an insight into the silage micropopulation and perhaps identify strains of interest for inoculum development. Strains which were found to dominate a good silage fermentation would be likely to effect preservation in an inoculated crop. The identification of these potential inoculants would be of enormous benefit in the screening of *L. plantarum* isolates. At present in the manufacture of commercial inoculants, it is assumed that *L. plantarum* strains

isolated from separate sources are different, but in this study identical isolates were observed in samples obtained from completely separate farms.

3.1 Chromosomal restriction endonuclease patterns

L. plantarum strains were isolated from various silages using the LP selection medium described in section 2.4 [Brophy, 1990]. Total DNA was isolated using the procedure described in section 2.11, restricted with *Eco*RI and analysed by gel electrophoresis. Results shown in Plate 3.1 indicate strain heterogeneity.

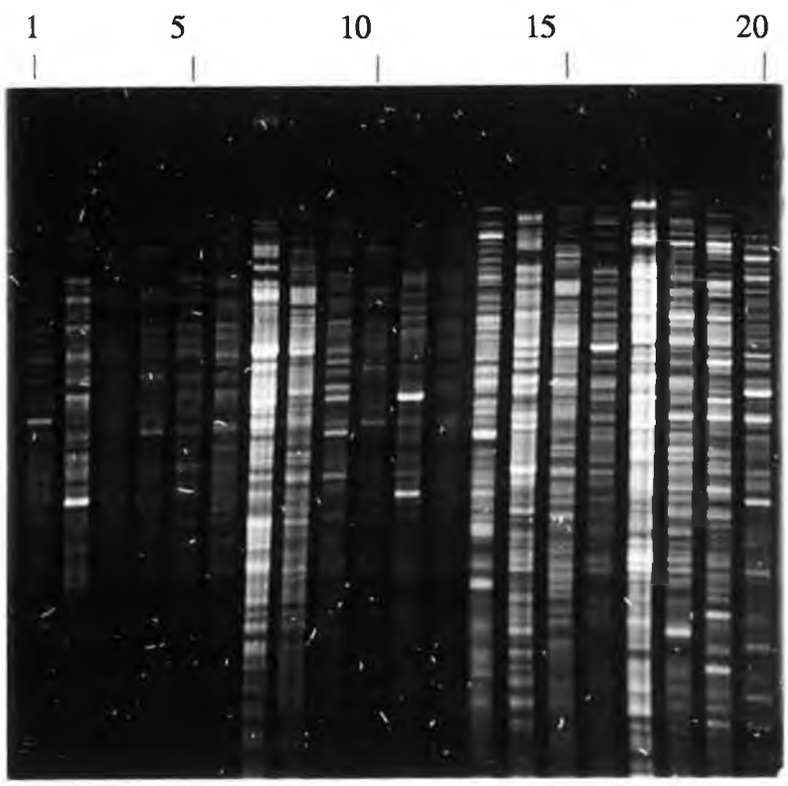


Plate 3.1. Chromosomal restriction endonuclease patterns (using *Eco*RI) of a variety of *Lactobacillus plantarum* strains isolated from grass silage. Lanes: (1) DCU101, (2) LP115, (3), AF1, (4) 1-2-5, (5) 4-1-4, (6) B6, (7) 1-2-7, (8) 1-1-49, (9) A15, (10) 1-3-12, (11) 4-1-2, (12) B4, (13)4-1-3, (14) AF2, (15) A13, (16) B2, (17) 1-3-33, (18) 1-3-3, (19) 1-3-13, (20) 1-2-19.

3.2 The use of rRNA as a direct probe to analyse strain heterogeneity

The initial attempts at rRNA fingerprint construction involved optimisation of the end-labelling, with γ-^{32}P, of *E. coli* 16S+23S rRNA and hybridisation to a range of *L. plantarum* silage isolates. Hybridisation to a northern blot of 16S+23S *E. coli* rRNA was performed as a positive control to ensure that the rRNA had not degraded and had labelled efficiently. The end-labelling procedure attaches one radioactive phosphate to the 5' end of the target polynucleotide at approximately 30% efficiency (BRL kit) and since 16S+23S rRNA are 1.5kb and 2.5kb in length, the resulting signal was quite weak (Plate 3.2). It did demonstrate that the various isolates examined had different rRNA fingerprints, each composed of between 7-9 bands.

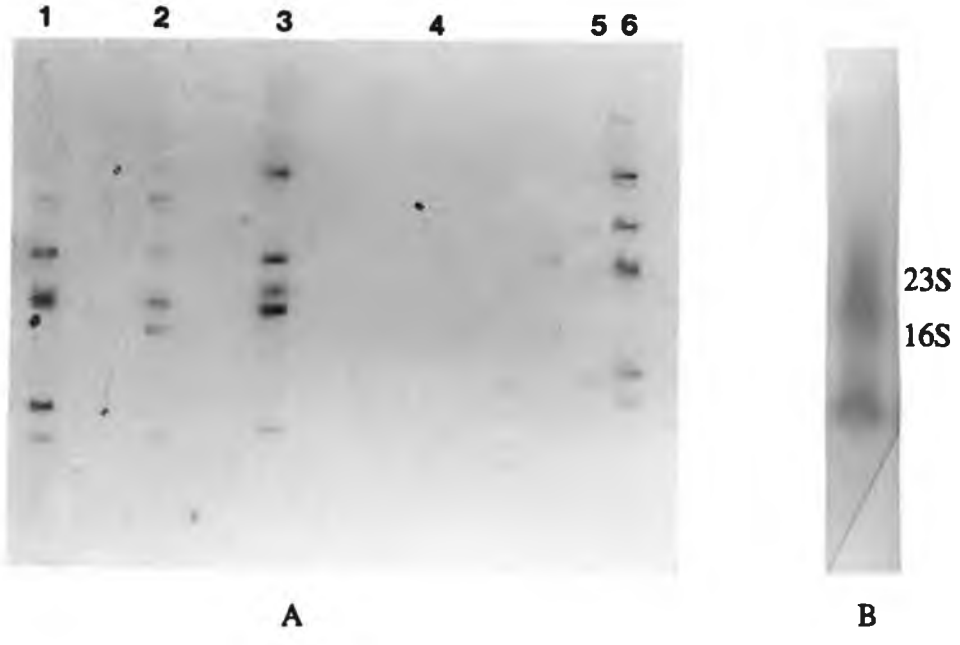


Plate 3.2. Ribosomal RNA patterns of selected *L. plantarum* strains (A) isolated from grass silage resulting from hybridisation with end-labelled *E. coli* 16S+23S rRNA. Lanes: (1) OP1.18, (2) OP2.5, (3) OP2.17, (4) λ/*Eco*RI, (5) OP2.23, (6) OP2.24. Hybridisation to a northern blot of 16S+23S *E. coli* rRNA (B) acted as a positive control.

3.3 The use of rRNA genes on plasmid pKK3535 as a fingerprinting tool

Due to the difficulties in handling rRNA and the limitations imposed by the end-labelling procedure, the recombinant plasmid pKK3535, containing the entire *E. coli* rRNA operon was obtained from Jürgen Brosius. A restriction map of pKK3535, reproduced from Brosius *et al.* (1981) is represented in Figure 3.1.

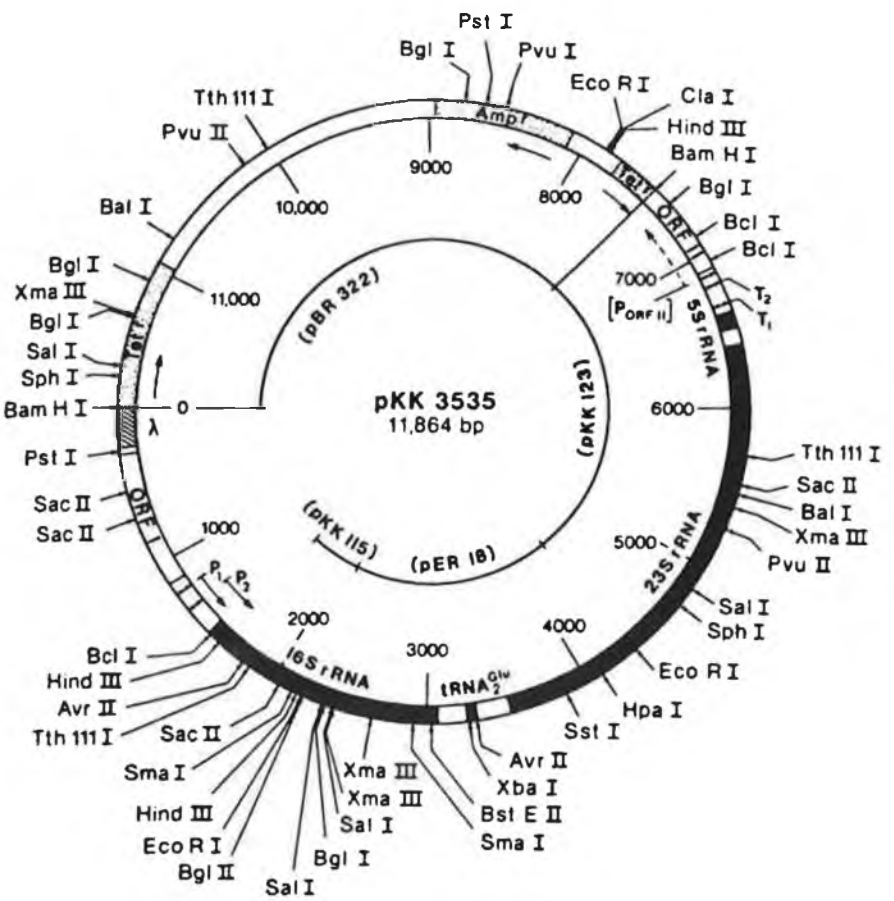


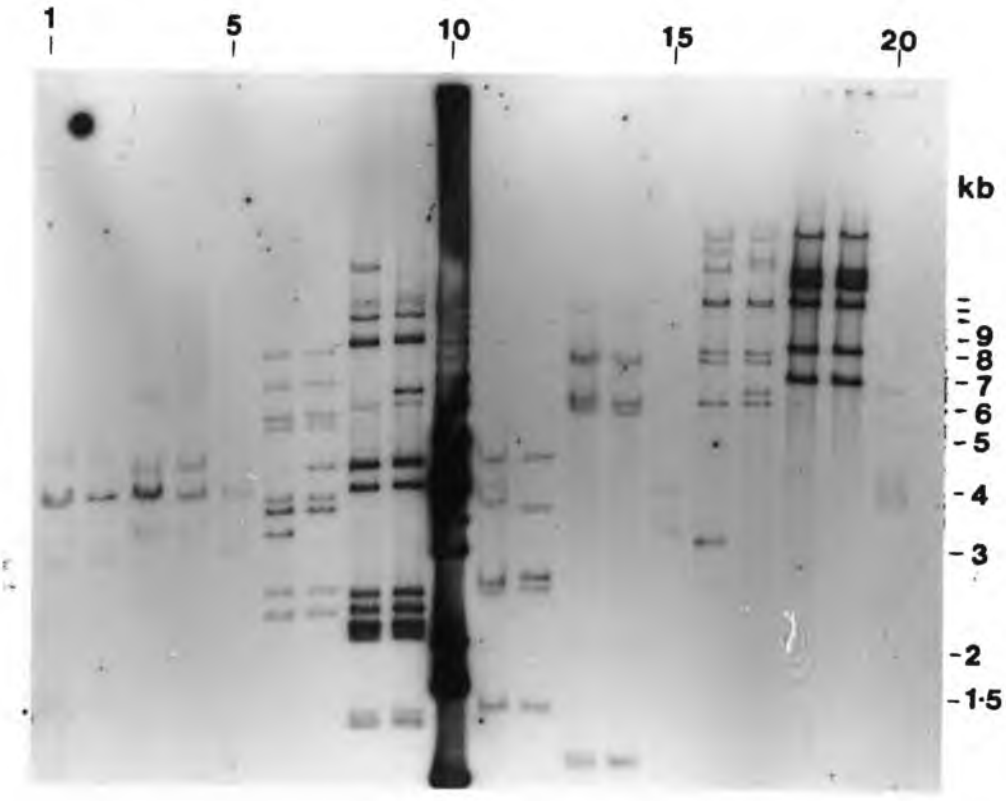
Figure 3.1. Restriction map of pKK3535 reproduced from Brosius *et al.*, (1981). Amp^r, ampicillin resistance; tet^r, tetracycline resistance; ORF, open reading frame.

Due to incomplete homology between *E. coli* and *L. plantarum* rRNA genes, the hybridisation conditions were varied to facilitate annealing and are described in detail in section 2.20.

Initially, a range of Gram-negative and Gram-positive species were hybridised with pKK3535 to investigate the possibility of using the recombinant plasmid as a fingerprinting tool. Total DNA from two strains of each species tested was restricted separately with *Eco*RI and *Bam*HI, and hybridised with pKK3535. The results are shown in Plate 3.3. *Eco*RI was chosen as it was routinely used in chromosomal restriction analysis. *Bam*HI was chosen because it has no recognition site within the rDNA operon of pKK3535 and so the resulting number of bands should reflect the number of copies of the operon present in *E. coli*.

The patterns following hybridisation were varied and interesting in that each genus and species had a completely unique fingerprint. The number of bands varied considerably for each species with the *L. plantarum* strains yielding nine *Eco*RI bands (ten bands in Plate 3.4b) and the *E. coli* strains yielding fourteen *Eco*RI generated rDNA bands. This reflects a report by Stull *et al.*, (1988) who detected 8-13 hybridisation bands using *Eco*RI cleaved chromosomal DNA of nine *E. coli* isolates hybridised with *E. coli* rRNA. The *Rhizobium* strains contained only five *Bam*HI generated rDNA bands. One 4kb band was common to *Rhizobium leguminosarum* biovar *viciae, Rhizobium meliloti* and *E. coli* in the *Eco*RI restriction (Plate 3.3, lanes 1,2,3,4,8 and 9). The *R. meliloti* and *E. coli* strains also shared a band, at approximately 4.5kb, present in one of the *L. plantarum* strains, LP115 (Plate 3.3, lanes 3,4,7,8 and 9). The *Bam*HI patterns yielded no common bands between these species.

The patterns of each pair of strains were not so variable and differentiation was dependent on the restriction enzyme used. The pairs of *Rhizobium* species could not be distinguished using either enzyme while the *L. plantarum* strains could be differentiated with both *Eco*RI (Plate 3.3, lanes 6 and 7) and *Bam*HI (Plate 3.3, lanes 16 and 17). It was interesting to note that *Pediococcus acidilactici* G24 shared no common bands with the *L. plantarum* strains.

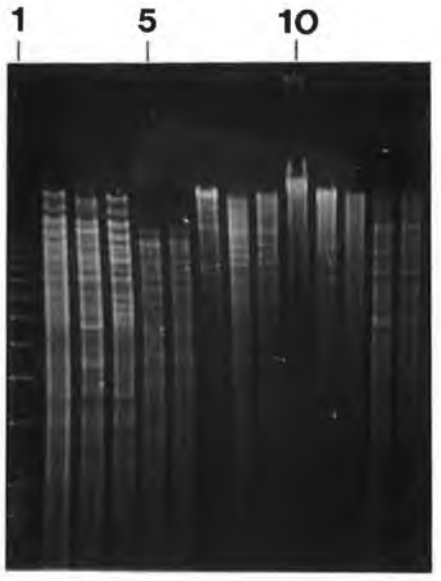


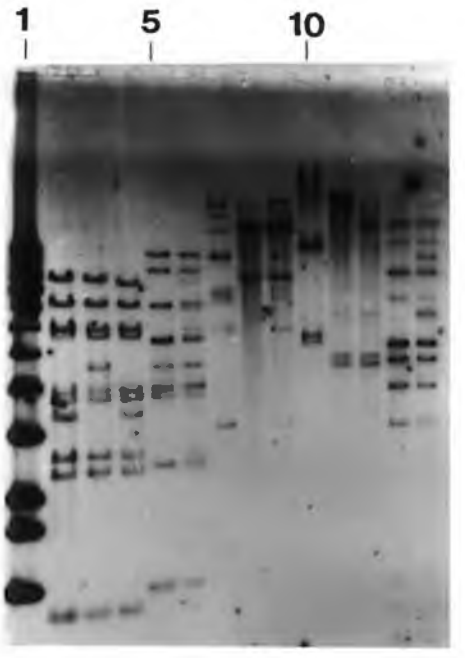
Lanes	Species/strain
1,11	*Rhizobium leguminosarum* biovar *vicae* J1300
2,12	*Rhizobium leguminosarum* biovar *vicae* VF39
3,13	*Rhizobium meliloti* 220-3
4,14	*Rhizobium meliloti* 102F34
5,15	*Pediococcus acidilactici* PLL08
6,16	*Lactobacillus plantarum* DCU101
7,17	*Lactobacillus plantarum* LP115
8,18	*Escherichia coli* JA221
9,19	*Escherichia coli* JM83
10	1kb ladder (top band 12kb)
20	*Pediococcus acidilactici* G24

Plate 3.3. rDNA patterns of different Gram-negative and Gram-positive species. Lanes 1-9 and lane 20 restricted with *Eco*RI and lanes 11-19 restricted with *Bam*HI.

The *E. coli* strains yielded the strongest signal due to their absolute homology with the probe DNA. Seven bands hybridised in the *Bam*HI restriction confirming that there are seven copies of the rRNA operon in *E. coli* and that there must be at least one *Bam*HI site between each copy. If one of the bands represented two or more copies of the operon, the intensity of the hybridisation signal would be stronger and this was not the case. Since the entire rRNA operon is appoximately 5.5kb, the smallest possible *Bam*HI fragment must be greater than 5.5kb as is reflected in the digests of the *E. coli* strains JM83 and JA221 (Plate 3.3, lanes 18 and 19). The presence of detectable differences between *E. coli* JM83 and JA221 observed in the *Eco*R1 rDNA patterns (Plate 3.3, 8 and 9) but not *Bam*HI rDNA patterns (Plate 3.3, lanes 18 and 19) demonstrated that enzyme choice is very important for the detection of differences at the strain level.

An interesting result was the differentiation between *Lactobacillus amylovorus* and *Lactobacillus amylophilus* based on their rDNA fingerprint with *Eco*RI, *Bam*HI and *Pst*I (plates 3.4B). These two species have numerous unique rDNA bands (Plate 3.4B, lanes 5+6, 8+9 and 13+14). However, with the exception of an extra top band in the *Eco*RI digest (Plate 3.4A, lanes 5+6) these two species are barely distinguishable from their chromosomal restriction patterns. Also of interest to note was the doublet between 3-4kb and a band at about 7kb which both these species have in common with *L. plantarum* strains DCU101 and LP115.





A B

Lanes	Species/strain
1	1kb ladder
2,4,7,10	*L. plantarum* DCU101
3	*L. plantarum* LP115
5,8,11,13	*L. amylovorus* NRRLB 4540
6,9,12,14	*L. amylophilus* NCIB 11546

Plate 3.4. Chromosmal restriction patterns (A) and rDNA patterns (B) following hybridisation with pKK3535 and the 1kb ladder of different *Lactobacillus* species. Lanes 2-6 restricted with *Eco*RI, lanes 7-9 restricted with *Bam*HI, lanes 10-12 restricted with *Xho*I (incomplete restriction) and lanes 13-14 restricted with *Pst*I.

3.4 Ribotyping of silage isolates

The rDNA fingerprinting technique was applied to *L. plantarum* isolates from six different, well preserved, grass silages, the sources of which are listed in Table 3.1. The isolates were selected on LP medium described in section 2.4 isolated and identified as described in section 4.2.

Sample	Grass type	Ensiled on	Silo type
A	Old pasture	1:8:'91	Baled
B	Old pasture	1:8:'91	Baled
AF	Old pasture	1:8:'91	Baled
1	Perennial Rye	9:9:'91	Bunker
2	Italian Rye	14:5:'91	Bunker
4	Perennial Rye	22:5:'91	Bunker

Table 3.1. Sources of grass silage isolates. No bacterial inoculants were added to the silages.

All of the isolates were restricted with *Eco*RI and hybridised with pKK3535. Some of the fingerprints observed are shown in plates 3.5 and 3.6. As is evident, a wide variety of fingerprint patterns were observed. Each of the *L. plantarum* isolates falls into one of 12 *Eco*RI patterns as listed in table 3.2.

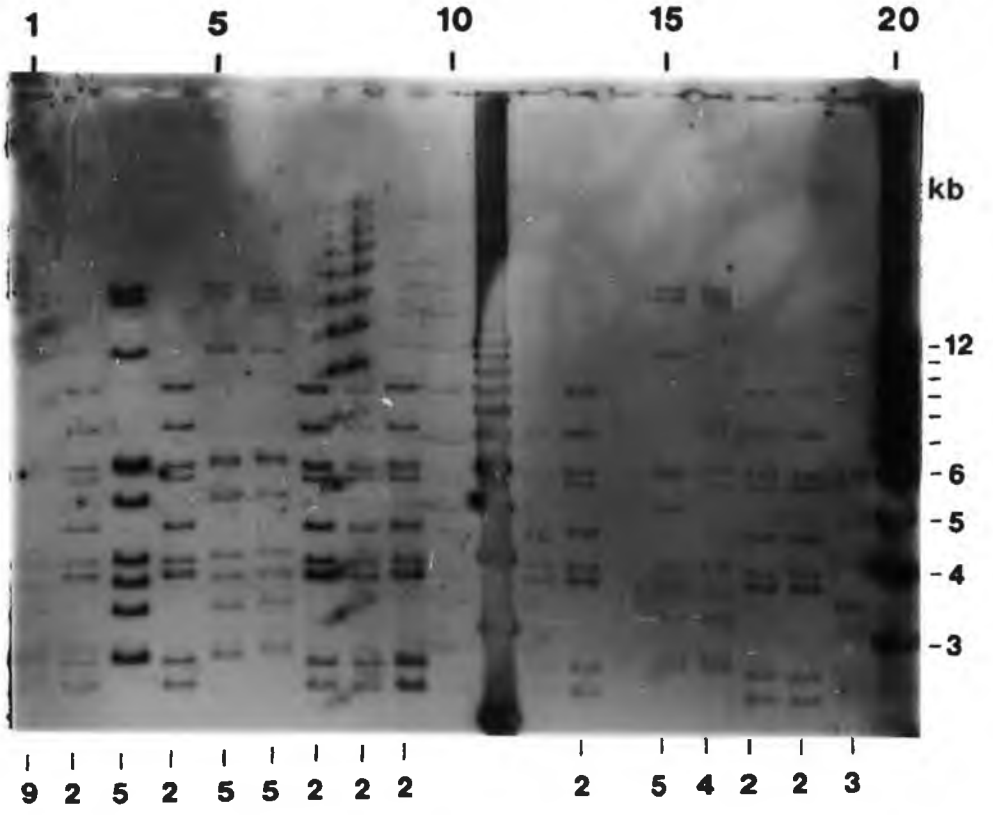


Plate 3.5 rDNA patterns (numbered below the autoradiograph) of a variety of *L. plantarum* isolates from grass silage, restricted with *Eco*RI. Lanes: (1) AF1, (2) AF2, (3) A13, (4) A15, (5) A22, (6) A27, (7) LP115, (8) A2, (9) B2, (10) B6, (11) 1kb ladder, (12) B4, (13) 1-3-33, (14) 2-1-1, (15) 1-1-49, (16) 1-2-7, (17) 1-2-19, (18) 4-1-2, (19) 4-1-3, (20) 1kb ladder.

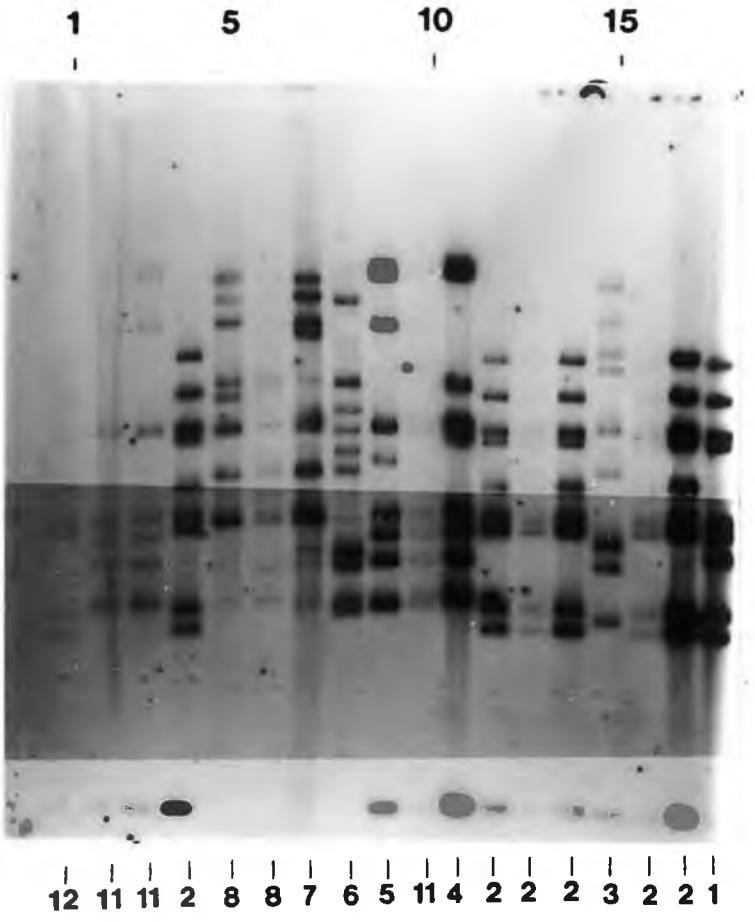


Plate 3.6. rDNA patterns (numbered below the autoradiograph) of a variety of *L. plantarum* isolates from grass silage, restricted with *Eco*RI. Lanes: (1) 1-3-3, (2) 1-3-12, (3) 1-3-13, (4) 1-3-33, (5) 2-1-1, (6) 2-1-3, (7) 2-1-8, (8) 2-1-9, (9) 1-1-49, (10) 1-2-5, (11) 1-2-7, (12) 1-2-19, (13) 4-1-1, (14) 4-1-2, (15) 4-1-3, (16) 4-1-4, (17) LP115, (18) DCU101.

rDNA pattern	*L. plantarum* silage Isolates
1	DCU101
2	A2, A4, A5, A7, A9, A10, A11, A15, A16, A20, A21, A28, A29, A33, AF2, AF3, B2, B4, B6, B8, B12, B16, B21, B23, B24, 1-2-19, 1-3-33, 4-1-1, 4-1-2, 4-1-4, LP115 (commercial inoculant)
3	4-1-3
4	1-2-7
5	A1, A3, A6, A12, A13, A17, A18, A22, A23, A26, A27, A30, A31, A32, 1-1-49.
6	2-1-9
7	2-1-8
8	2-1-1, 2-1-3
9	A19, AF1
10	A14
11	A8, B1, B5, B7, B11, B22, 1-2-5, 1-3-12, 1-3-13
12	1-3-3

Table 3.2. Grouping of *L. plantarum* strains based on their *Eco*RI rDNA pattern.

To determine whether isolates with the same rDNA pattern were the same strains, chromosomal restriction patterns were compared and where possible, isolates having the same chromosomal restriction pattern were placed into groups. In each case, isolates with identical chromosomal restriction patterns yielded the same rDNA fingerprint. However, some of the isolates with the same rDNA patterns had different chromosomal patterns and nineteen were further examined for more suitable restriction site polymorphisms (see section 3.5 below). Isolates with unique rDNA fingerprints such as *L. plantarum* strains 4-1-3, 1-2-7, 2-1-9, 2-1-8, A14 and 1-3-3 also had unique chromosomal restriction patterns. Most of the identical strains were isolated from the same source. There were however, identical *L. plantarum* strains

isolated from different silages from completely separate farms (1-1-49 and A18 or AF2 and A16).

3.5 Selection of suitable restriction enzymes to highlight rDNA differences

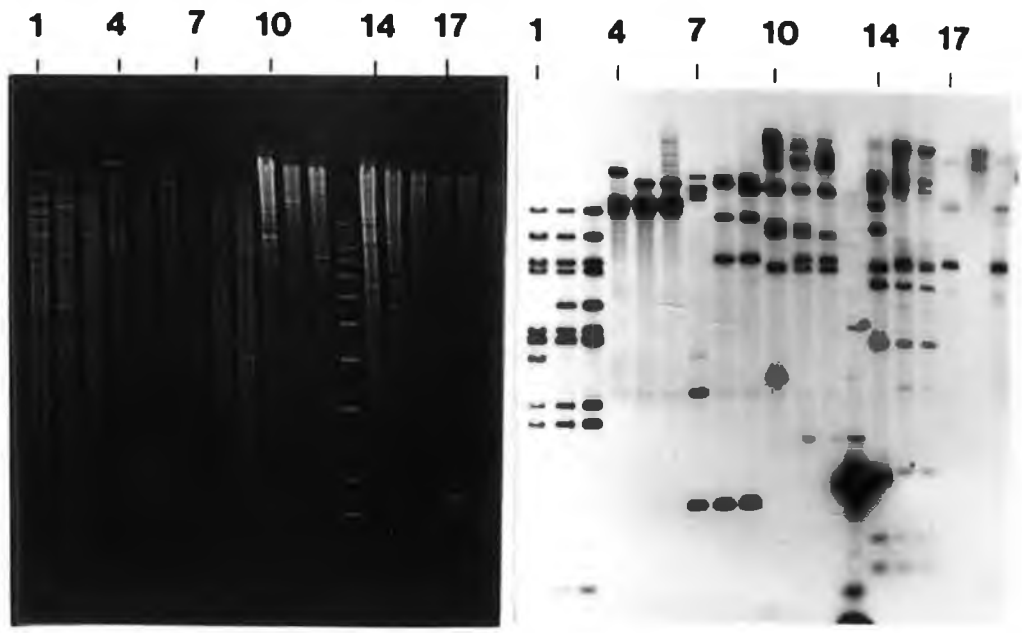
To determine the choice of restriction enzyme to further subdivide the nineteen strains which had the same rDNA patterns yet different chromosomal restriction patterns, a range of potential enzymes were screened for their usefulness including *Sal*I, *Hind*III, *Bam*HI, *Sma*I, *Pst*I, *Kpn*I, *Eco*RI/*Pst*I, *Apa*I, *Xho*I, *Bgl*II, *Taq*I, *Hae*III, *Hpa*I, *Hinf*I and *Xba*I. Two of the autoradiographs and their corresponding gels are shown in plates 3.7 and 3.8. In these autoradiographs, the patterns of *L. plantarum* strains DCU101, LP115 and B2 were compared. *L. plantarum* B2 and LP115 had identical *Eco*RI rDNA patterns and so were used to find the most suitable enzyme to facilitate differentiation. To ensure that the resulting enzyme, which differentiated *L. plantarum* B2 and LP115, also differentiated *L. plantarum* DCU101, this strain was also included. As is evident from the results, *L. plantarum* B2 and LP115, while originating from completely separate sources, have many rDNA bands in common for all of the enzymes examined. However, they could be differentiated using *Hind*III, *Bam*HI, *Kpn*I, *Eco*RI/*Pst*I and *Bgl*II. Because *Bam*HI yielded a good spread of bands and the unique bands were strong, this enzyme was chosen to differentiate the nineteen *L. plantarum* isolates of similar *Eco*Rl patterns.

The enzymes, *Taq*I, *Hae*III, *Hpa*I and *Hinf*I, having 4bp recognition sites, were of little use because of the number of bands and size of bands generated, which couldn't be adequately separated under the conditions used. The increase in agarose concentration to 1.2% as was tried in part of the gel in plate 3.8, resulted in a reduced transfer efficiency to the filter and a subsequent reduction in signal. There was no differentiation between *L. plantarum* B2 and LP115 in hybridisation to *Hpa*I digests (lanes 20-22) or between *L. plantarum* DCU101 and LP115 in hybridisation to *Taq*I digests (results not shown).

An attempt was made to correlate the number and size of rDNA bands

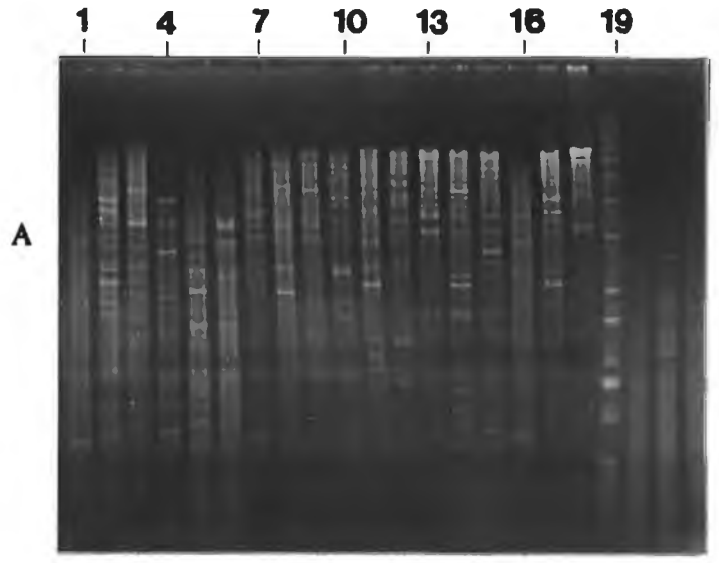
with the recognition site of the enzyme. If a correlation could be found, future restriction enzyme screening would be facilitated by the knowledge of its recognition site and the (G+C) mol% content of the target DNA. Table 3.3 lists the enzymes used, their recognition sites and the resulting number of bands produced following hybridisation. The GenBank (release 76, June, 1993) was searched for available *L. plantarum* ribosomal RNA sequences but resulted in only one 5S rRNA and two 16S rRNA sequences. These sequences were imported into the Seqaid™ programme (section 2.24.8) and a restriction site search was carried out on all three sequences. Unfortunately no 23S rRNA sequence was listed for *L. plantarum*, prohibiting a complete search. The resulting number of relevent sites are listed in Table 3.3. There seems to be no correlation between the G+C% content of enzyme target sites and the usefulness of the enzyme in strain discrimination.

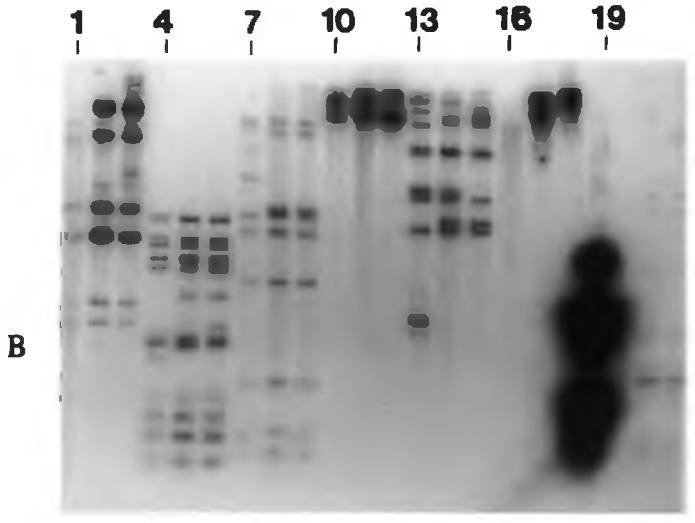
Restriction of the nineteen *L. plantarum* isolates having identical *Eco*RI rDNA patterns, with *Bam*HI resulted in differentiation of the isolates as listed in Table 3.6.



Lane	Restriction enzyme
1-3	*Eco*RI
4-6	*Sal*I
7-9	*Hind*III
10-12	*Bam*HI
13	1kb ladder
14-16	*Sma*I
17-19	*Pst*I

Plate 3.7. Chromosomal restriction patterns (A) and rDNA patterns (B) resulting from variation in restriction enzyme. *L. plantarum* strains DCU101, LP115 and B2 were loaded sequentially.





Lane	Restriction enzyme
1-3	*Kpn*I
4-6	*Eco*RI/*Pst*I
7-9	*Sma*I
10-12	*Bgl*II
13-15	*Bam*HI
16-18	*Xba*I (not fully restricted)
19	1kb ladder
20-22	*Hpa*II

Plate 3.8. Chromosomal restriction patterns (A) and rDNA patterns (B) resulting from variation in restriction enzyme. *L. plantarum* strains DCU101, LP115 and B2 were loaded sequentially. Lanes 19-22 contained 1.2% agarose.

Enzyme	Recognition site	G+C%	Bands	No. of rDNA sites	
				16S	5S
*Apa*I	G G G C C C	100%	7	1	0
*Bam*HI*	G G A T C C	66.6%	10	0	0
*Bgl*II*	A G A T C T	33.3%	5	0	0
*Eco*RI	G A A T T C	33.3%	9-10	0	0
*Hae*III	G G C C	100%	TMTD	5(6§)	?
*Hind*III*	A A G C T T	33.3%	6	0	1
*Hinf*I	G A N T C	50%	TMTD	2	0
*Hpa*II	C C G G	100%	>8	4	0
*Kpn*I*	G G T A C C	66.6%	7-8	0	0
*Pst*I	C T G C A G	66.6%	5-7	1	0
*Sal*I	G T C G A C	66.6%	4	0	0
*Sma*I	C C C G G G	100%	10-12	1§	0
*Taq*I	T C G A	50%	TMTD	3	0
*Xho*I	C T C G A G	66.6%	4-5	0	0

* The enzymes which were most useful for strain differentiation.

§ These sites only occurred in one of the *L. plantarum* 16S rRNAs analysed.

The arrow (↓) indicates the site of actual restriction.

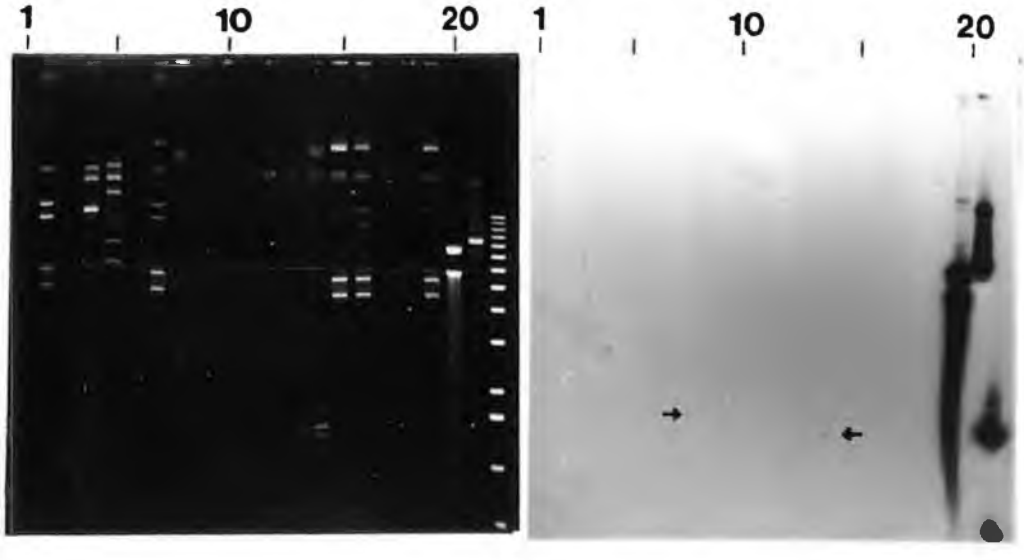
TMTD; too many to differentiate

The G+C% content of the *L. plantarum* 16S rDNA sequence extracted from the GenBank is 51.3%.

Table 3.3. Restriction enzyme recognition sites and number of rDNA bands produced on hybridisation between pKK3535 and digests of total DNA from *L. plantarum* isolates.

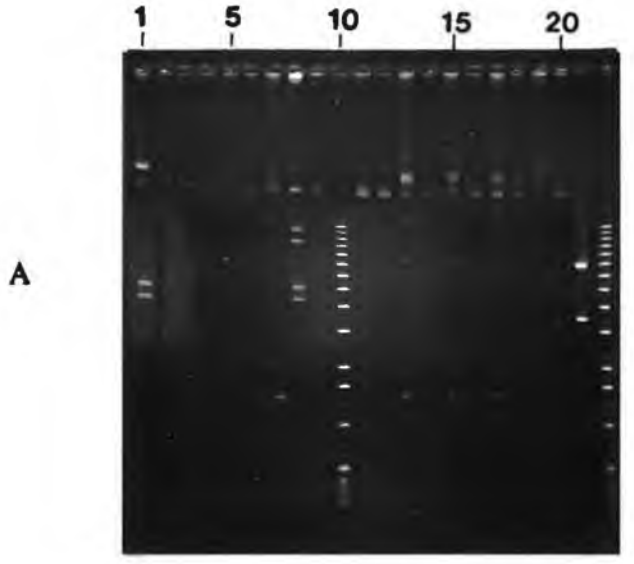
3.6 Plasmid profiling of silage isolates

Plasmid analysis was also carried out on all of the isolates examined using the Anderson and M^c Kay procedure (section 2.12). The isolates were grouped according to their plasmid profiles some of which are shown in Plate 3.9 and 3.10.

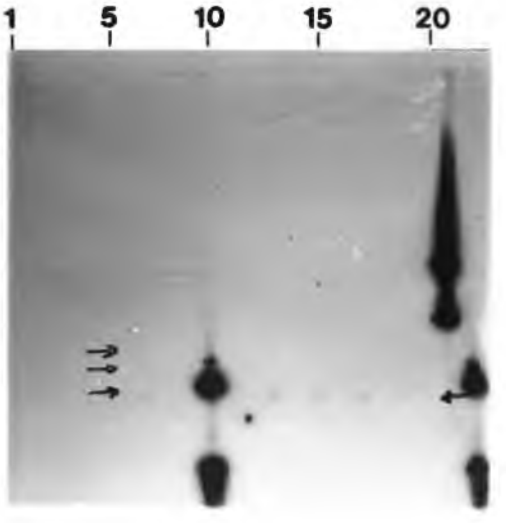


A B

Plate 3.9. Plasmid profiles (A) and autoradiograph following hybridisation with pKK3535 (B) of a variety of *L. plantarum* grass silage isolates. The plasmids pGB100 (5.795 kb) and pKK3535 (11.864 kb) were included as size markers and positive hybridisation controls. Lanes: (1) A1, (2) A2, (3) A3, (4) A4, (5) A5, (6) A6, (7) A7, (8) A8, (9) A9, (10) A10, (11) A11, (12) A12, (13) A13, (14) A14, (15) A15, (16) A16, (17) A17, (18) A18, (19) A20, (20) pGB100, (21) pKK3535, (22) 1kb ladder.



A



B

Plate 3.10. Plasmid profiles (A) and autoradiograph following hybridisation with pKK3535 (B) of a variety of *L. plantarum* grass silage isolates. The 1kb ladder was included as a positive hybridisation control. Lanes: (1) A20, (2) A21, (3) A22, (4) A23, (5) A28, (6) A30, (7) AF1, (8) AF2, (9) AF3, (10) 1kb ladder, (11) B3, (12) B4, (13) B5, (14) B6, (15) B7, (16) B8, (17) B9, (18) B10, (19) B11, (20) B16, (21) pGB100, (22) 1kb ladder.

It was impossible to assign groups to plasmids which were larger than the chromosomal DNA and the molecular weight markers as with different electrophoresis conditions these bands couldn't be compared. Therefore, they were collectively assigned the subgroup letter E. It is also important to point out that plasmid profiling yields no information about the individual plasmids. Two different plasmids may in fact have the same molecular weight and therefore be assigned to the same plasmid group yet have in actual fact nothing in common. The plasmid profiles of selected isolates were hybridised with pKK3535 to ensure that the indigenous plasmids shared no homology to either the pBR322 or rDNA elements of pKK3535 and the results are listed in Table 3.4.

Silage A			
A14 (+)	A21 (-)	A23 (-)	A30 (-)
A20 (-)	A22 (-)	A28 (-)	A33 (-)
Silage AF			
AF1 (+)	AF2 (-)	AF3 (-)	
Silage B			
B3 (-)	B8 (-)	B15 (-)	B21 (-)
B4 (-)	B9 (+)	B16 (-)	B22 (-)
B5 (+)	B10 (-)	B18 (-)	B23 (-)
B6 (-)	B11 (+)	B19 (-)	B24 (-)
B7 (+)	B12 (-)	B20 (-)	
Silages 1,2 and 4.			
1-2-19 (+)	2-1-1 (-)	2-1-9 (+)	4-1-3 (-)
1-3-3 (-)	2-1-8 (+)	4-1-2 (-)	4-1-4 (-)

Table 3.4. Isolates which were tested for hybridisation with pKK3535, the results are in brackets.

The plasmids which contained homology to pKK3535 yielded a very weak signal following four weeks exposure to autoradiography film and in all cases homology was to the smallest plasmid band. The plasmid profiles and autoradiographs are shown in Plates 3.9 and 3.10. Although the *Eco*RI restriction patterns of the plasmids were unknown and therefore a particular rDNA band could not be attributed to its homology, it was assumed that the signals were too weak to interfere with the rDNA patterns as four weeks were required before a signal was detected. This assumption held for the *L. plantarum* isolate, 1-2-19, which yielded an rDNA pattern of 2 despite one of its plasmids (plasmid profile T) having some homology with pKK3535 and isolate AF2 which also yielded an rDNA pattern of 2 and whose plasmids (plasmid profile D) had no homology with pKK3535.

3.7 Analysis of *L. plantarum* strain heterogeneity in the silage population

All of the isolates were assigned a group on the basis of chromosomal restriction pattern, rDNA and plasmid profile. So for example the *L. plantarum* isolate A1 was given the code C5, meaning chromosomal restriction pattern group C, rDNA group 5 and no detectable plasmid profile. These results are summarised in Table 3.5.

It was then possible to analyse the strain heterogeneity of the *L. plantarum* subpopulation isolated from different silage samples. Silage A contained predominately two strain types: groups C5 and A2D which comprised about 60% of the isolates from this source. It was of interest to note that despite numerous attempts, no plasmids could be isolated from any of the members of the C5 strain group.

Strain	Chromosomal pattern*	rDNA	plasmid
A1	C	5	none
A2	A	2	A
A3	C	5	none
A4		2	B
A5		2	C
A6	C	5	none
A7	A	2	D
A8		11	J
A9	A	2	D
A10	D	2	E
A11	not A or C	2	K
A12	C	5	none
A13	C	5	none
A14	not A or C	10	I
A15	A	2	D
A16	A	2	D
A17	C	5	none
A18	C	5	none
A19		9	G
A20	A	2	D
A21	not A or C	2	none
A22	C	5	none
A23	C	5	none
A24			K
A26	C	5	none
A27	C	5	none
A28	D	2	E
A29	not A or C	2	L
A30	C	5	none
A31	C	5	none
A32	C	5	none
A33	B	2	M
AF1		9	G
AF2	A	2	D
AF3	D	2	E
B1	G	11	none[a]
B2	not D	2	N
B3	B	? (2)	E
B4	D	2	E
B5	G	11	H
B6	D	2	E
B7	G	11	H
B8	D	2	E
B9	G	? (11)	H
B10	D	? (2)	E
B11	G	11	H

B12	nor G or D	2	O
B13	G	? (11)	P
B14	G	? (11)	H
B15	G	? (11)	P
B16	D	2	E
B17	D	? (2)	E
B18	D	? (2)	Q
B19	D	? (2)	E
B20	D	? (2)	Q
B21	not D	2	R
B22		11	S
B23	D	2	E
B24	D	2	E
1-1-49	C	5	none
1-2-5	F	11	X
1-2-7		4	Z
1-2-19		2	T
1-3-3		12	U
1-3-12	F	11	X
1-3-13	F	11	X
1-3-33		2	none
2-1-1		8	F
2-1-3		8	none
2-1-8		7	T
2-1-9		6	T
4-1-1		2	E
4-1-2		2	Y
4-1-3		3	E
4-1-4		2	V

* only chromosomal patterns common to more than one isolate were assigned a letter.

none; no plasmids found despite numerous attempts.

? ( ) rDNA pattern unknown, but the most probable pattern based on chromosomal restriction and plasmid profile patterns is given in brackets.

[a] no plasmid was found for this isolate however, it was placed in the strain group, G11H due to its similarity to the other isolates in this group.

Table 3.5. Summary of rDNA, chromosomal and plasmid patterns for the *L. plantarum* silage isolates.

Isolates with identical *Eco*RI rDNA pattern yet different chromosomal pattern were analysed further using *Bam*HI rDNA fingerprints and grouped accordingly. The results are listed in Table 3.6.

Isolate	Strain type[a]	*Eco*RI rDNA pattern	*Bam*HI rDNA pattern
A16	A2D	2	6
AF2	A2D	2	6
B2	2N	2	1
B12	2O	2	2
4-1-1	2E	2	9
4-1-4	2V	2	4
1-2-19	2T	2	*
4-1-2	2Y	2	*
1-3-33	2none	2	3
B4	D2E	2	10
AF3	D2E	2	twtd
A18	C5none	5	5
A22	C5none	5	5
A23	C5none	5	5
1-1-49	C5none	5	5
2-1-1	8F	8	#
2-1-3	8none	8	8
A19	9G	9	7
AF1	9G	9	7

[a] strain type results from examination of *Eco*R1 chromosomal restriction endonuclease, rDNA and plasmid patterns, see table 3.5.

* these two strains have differing *Bam*HI patterns however the patterns are not clear enough to compare with the other isolates.

twtd; too weak to determine the pattern

this pattern is different to *Bam*HI rDNA pattern 8 but is too weak to identify clearly.

Table 3.6 Differentiation of isolates using *Bam*HI rDNA patterns.

Strain	Isolate	rDNA	Plasmid	C/somal
C5	A1	✓		
D2E	A28	✓		✓
10I	A14	✓		
9G	AF1	✓		
3E	4-1-3	✓		
4Z	1-2-7	✓		
6T	2-1-9	✓		
7T	2-1-8	✓		
12U	1-3-3	✓		
2T	1-2-19	✓		
A2A	A2		✓	
A2D	A7		✓	
2B	A4		✓	
2C	A5		✓	
2K	A11		✓	
2L	A29		✓	
B2M	A33		✓	
G11H	B5		✓	
F11X	1-2-5		✓	

Table 3.7. Usefulness of the three different typing methods in strain identification of a subsample of the isolates. The tick (✓) indicates the typing method required to differentiate each strain.

	Silage Sample					
Strain	A (n=32)	B (n=24)	AF (n=3)	1 (n=8)	2 (n=4)	4 (n=4)
C5	14(43.75%)	0	0	1(12.5%)	0	0
A2D	5 (15.65%)	0	1(33.33%)	0	0	0
D2E	2 (6.25%)	9 (37.5%)	1(33.33%)	0	0	0
9G	1 (3.13%)	0	1(33.33%)	0	0	0
2K	2 (6.25%)	0	0	0	0	0
G11H	0	6 (25%)	0	0	0	0
G-P	0	2 (8.33%)	0	0	0	0
D-Q	0	2 (8.33%)	0	0	0	0
F11X	0	0	0	3(37.5%)	0	0
others	8 (25%)	5 (20.85%)	0	4 (50%)	4(100%)	4(100%)

Table 3.8. Strain heterogeneity among *L. plantarum* isolates from the six different silage samples.

3.8 Analysis of *L. plantarum* strain heterogeneity at the onset of the silage fermentation

To investigate the strain heterogeneity among *L. plantarum* isolates at the onset of fermentation, samples from day 0 (day of ensiling) and day 2 (after 48h fermentation) of an uninoculated grass silage fermentation were analysed. The samples were obtained from laboratory silos and the treatments are listed in Table 4.1. Since inoculants should be comprised of fast growing, homofermentative lactic acid bacteria and due to the succession of the silage micropopulation (section 1.3), day 2 was chosen as the best inoculum source. At this stage of the fermentation the pH had fallen from 6.28 to about 5.37 on day 2 (section 4.1) and the *L. plantarum* numbers had risen from $< 2x10^3$ cfu/g to $3.53x10^5 \pm 2.78$ cfu/g forage (section 4.1). Fifty three of the *L. plantarum* isolates tested yielded clean DNA which was restricted with *Eco*RI. Plate 3.11 shows some of the resulting electrophoresis patterns.

At first glance, they all appeared to be the same strain which was identical to *L. plantarum* LP115, a commercial inoculant. However *L. plantarum* isolates 2-53 (Plate 3.11A, lane 16) and 2-65 (Plate 3.11B, lane 11) seemed to contain different chromosomal restriction patterns yet on rDNA analysis contained the same fingerprint as *L. plantarum* LP115. The plasmid profiles for these two isolates were identical and differed to that of *L. plantarum* LP115 (Plate 3.12, lanes 1 and 6). There was no further ribotyping required to differentiate these strains from *L. plantarum* LP115.

The *L. plantarum* isolates 2-55, 2-58 and 2-64 (Plate 3.11A, lane 17; Plate 3.11B, lanes 5 and 10) seemed to be missing the top electrophoresis band and so plasmid profiles were compared to that of an LP115-like isolate, 2-62. *L. plantarum* isolates 2-55 and 2-64 contained identical plasmid profiles (Plate 3.12, lanes 2 and 5) which differed from those of 2-58 and 2-62 which were also identical to each other (Plate 3.12, lanes 3 and 4). Neither *L. plantarum* isolates 2-55 and 2-64 were able to ferment raffinose but did ferment lactose, mannitol and sorbitol. The reason for this annomaly in fermentation of raffinose could be linked to the curing of the top

electrophoresis band which may have been part of an extrachromosomal element. The identical rDNA patterns and almost identical chromosomal restriction patterns seems to eliminate the possibility that these isolates were in actual fact *Lactobacillus casei* species which have also been reported in silage and do not ferment raffinose. If the raffinose fermentation in these isolates was linked to a plasmid, the traditional classification methods based on fermentation profiles would seem fundamentally flawed as plasmids can be exchanged or lost from the host. Ahrné and Molin (1991) raised this objection in their study on spontaneous mutations altering the raffinose metabolism of *L. plantarum*. In that case however, *L. plantarum* strains which had grown poorly on raffinose mutated in high frequency to grow well on raffinose plates. There was no link made between plasmids and the increased raffinose metabolism.

The objective of the analysis of this silage sample was to investigate the strain heterogeneity at the onset of fermentation, but based on chromosomal restriction analysis $>79.3\%$ of the isolates appeared to be *L. plantarum* LP115. The source of this isolate could not have been from the inocula ensiled that same day because the control silos were always set up first, the inoculation table was swabbed with methylated spirits before ensiling and no other inoculant strains were found in the control silage. The most likely explanation was the inoculation of the grass from the farm machinery since *L. plantarum* LP115 was used as an inoculant previously. The three *L. plantarum* isolates from day 0 were also *L. plantarum* LP115 which must have been present from the onset of fermentation.

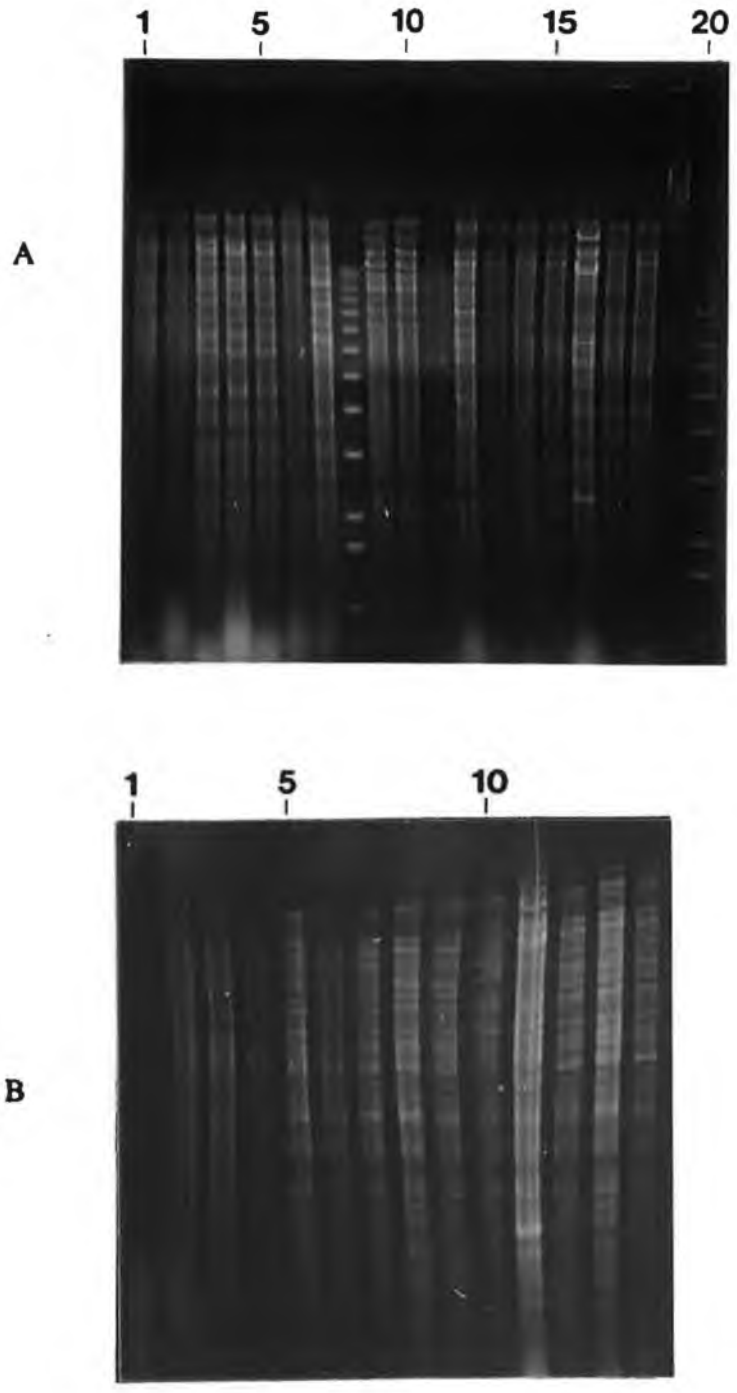


Plate 3.11 Chromosomal restriction analysis using *Eco*RI of a subsample of the *L. plantarum* silage isolates from Day 2. Lanes for Gel A: (1) 2-41, (2) 2-42, (3) 2-43 (4) 2-44, (5) 2-45, (6) 2-46, (7) 2-47, (8) 1kb ladder, (9) DCU101, (10) DCU101, (11) LP115, (12) 2-48, (13) 2-49, (14) 2-50, (15) 2-52, (16) 2-53, (17) 2-55, (18) 2-56, (19) 2-57, (20) 1kb ladder.
Lanes for Gel B: (1) 1kb ladder, (2) DCU101, (3) DCU101, (4) LP115, (5) 2-58, (6) 2-59, (7) 2-61, (8) 2-62, (9) 2-63, (10) 2-64, (11) 2-65, (12) 2-66, (13) 2-67, (14) LP115.

Lanes	*L. plantarum* isolate
1	2-53
2	2-55
3	2-58
4	2-62 (LP115-like)
5	2-64
6	2-65

Plate 3.12 Plasmid profiles of a subsample of the *L. plantarum* silage isolates from Day 2.

3.9 Discussion

This study had two main aims: to evaluate the relative sensitivities of three typing methods in detecting differences between *L. plantarum* strains and to determine whether strain dominance exists in a heterogenous mixed microbial population such as silage.

DNA restriction patterns, rDNA patterns and plasmid profiling are known to be useful methods for epidemiological investigations. Higher sensitivities have been demonstrated as compared to biochemical characteristics, phage typing or antimicrobial susceptibility testing [Altwegg *et al.*, 1989; Martinetti & Altwegg, 1990]. Chromosomal restriction endonuclease patterns are difficult to analyse due to the limited resolution of the high number of bands present when total bacterial DNA is separated on the gel, however they are useful for strain differentiation and grouping. In an effort to reduce the number of bands to be analysed, rDNA fingerprinting was investigated. Plasmid profiling and hybridisation were carried out to ensure that the presence of extrachromosomal elements would not interfere with either of the other two techniques.

In this study chromosomal restriction endonuclease patterns generated by *Eco*RI cleavage were used primarily to differentiate isolates. Isolates with identical chromosomal patterns were assumed to be the same strain. Conversely, isolates with differing chromsomal pattern, regardless of the results from the other two typing methods, were assumed to be different strains. Only one difficulty arose using chromosomal fingerprinting as a tool for differentiation regarding the two species *L. amylovorus* and *L. amylophilus*. On *Eco*RI, *Bam*HI and *Pst*I restriction, the chromosomal patterns of these two species were practically identical (Plate 3.4A, lanes 5+6, 8+9 and 13+14) with the exception of an extremely faint high molecular weight band on electrophoresis of the *Eco*RI digests (Plate 3.4A, lanes 5+6). However, rDNA analysis demonstrated definite differences in the banding patterns between *L. amylovorus* and *L. amylophilus* (Plate 3.4B, lanes 5+6, 8+9 and 13+14) allowing species differentiation. Chromosomal patterns have the disadvantage that patterns generated under differing electrophoresis

conditions are difficult to compare and electrophoresis conditions have to be optimised to yield clear, well separated and well defined banding patterns. Any blurred or hazy chromosomal restriction patterns obstruct strain differentiation. Because DNA fingerprints are highly sensitive to minor genomic variations in nucleotide sequences, they offer precise means of characterising species and of identifying individual strains of closely related bacteria when more traditional methods are unsuitable or unavailable.

Ribotyping involves hybridisation of rRNA or rRNA genes with chromosomal DNA cleaved with restriction endonucleases. Restriction endonucleases cleave the DNA within or between the rRNA operons and following hybridisation to an rRNA probe result in banding patterns. The patterns are comprised of about 3-20 bands depending on the number of restriction sites within or flanking the rRNA genes. Disproportionate intensities of the individual hybridisation signals within the patterns occur if fragments carry only part of the target region of the probe. Ribotyping offers the advantages that the technique is applicable to any microorganism, it is reproducible, the cloned rRNA is available and can be used as a broad spectrum probe, the patterns are relatively simple and pattern matching in the future can be computerised. A major advantage of rDNA typing is that the patterns have been shown to be stable [Stull *et al.*, 1988] and that the results are not influenced by the physiological activity of the organisms or the culture conditions [Köhler *et al.*, 1991].

The initial ribotyping experiments involved examination of a variety of Gram-positive and Gram-negative species to analyse the heterogeneity of banding patterns for the restriction endonucleases *Eco*RI and *Bam*HI and to assess their usefulness in strain and species differentiation. Each species had its own unique rDNA fingerprint and the pairs of *L. plantarum* and *E. coli* could be differentiated using *Eco*RI. Due to the low number of strains for each species analysed, species or genus specific bands were not identified. However, many of the strains shared common rDNA bands. *R. meliloti* and *E. coli* strains shared a band at approximately 4.5kb also present in one of the *L. plantarum* strains. This could not be attributed to an internal restriction

fragment within the rRNA operon and it outlines the danger of assigning taxonomic relevance to shared rDNA bands. While probed RFLP's are useful markers to separate organisms from one another, one has to be careful to use similar or identical patterns in groups of genetically related organisms. Fragments of comparable lengths are not necessarily homologous. This approach is more likely to be of relevance among groups of closely related strains.

The investigation of *Lactobacillus* genus specific bands was too large a task to be covered in this work and would be of little direct application to the investigation of the silage micropopulation. The presence of a common doublet at 3-4kb and the 7kb band between the *L. plantarum* strains and *L. amylovorus* and *L. amylophilus* suggests relatedness. However, without further extensive investigation, they cannot be called genus specific bands. On investigation of a large number of *L. plantarum* isolates, the presence of these "characteristic" bands was not always observed. This again emphasises that taxonomic relatedness should not be attributed to rDNA banding patterns especially since different sized bands could result from only a point mutation in the restriction site of interest. To date, there have been no extensive studies on genus or species specific rDNA banding patterns among *Lactobacillus* species or the lactic acid bacteria.

Thomson-Carter (1989) attempted to identify staphlococcal genus specific rDNA bands using *Hind*III and *Eco*RI cleavage, however although similarities were apparent, each species had its own characteristic banding pattern. Kanaujia *et al.* (1991) described the identification of species specific rDNA patterns for *Mycobacterium fortuitum-chelonei* complex using *Eco*RI but only six strains were included in his study. Hernandez and Owen (1991) examined rDNA patterns resulting from *Hae*III restriction of twelve thermophilic campylobacters identified as *C. jejuni* and *C. coli*. Nine of the twelve strains had different ribopatterns while three were identical. No single band was characteristic of either species however a few bands occurred repeatedly in 70% of the strains. As results emerge from other laboratories perhaps rRNA fingerprinting will become more valuable for genus and species

identification.

Plasmid profiling was carried out on all of the strains to ensure that interpretation of chromosomal restriction patterns was not affected by varying plasmid content. The plasmids were hybridised with pKK3535 at 64°C and homology was observed in a number of strains. This was not expected as there have been no reports to my knowledge of *L. plantarum* indigenous plasmids having homology with pBR322 or its derivatives. Bouia *et al.* (1989) reported the sequencing and subsequent analysis of a small (2093bp), cryptic plasmid (pLP1) from *L. plantarum* CCM 1904 and discovered that the Open reading Frame (ORF) exhibited complementarity with the 3' end of the 16S rRNA from *Bacillus subtilis*. They also reported the presence of pLP1 related plasmids in several LAB species. This suggests the possibility that the plasmids which hybridised weakly to pKK3535 in this study (small plasmids of the profiles I,G, H, and T) also contain ORF's which have some homology with rRNA genes present on the probe. They may also be related to the pLP1 plasmid. Due to their very weak signal they did not appear to have affected the rDNA patterns.

The plasmid content of the *L. plantarum* strains examined varied considerably, with the strain group, C5, containing no plasmids despite numerous attempts at their isolation. Plasmids may exist in strains of the group C5 but at very low copy number not detectable under the conditions used. It was of interest to note that this strain group were the most dominant in silage A and perhaps the absence of extrachromosomal DNA gave this strain a competitive advantage.

There were incidents of *L. plantarum* isolates with identical chromosomal restriction patterns containing different plasmid profiles including A2 with A9 and B14 with B15 however these profiles were quite similar. There were also *L. plantarum* isolates which had different chromosomal patterns but the same plasmid profile T ; 2-1-8, 2-1-9, and 1-2-19 (coincidently all contain a plasmid which hybridised to pKK3535). Since plasmids are extrachromosomal elements that can be exchanged between strains, this was not unexpected. Once again it is important to note that

plasmid profiles may be identical but the plasmids themselves could be quite different. Extensive restriction analysis or hybridisation studies would have to be carried out to verify plasmid identity. In the chromosomal restriction patterns, the plasmids would have been restricted while the plasmid profiles were undigested so differences in restriction sites in the plasmids could affect the chromosomal patterns. While plasmids in Gram-negative bacteria are permissive and therefore less amenable to taxonomic studies, the plasmids of *L. plantarum* have been found to be quite stable [Hill and Hill, 1986]. In this study it was observed that plasmid profiling is quite sensitive to strain variation, however low plasmid carriage frequency in some species precludes its widespread use in strain typing.

The three typing methods resulted in the following conclusions; *L. plantarum* isolates with identical chromosomal restriction patterns always yielded identical rDNA fingerprints and almost always contained the same plasmid profile, with the exception of A2A and A2D strains. Conversely, *L. plantarum* isolates with unique rDNA fingerprints also had unique chromosomal restriction patterns and almost always had unique plasmid profiles with the exception of the plasmid profile T, present in *L. plantarum* strains of *Eco*RI rDNA patterns, 2,6 and 7.

Altwegg *et al.* (1989) described *Salmonella typhi* strains with identical *Sma*I and *Pst*I chromosomal patterns yielding different rDNA fingerprints but no possible explanation was suggested. Martinetti and Altwegg (1990) also described the phenomenon of identical chromosomal restriction patterns yet different rDNA patterns for *Salmonella enteritidis* however no electrophoresis photographs were given. Perhaps similar plasmid patterns masked chromosomal differences, or the high number of chromosomal bands precluded strain differentiation. Alternatively one point mutation could not concievably alter a chromosomal restriction pattern while it may affect the rDNA pattern more dramatically. In their study, the small indigenous plasmids of 3.3kb had homology with the pBR322 part of pKK3535 which may have affected the results.

In this study, the observance of different rDNA patterns yet identical

chromosomal restriction patterns between two *L. plantarum* strains was not observed. However the chromosomal restriction patterns of the two species *L. amylovorus* and *L. amylophilus* were practically identical while their rDNA patterns were different. They are the only two species of *Lactobacillus* that can routinely degrade starch [Kandler & Weiss, 1986], yet according to Collins *et al.* [1991], while both are assigned to the Delbrueckii phylogenetic group, they are quite distantly related. These two species were differentiated with each of the three enzymes tested, *Eco*RI, *Bam*HI and *Pst*I using rDNA fingerprinting. The GenBank (release 76) was searched for rRNA sequences from *L. amylovorus* and *L. amylophilus* but only 16S sequences were available. Comparison of the restriction sites within the 16S rRNA genes between these two species revealed single sites which were present in *L. amylovorus* but not in *L. amylophilus* (*Pst*I, *Bcl*I and *Sma*I) which would account for the variance in rDNA pattern. No *Eco*RI or *Bam*HI sites were present in the 16S rDNA of either species.

In this study there were a number of *L. plantarum* isolates with similar rDNA patterns yet different chromosmal restriction patterns and plasmid profiles. An attempt was made to find a more suitable restriction enzyme to differentiate these strains. Numerous authors have cited the importance of restriction enzyme choice including Yogev *et al.* (1988), Köhler *et al.* (1991) and Altwegg *et al.* (1989) with Martinetti and Altwegg (1990) screening 14 restriction enzymes to differentiate *Salmonella enteritidis* strains. Eventually two enzymes, *Sma*I and *Sph*I were found to give the desired sensitivity. They also observed that the enzymes which generated large fragments were the most useful for strain differentiation. In this study, no correlation between the size of fragments generated or %G+C content of the recognition site of the enzyme and strain discrimination could be found. The *Eco*RI/*Pst*I restriction which yielded 11-12 fragments smaller than 7kb discriminated between *L. plantarum* strains B2 and LP115 just as efficiently as restriction with *Bgl*II which resulted in 5 fragments all larger than 12kb. Similarily restriction with *Sma*I which has a recognition sequence of CCCGGG (100% G+C content) yielded no fewer fragments than restriction

with *Eco*RI although *Lactobacillus* is classified in the low G+C content subgroup of the Gram-positive Eubacteria (section 1.5). *Lactobacillus plantarum* has a G+C mol% of 45 ± 1 [Kandler & Weiss, 1986]. It would be beneficial to researchers if some correlation could be found to elimate the need to screen restriction enzymes prior to implementation of rDNA typing. The *L. plantarum* 16S rRNA sequences extracted from the GenBank have a % G+C content of 51.3% and so there should be no bias towards enzymes with a high AT content cutting within this operon. This is reflected in the recognition sites of some of those enzymes which do cut in the 16S rRNA; *Sma*I (CCCGGG), *Pst*I (CTGCAG), *Apa*I (GGGCCC), *Taq*I (TCGA), *Hinf*I (GANTC), *Hae*III (GGCC) and *Hpa*II (CCGG). Unfortunately the 23S rRNA of *L. plantarum* either has not yet been sequenced or has not been submitted to the GenBank (release 76, April 1993).

A very weak band in each lane at approximately 2.5kb resulted following hybridisation of pKK3535 with the electrophoresed DNA described in plate 3.7. It occurred in all of the restrictions at exactly the same place. The seven different restriction enzymes had no effect on the position or intensity of the band and so it was assumed to be contaminating rRNA and was disregarded. rRNA is usually electrophoresed in denaturing gels and so migration in a non-denaturing gel cannot be related to its size [Maniatis *et al.*, 1982]. It was not observed in any of the other autoradiographs and so did not affect the description of the isolates.

The second restriction enzyme chosen for ribotyping, *Bam*HI, further differentiated the nineteen *L. plantarum* strains of similar rDNA yet different chromosomal pattern into ten groups indicating once again that the choice of restriction enzyme is of vital importance in the sensitivity of this technique. All of the isolates with identical rDNA yet different chromosomal patterns could be differentiated with a *Bam*HI rDNA pattern. Those that were in doubt due to the weak signal on the autoradiograph did appear to be differentiated but this could not be conclusively demonstrated. Conversely those with identical rDNA and chromosomal pattern yielded identical *Bam*HI patterns. The disadvantages of using *Bam*HI for preliminary differentiation are the

requirement for very clean, total DNA and a much longer incubation time with the restriction endonuclease (the digests were often carried out over 2-3 days with the addition of enzyme every 5 hours while *Eco*RI restricted routinely within 4 hours). The resulting bands following hybridisation with pKK3535 are larger and more difficult to separate adequately especially if the bands are diffuse.

In summary, the first aim to evaluate the effectiveness of three typing methods has led to the conclusions that provided the correct restriction enzyme is chosen, rDNA patterns can be highly sensitive to strain differences and are extremely useful when combined with plasmid profiling and chromosomal restriction analysis. Because the first two procedures involve visual comparison of fewer bands, initial strain differentiation should combine these two techniques. However, since rDNA patterns involve chromosomal restriction analysis as a by product of the technique, these patterns can be consulted to provide extra sensitivity between strains of dubious identity. These results reflect the latest reports in the literature. Martinetti and Altwegg (1990) observed that ribotyping was the most sensitive technique for strain discrimination yet chromosomal restriction analysis and plasmid profiling made a valuable contribution. Altwegg *et al.* (1988) found that rDNA typing and chromosomal restriction patterns were highly discriminatory. Restriction analysis yielded different patterns for all of the *Aeromonas* strains tested including those that were inseparable by rDNA typing.

The problem of visual comparison of many bands might be overcome if an internal standard, that would allow normalisation of patterns resulting from different electrophoresis conditions and the automated analysis of rDNA patterns using a laser-scanning densitometer, were incorporated. All of the materials for rDNA typing are commercially available and with the development of non-radioactive probes, the need for a specialised laboratory would be eliminated. In this study, non-radioactive digoxygenin labelled pKK3535 and 1kb ladder was hybridised to *Eco*RI restricted chromosomal DNA from *L. plantarum*, however under the hybridisation conditions recommended by the manufacturer, with the reduction of temperature from

68°C to 64°C and washes carried out at room temperature for a total of 30 minutes, only the 1kb markers yielded a signal. Perhaps with a further reduction in the stringency of hybridisation, this would be successful in the future. Digoxygenin labelled oligonucleotide probes and a PCR amplified fragment (528bp) were used successfully by Köhler *et al.* (1991) by reducing the hybridisation temperature and decreasing the stringency of the washes. Gustaferro & Persing (1992) described a novel procedure for direct covalent coupling of a horseradish peroxidase - polyethyleneimine complex to rRNA followed by enhanced chemiluminescence. They compared labelling of rRNA using this method with conventional isotopic labelling for ribotyping of *Staphylococcus aureus* isolates. They reported excellent resolution with distinctive hybridisation patterns observed after 100 minutes of exposure for their chemiluminescent probe.

The ability to differentiate all strains by ribotyping makes it an attractive technique. For characterising strains collected over extended periods of time, ribotyping may provide more dependable discrimination than plasmid profile analysis, since the genes encoding rRNA are chromosomally located and are not readily lost. In comparison with differentiation methods based on DNA, rRNA or *in situ* colony hybridisation to specific probes, the rRNA approach is experimentally more laborious and expensive but no strain specific rRNA-targeted probes have been designed and possibly strain differences at the rRNA level may not be sufficient for probe development. Furthermore, it would be rather expensive to develop specific probes for each of the many strains based on rRNA sequences.

The second aim of this study was to examine the strain heterogeneity in grass silage samples and to determine whether a dominant strain existed. In the silage sample A, two main strains were identified; C5 and A2D comprising 43.75% and 15.65% respectively of the isolates. In silage B, the strains D2E and G11H comprised 37.5% and 25% respectively of the isolates. The four other silage samples were analysed to provide a good range of different strains for use as potential silage inoculants. It was therefore apparent that at the end of the silage fermentation a few main strains

dominated the *L. plantarum* population and these are probably more suited to the conditions experienced during the latter stages of the fermentation such as low pH and high acetate concentration. The isolates chosen for silo trials were selected from those strains that were both dominant and recessive in the silages to try to correlate dominance with inoculum performance and efficient grass preservation. The results are described in chapter 4.

It would have been of interest to examine the strain heterogeneity at the onset of the silage fermentation, perhaps after two days, as an inoculant is required to be active particularly at the early stages of ensilage to ensure a good reduction in pH. Unfortunately the attempt made in this study seems to have been affected by the presence of the commercial inoculant *L. plantarum* LP115. There have been no reports to my knowledge on the analysis of strain heterogeneity in grass silage and further studies of this nature might yield an insight into the complex microbial population shifts that occur during ensilage. This in turn might help us to comprehend the causes for inoculum success and failure and subsequently enable us to choose successful silage inocula in the future.

Chapter 4

Selection and Evaluation of potential *Lactobacillus plantarum* Grass Silage Inoculants

4.1 Assessment of *L. plantarum* as a grass silage inoculant

To investigate the efficacy of *L. plantarum* as a grass silage inoculant, laboratory silo trials were carried out on two strains, *L. plantarum* LP115 - a commercial silage inoculant and *L. plantarum* DCU101. The silo type used was as described by O'Kiely and Wilson (1991) and has been shown to reflect farm scale silo conditions (Plate 4.1 and Figure 4.1).

All of the inoculants described in this chapter, unless otherwise specified, were prepared from fresh overnight MRS broth cultures which were diluted with sterile Ringers diluent to yield the required inoculation rate. Italian Ryegrass (*Lolium multiflorum*) was freshly cut by a double chop forage harvester and the treatments in each experiment were ensiled in triplicate within seven hours. The chopped grass was inoculatedon a table which had been swabbed with methylated spirits. Gloves were changed between treatments to prevent strain carryover. The methods for inoculum preparation and ensiling are described in detail in section 2.25.

Plate 4.1. Laboratory silo of the type used in the inoculant trials.

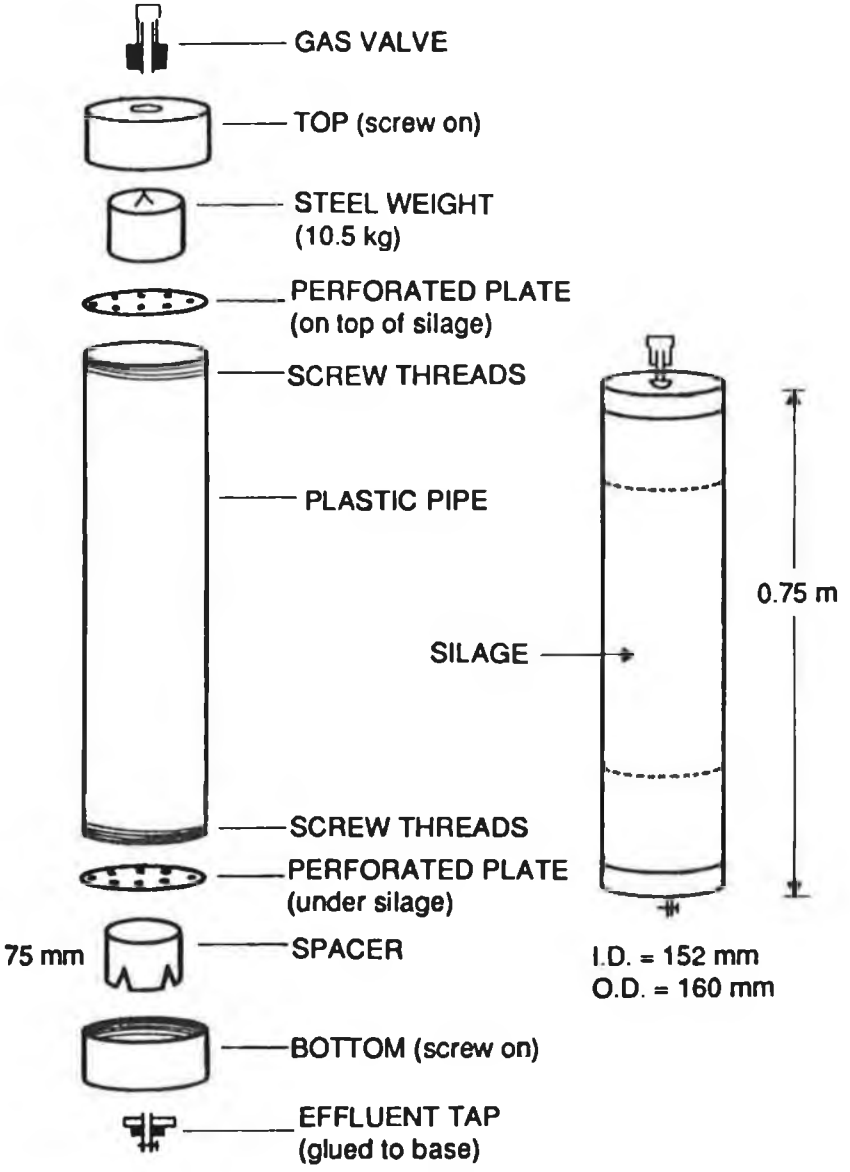


Figure 4.1 A schematic represention of the laboratory silo used in the inoculant trials (reproduced from O'Kiely and Wilson 1991)

4.1.1 The effect of *L. plantarum* inoculation on the rate of pH decrease

The treatments used to assess the influence of different strains of *L. plantarum* on pH decrease are described in Table 4.1 and the pH results over the seven day ensiling period are presented in Table 4.2.

Treatment	Composition of inoculants
1	Sterile diluent
2	LP115
3	DCU101
4	LP115 + DCU101 (1:1)
5	LP115 + DCU101 (9:1)
6	LP115 + DCU101 (1:9)

Table 4.1 : Composition of inoculants. All the inoculants were *L. plantarum* and were applied at a total rate of $1x10^6$ cfu/g grass. Each of the treatments was carried out in triplicate (total of 54 silos).

Treatment	Day 2	Day 4	Day 7
1	5.37 ± 0.07	4.54 ± 0.03	4.02 ± 0.04
2	4.69 ± 0.13	4.04 ± 0.00	3.80 ± 0.04
3	4.74 ± 0.05	4.07 ± 0.06	3.84 ± 0.02
4	4.68 ± 0.04	4.08 ± 0.03	3.83 ± 0.04
5	4.72 ± 0.03	4.07 ± 0.04	3.81 ± 0.01
6	4.70 ± 0.09	4.07 ± 0.07	3.81 ± 0.01

Table 4.2 : pH change over the seven day fermentation period. The standard deviations are written in small numbering. The initial pH of the grass was 6.28. Treatments as described in Table 4.1.

As is evident from Table 4.2, both of the inoculant strains caused a more rapid pH decrease and resulted in a lower final pH than the uninoculated control. The *L. plantarum* strain, LP115 (Treatment 2) appeared to be the most efficient inoculum. However when the variability of the pH results (± 0.04) are taken into consideration, the difference is not significant. The results from the combination of DCU101 and LP115 illustrated that there was no antagonistic action between these competing strains and there were no significant pH differences between the ratios.

4.1.2 Enumeration of Lactic acid bacteria (LAB) during ensilng

To assess the lactic acid bacterial population, cell counts on MRS were performed over the fermentation period and the results are presented in Table 4.3. The LAB population on the day of ensiling was about 2.7 $\pm$ 2.3 x10^5 cfu/g of grass and so the inoculation level of 1x10^6 cfu/g was sufficient to result in a two to ten fold (due to the standard deviation) excess over the indigenous population. This reflected previous reports on the recommended inoculation rate for grass silage to ensure an adequate starting population of inoculated bacteria. The large variability in LAB numbers on the day of ensiling was due to the difficulty in adequately mixing 10ml of inoculum into seven kg of dry grass. As the fermentation proceeds and the dry matter content is reduced, the cell counts become more accurate. Changes in the LAB numbers for treatments 1 and 3 are represented graphically in Figure 4.2. The dominance of the inoculum over the indigenous population can be seen.

Treatment	Day 0 (10^6 cfu/g)	Day 2 (10^9 cfu/g)	Day 7 (10^8cfu/g)
1	0.27 $\pm$ 0.23	0.63 $\pm$ 0.01	1.45 $\pm$ 0.24
2	2.48 $\pm$ 1.93	1.46 $\pm$ 0.52	4.19 $\pm$ 0.25
3	1.28 $\pm$ 0.60	1.50 $\pm$ 0.80	2.72 $\pm$ 0.19
4	2.84 $\pm$ 0.92	1.26 $\pm$ 0.68	2.17 $\pm$ 0.00
5	1.25 $\pm$ 0.50	1.42 $\pm$ 0.36	4.30 $\pm$ 0.12
6	1.38 $\pm$ 0.57	1.26 $\pm$ 0.87	3.52 $\pm$ 0.56

Table 4.3. Lactic acid bacterial population over the seven day ensilage period enumerated on MRS medium, incubated at 30°C for 48 hours. Treatments as described in Table 4.1.

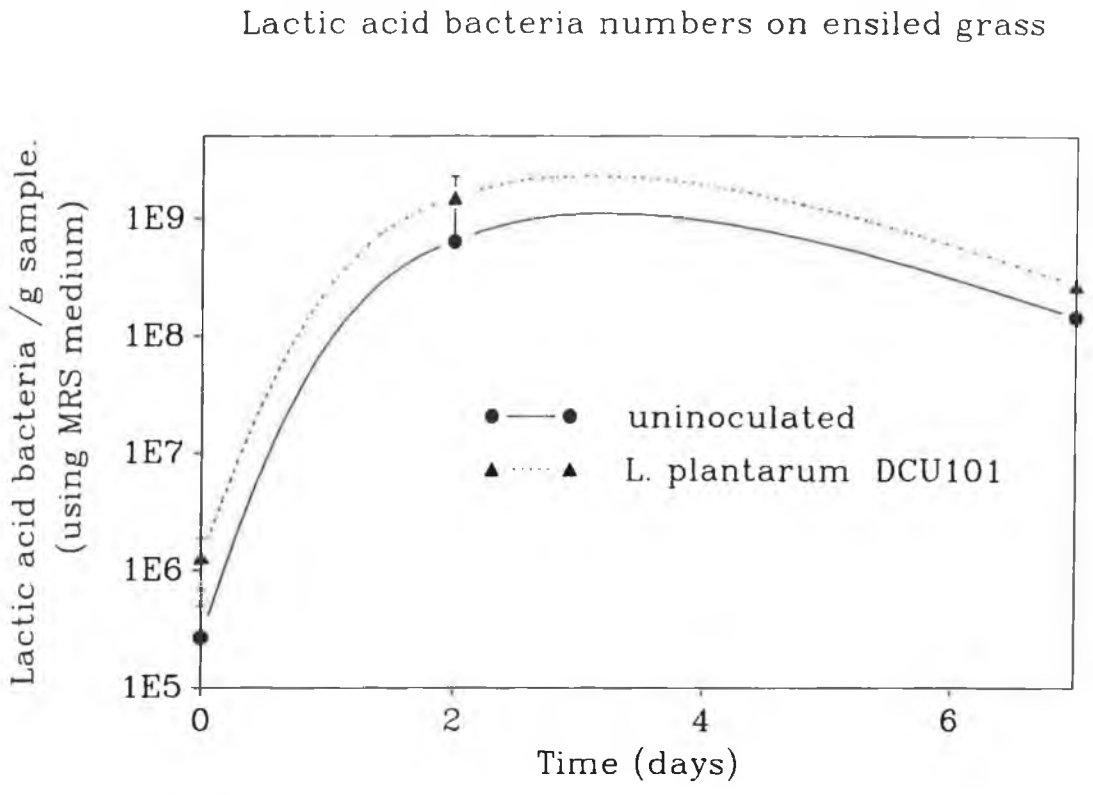


Figure 4.2. Lactic acid bacterial population over the seven day ensilage period enumerated on MRS medium incubated at 30°C for 48h. *L. plantarum* DCU101 was inoculated at a rate of $1x10^6$ cfu/g grass.

4.1.3 Enumeration of *L. plantarum* population during ensilage

The *L. plantarum* population was enumerated using LP medium [Brophy, 1990] containing sorbitol as a fermentable carbohydrate source. The inclusion of the pH indicator bromocresol green allows the differentiation of yellow, sorbitol utilising, homofermenters from the heterofermenters which remain green in colour. Selection on the basis of acid production from sorbitol eliminates leuconostocs, pediococci, *Enterococcus faecalis* and all lactobacilli

except *Lactobacillus salivarius, Lactobacillus casei, Lactobacillus coryneformis* subsp. *coryneformis* and *L. plantarum*. However of these, *L. plantarum* is the only microorganism to utilise both raffinose and ribose [Kandler & Weiss, 1986]. In each experiment a minimum of 250 yellow colonies were cross checked on LP containing raffinose or ribose for validation as *L. plantarum*.

The results of the enumeration for treatments 1 to 6 are presented in Table 4.4 and for treatments 1 and 3 may be compared graphically with corresponding pH decreases in Figure 4.3. The indigenous *L. plantarum* numbers were less than $2x10^3$ cfu/g of grass and by Day 7 had only reached a level of about $2x10^6$ cfu/g of forage. The *L. plantarum* numbers in the inoculated silos increased rapidly from $1x10^6$ cfu/g to about $1.5x10^9$ cfu/g after 48 hours which corresponded to a dramatic pH decrease. The difference between treatments 1 and 3 in the drop in pH was more significant after 2 days, indicating that inoculation with *L. plantarum* DCU101 was beneficial in the critical early stages of the fermentation. The numbers leveled off by Day 7 to about $3x10^8$ cfu/g as the population began to shift towards the heterofermentative LAB.

The final pH (4.02) of the uninoculated control silage resulted from the non-*L. plantarum* lactic acid bacterial population present on the grass. These microorganisms would not have been counted on the LP selection medium. However, the total LAB counts on MRS indicate their numbers. Growth of microorganisms such as the homofermentative pediococci or the heterofermentative leuconostocs is inhibited on LP medium yet their metabolic activity would contribute to the reduction in pH. On comparison of the *L. plantarum* with the LAB cell counts, the inoculants appear to have completely dominated the indigenous population.

Treatment	Day 0 (10^6 cfu/g)	Day 2 (10^9 cfu/g)	Day 7 (10^8cfu/g)
1	$< 2x10^3$ cfu/g	$3.53x10^5 \pm 2.78$	$2.11x10^6 \pm 1.41$
2	1.79 ± 1.12	1.53 ± 0.73	3.72 ± 0.34
3	1.94 ± 0.95	1.26 ± 0.76	2.75 ± 0.64
4	3.05 ± 1.42	1.06 ± 0.04	3.44 ± 1.36
5	1.46 ± 0.67	1.56 ± 0.17	4.24 ± 1.20
6	1.54 ± 0.89	2.25 ± 2.15	3.47 ± 0.47

Table 4.4. *Lactobacillus plantarum* population over the seven day fermentation period as enumerated on LP medium, incubated at 30°C for 48 hours. Treatments as described in Table 4.1.

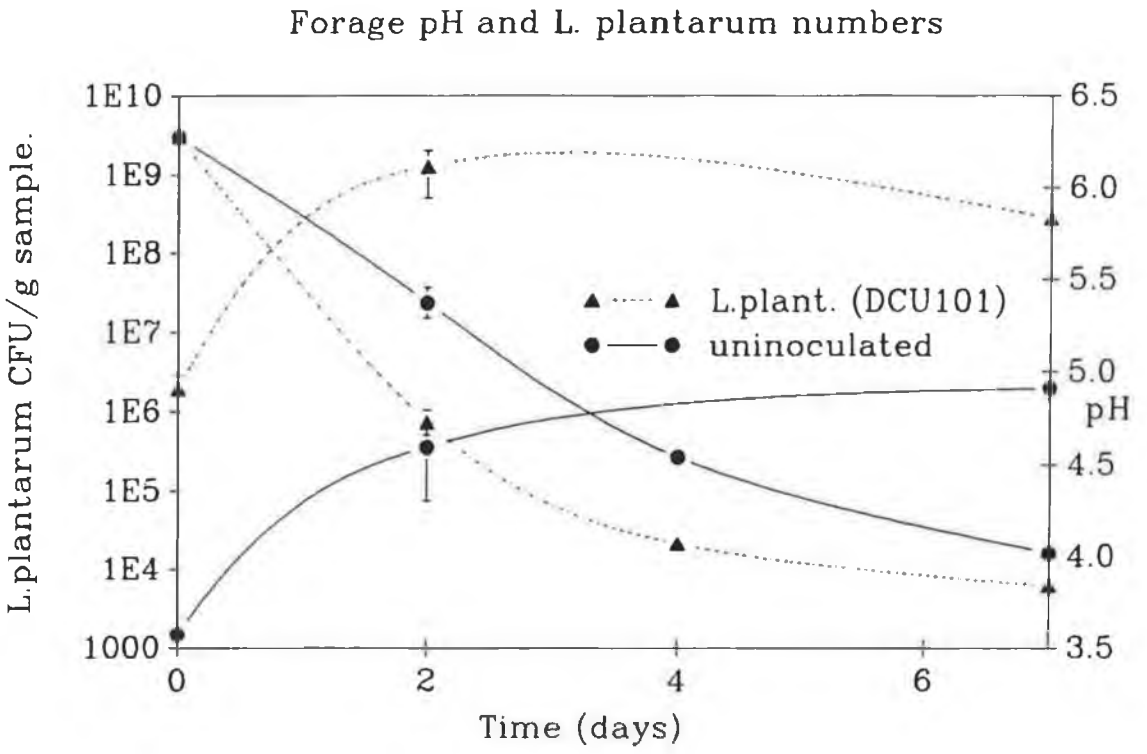


Figure 4.3. Changes in *L. plantarum* population and pH over the seven day ensilage period.

4.1.4 Enumeration of the Coliform - Aerogenes population during ensilage

Enterobacterial activity during the initial fermentation period reduces the available water soluble carbohydrate for the LAB. Furthermore, endotoxins that remain in the silage may have a negative influence on animal health. The efficient suppresion of enterbacterial growth is therefore desirable.

The Coliform - Aerogenes population was monitored over the fermentation period and the results are presented in Table 4.5. Inoculation with *L. plantarum* caused a reduction in the Coliform - Aerogenes numbers when compared with the uninoculated control silos. Enterobacteria are inhibited in silage by a low pH and Figure 4.4 illustrates this graphically as the pH falls over the ensilage period.

Treatment	Day 0	Day 2 (10^7 cfu/g)	Day 7 (cfu/g)
1	> $1x10^7$ cfu/g	6.68 ± 1.10	$3.45x10^3$ ± 3.29
2	"	2.73 ± 0.81	< 10^2
3	"	3.45 ± 1.43	< 10^2
4	"	2.82 ± 0.45	< 10^2
5	"	1.40 ± 0.18	< 10^2
6	"	2.76 ± 2.39	< 10^2

Table 4.5. Coliform - Aerogenes population over the seven day fermentation period enumerated on overlaid VRBA after 16 hours incubated at 37°C. Treatments as described in Table 4.1.

In summary, both *L. plantarum* inoculants were capable of domination over the indigenous population and effecting a more rapid reduction in pH with a subsequent reduction in coliform contamination.

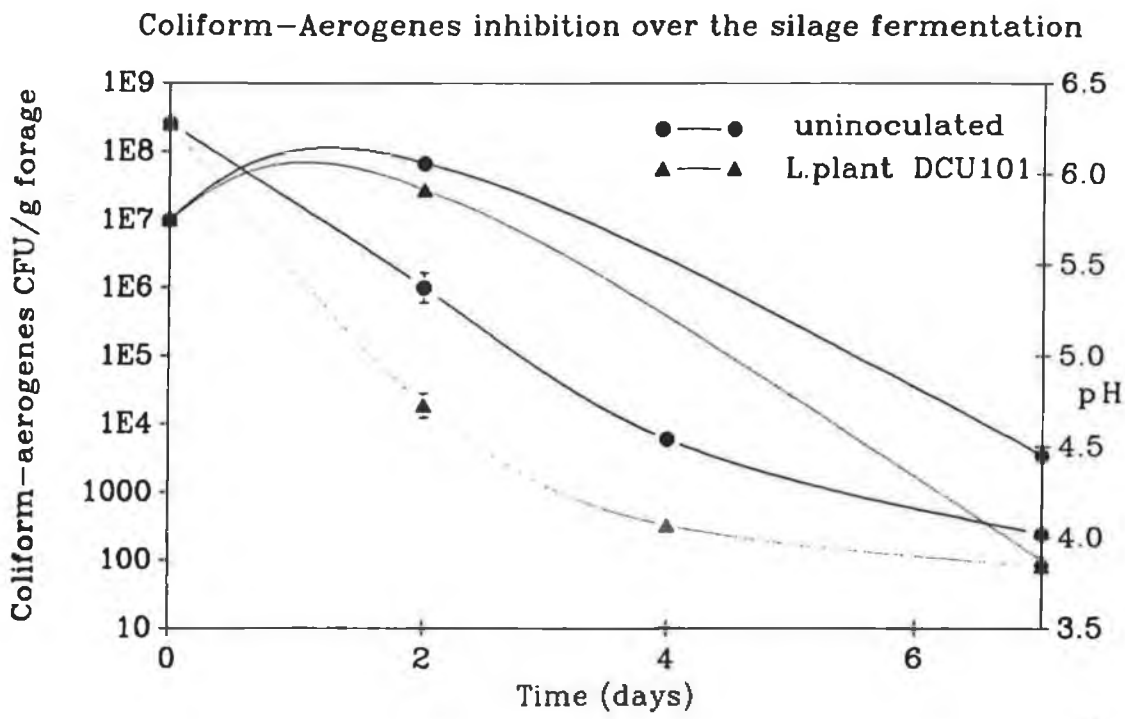


Figure 4.4. Changes in the coliform - aerogenes population and pH over the seven day ensilage period in inoculated and uninoculated silage. The initial level of coliform - aerogenes contamination may have been higher.

4.2 Selection of *L. plantarum* strains for use as silage inoculants.

Having demonstrated a beneficial effect from *L. plantarum* inoculation, experiments were conducted with the aim of selecting a strain most suited to grass silage conditions. Poor inoculant performance has been attributed to the selection of strains unsuited to the silage microenvironment. Each particular plant species provides a unique environment in terms of competing microorganisms; natural plant antagonists, substrate type, availability and concentration and various physical factors [Daeschel *et al.*, 1987]. Wieringa and Beck (1964) considered lack of proteolytic activity to be an essential feature of an inoculant and Woolford and Sawczyc (1984a) emphasised domination of the epiphytic microflora along with the establishment of a low pH as the most important criteria.

With these criteria in mind, a novel competition assay to assess the potential of strains to compete and dominate during ensilage, was developed and applied.

4.2.1 Sources of *L. plantarum* strains

The initial search for a dominant indigenous *L. plantarum* strain involved examination of the *L. plantarum* population present on silage. It was thought that as the initial pH reduction is a vital stage of the fermentation, the *L. plantarum* strains present on the silage on day 2 would be the most competitive and actively growing, reflected in the huge population increase over the initial 48 hours. Fifty three *L. plantarum* isolates were purified and analysed using chromosomal restriction endonuclease patterns and rDNA fingerprinting to examine the strain heterogeneity with a view to choosing the most dominant strain. The results are presented in chapter 3, plates 3.11 and 3.12. The most dominant strain (79.3%) appeared to be LP115 - a commercial inoculant.

Six different grass silages were then obtained none of which had been inoculated. These silages are decsribed in Table 4.6.

Sample	Grass type	Ensiled on	Silo type	*L. plantarum*[a]
A	Old pasture	1:8:'91	Baled	$3x10^3$ cfu/g
B	Old pasture	1:8:'91	Baled	$3x10^3$ cfu/g
AF	Old pasture	1:8:'91	Baled	$3x10^2$ cfu/g
1	Perennial Rye	9:9:'91	Bunker	$8x10^6$ cfu/g
2	Italian Rye	14:5:'91	Bunker	$2x10^4$ cfu/g
4	Perennial Rye	22:5:'91	Bunker	$9x10^3$ cfu/g

[a] The *L. plantarum* numbers are only approximate.

Table 4.6. Description of silages from which *L. plantarum* was isolated. No bacterial inoculants were added to the silages. The samples were obtained on 16:1:'92 for silages A, B and AF and on 13:12:'91 for silages 1,2 and 4.

L. plantarum isolates were selected on LP medium containing raffinose as a fermentable carbohydrate and a number of yellow colonies were replica plated onto LP containing sorbitol, ribose, lactose or mannitol. All of the colonies yellow on raffinose were also yellow on lactose and mannitol. However, of the isolates from silages 1,2 and 4, some were green on sorbitol as outlined in Table 4.7. This emphasised the need to confirm the *L. plantarum* identification by cross checking on the other media.

Sample	raffinose	sorbitol	lactose	mannitol	ribose
A	33	33	33	33	33
B	24	24	24	24	24
AF	3	3	3	3	3
1	250	26	250	250	26 tested +
2	16	4	16	16	4 tested +
4	5	4	5	5	4 tested +

Table 4.7. Identification of *L. plantarum* isolates. The initial selection was carried out on LP medium containing raffinose as a fermentable carbohydrate. Isolated that were yellow on raffinose were subsequently tested for a yellow appearance on LP medium with other carbohydrates.

Seventy five of the *L. plantarum* isolates were examined in more detail using chromosomal restriction patterns, rDNA fingerprints and plasmid profiling, as described in chapter 3, to determine the number of different strains. The results are presented in Table 3.5.

4.3 **Biochemical analysis of *L. plantarum* strains assessed as potential silage inoculants.**

4.3.1 Proteolytic activity

Twenty two of the *L. plantarum* isolates (Table 4.8) were assayed for proteolytic activity using MRS agar containing skimmed milk (section 2.?). *Bacillus cereus*, a known proteolytic bacterium was included as a positive control. Proteolytic activity has been reported for some strains of *L. plantarum* [Broome & Hickey, 1991; Khalid & Marth, 1990]. However, none of the silage isolates tested exhibited any proteinase production.

4.3.2 Amylase activity

Distinct starch degradation leading to clearing on starch plates is only observed in a few species of *Lactobacillus*; *L. amylovorus* and *L. amylophilus* [Kandler & Weiss, 1986]. However, many strains of *Lactobacillus acidophilus, Lactobacillus crispatus* and *Lactobacillus gasseri* are able to ferment starch [Johnson *et al.*, 1980]. There has also been a recent report of a strain of *L. plantarum* isolated from cassava waste, exhibiting α-amylase activity [Giraud *et al.*, 1991] and so the twenty two silage isolates were tested for their ability to utilise starch. *L. amylovorus* was included as a positive control strain. None of the isolates displayed amylase activity.

All of the isolates were tested for endoglucanase activity but, as expected, none was detected.

4.3.3 Bacteriocin activity

Bacteriocins produced by *L. plantarum* have been reported recently. These include Plantaricin A, active against *Lactobacillus* spp., *Pediococcus* spp., *Leuconostoc* spp. and *E. faecalis* [Daeschel *et al.*, 1990], Plantaricin B, active against *L. plantarum*, *Leuconostoc mesenteroides* and *Pediococcus damnosus* [West & Warner, 1988] and Plantaricin S, active against *Lactobacillus* spp., *Leuconostoc* spp., *Lactococcus* spp. and *Pediococcus* species [Jimanez-Diaz *et al.*, 1990]. Okereke & Montville (1991) reported the bacteriocin inhibition of *Clostridium botulinum* spores by three strains of *L.*

plantarum. Strains exhibiting bacteriocin production may have a competitive advantage over the indigenous silage population and would therefore be desirable as inoculants.

The twenty two strains of *L. plantarum* were tested for bacteriocin activity against (a) each other, *L. plantarum* DCU101 and *L. plantarum* LP115 as described in section 2.26.1 using the *P. acidilactici* strain, pLL04 as a positive control. No activity was detected. Resistance to the pediocin produced by pLL04 was also examined but none was detected.

4.3.4 Action on organic acids

The buffering capacity of organic acids has a significant influence on the pH of silage and when these acids are metabolised by lactic acid bacteria, acidity is reduced with a subsequent retardation in the rate of pH decrease. Dissimilation of organic acids also results in undesirable losses in dry matter [Daeschel *et el.*, 1987]. The utilisation of citric, succinic and gluconic acid as fermentable carbohydrate sources by the 22 isolates was examined. no utilisation was detected.

A2	1-1-49	2-1-8
A13	1-2-5	2-1-9
A15	1-2-7	4-1-1
AF1	1-2-19	4-1-2
AF2	1-3-3	4-1-3
B2	1-3-12	4-1-4
B4	1-3-13	DCU101
B6	1-3-33	Bactensil

Table 4.8. *L. plantarum* isolates screened for protease and amylase activity, organic acid fermentation, and bacteriocin production. All were negative.

As the strains were isolated from well preserved Irish grass silages, it was assumed that all would be ideally suited to the silage microenvironment and so differences in growth characteristics displayed in laboratory medium under ideal conditions would not be reflected in the silo. Growth rate under laboratory conditions was, therefore not taken into consideration in choosing strains for assessment. Based on the selection procedure and strain differentiation a number of different *L. plantarum* strains from different sources were chosen for comparative assessment under ensilage conditions. The development of an assay to determine the competitive ability of each test strain when challenged with the epiphytic microbial population is described below.

4.4 The Evaluation of potential *L. plantarum* silage inoculants using a novel competition assay

It is widely agreed that in selecting bacteria for silage inoculants, the most important criterium is the ability of the inoculant strain to dominate the fermentation thereby causing a rapid fall in pH. Silage inoculants are generally applied at 10^5-10^6 cfu/g grass and must be suffiently competitive to dominate the epiphytic population. This may be as high as 10^6 cfu/g at ensilage and is usually predominated by heterofermentative bacteria which are less efficient at lactic acid production. Although a number of authors have cited uncompetitiveness of inoculant strains as a possible explanation for inoculant failure [Moon *et al.*, 1981; Ely *et al.*, 1982], no system for assessing competitiveness based on the variability of the silage microenvironment has been proposed. Such a system was therefore developed for *L. plantarum* to screen several potential silage inoculants. The assay involved co-inoculation of herbage with equal numbers of the test strain and the standard competitive strain *L. plantarum* DCU101. Subsequently, the ratio of the strains is followed by enumerating *L. plantarum* DCU101 using a strain specific DNA probe. This allows the competitive ability of the test strain to be evaluated.

4.4.1 Enumeration of *L. plantarum* DCU101 in Co-inoculation Experiments.

Enumeration of *L. plantarum* DCU101 in the mixed inoculation experiments combined initial selection on LP medium and hybridisation with the *L. plantarum* DCU101 specific DNA probe, pGB100. The recombinant plasmid, pGB100 [Brophy, Ph.D thesis, DCU, 1990] contains p101a, a cryptic plasmid (2.2kb) from *L. plantarum* DCU101, cloned into pAT153, a multicopy *E. coli* vector. pGB100 was used in colony and Southern hybridisation experiments to determine its specificity and, therefore, usefulness as a strain specific DNA probe. Of the thirteen *L. plantarum* strains used in the two co-inoculation trials, only one strain, *L. plantarum* DCU101 had homology with the probe.

Colony Lysis procedure

To facilitate the screening of a significant number of *L. plantarum* colonies, the colony lysis technique had to be optimised. This involved varying the growth and lysis conditions. The final protocol is described below. The 20x20 cm (approximately) trays facilitated the growth of about 600 well spaced colonies on each nitrocellulose square. Both of the recombinant plasmids pKK3535 and pGB100 were used in the initial optimisation hybridisation experiments to confirm lysis.

Following incubation at 30°C for 48 hours on MRS medium, the nitrocellulose filter containing the colonies, was transferred sequentially onto 3MM Whatman paper saturated with the following solutions.

1.	50mM Tris, pH 8.0	5 mins	rt*
2.	10mg/ml lysozyme in 6.7% sucrose/TE pH 8.0	3 hours	37°C
3.	20% SDS	10 mins	rt
4.	0.5M NaOH / 1.5M NaCl	5 mins	rt
5.	0.5M Tris / 1.5M NaCl, pH 8.0	5 mins	rt
6.	2x SSPE, pH 7.4	5 mins	rt

* rt, room temperature

4.4.2 Selection of dominant *L. plantarum* strains using the competition assay

The *L. plantarum* test strains used in the first co-inoculation experiment with the exception of *L. plantarum* LP115 were selected from four separate silages and were all genotypically unique as demonstrated by chromsomal restriction analysis, rDNA fingerprinting and plasmid profiling. A range of naturally dominant strains and those found in low number were selected to examine the fidelity of the dominant performance under different ensilage conditions. The chromosomal restriction patterns and the results of hybridisation with pGB100 are given in Plate 4.2.

To ensure that the strain mixtures were compatible, all were screened for the absence of antagonistic agents that would inhibit the standard strain *L. plantarum* DCU101. As noted in section 4.3.3, no such agents were detected. This implied that any changes in the strain ratio during the co-inoculation experiments would be as a result of superior adaptation to silo conditions by one of the strains.

The performance of the test strains in the silo was assessed by comparing their prograss when co-inoculated individually with *L. plantarum* DCU101. The mixed inoculation treatments and controls are listed in Table 4.9.

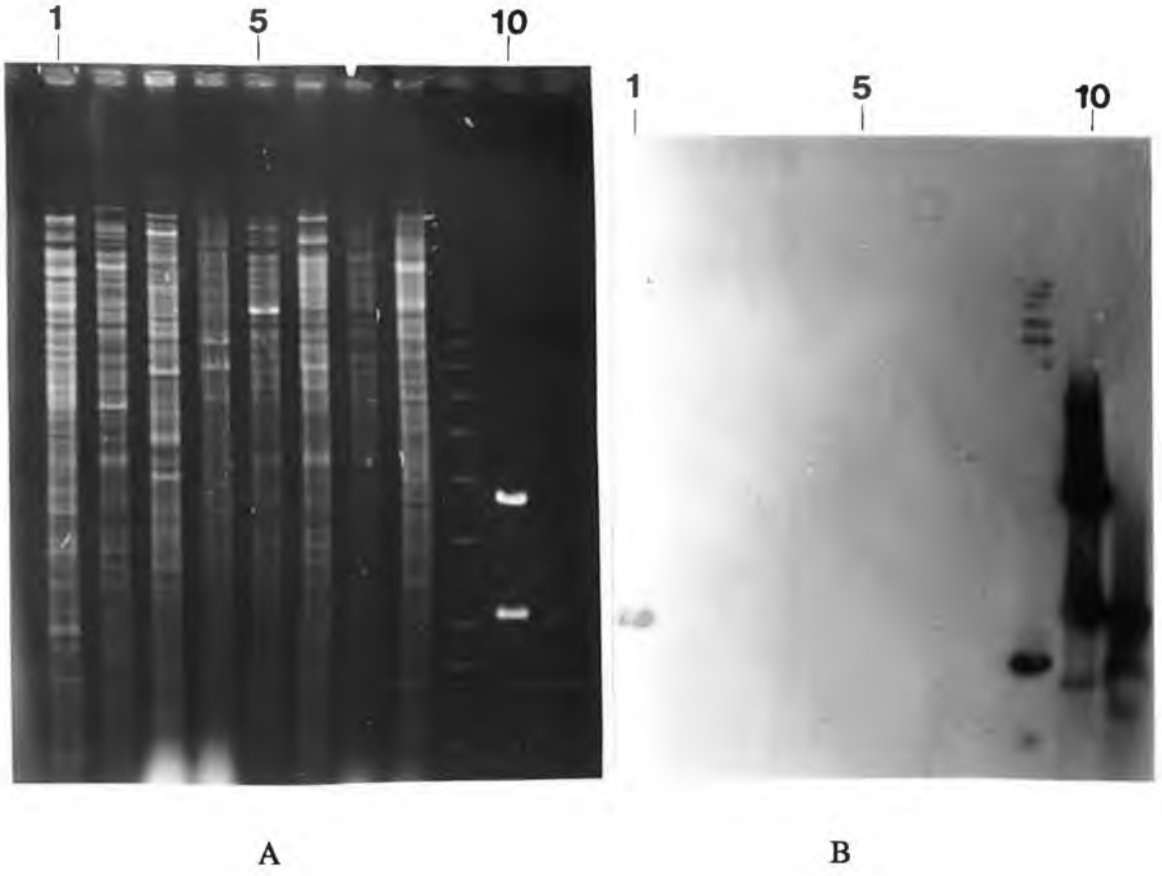


Plate 4.2 Chromosomal restriction (*Eco*RI) patterns of the *L. plantarum* strains used in the first co-inoculation experiment (A) and the resulting autoradiograph on hybridisation with pGB100 (B). Lanes: (1) DCU101, (2) LP115, (3) 4-1-3, (4) AF2, (5) B2, (6) 1-3-33, (7) B4, (8) 1-1-49, (9) 1kb ladder (BRL), (10) pGB100, (11) p101a. Hybridisation to the 1kb ladder is due to partial homology between pAT153 (the replicon of pGB100) and fragments of the ladder.

Treatment	Composition of inoculants	Actual ratio inoculated
1	sterile diluent	na
2	DCU101	$0.9x10^6$ cfu/g
3	DCU101 + LP115	1 : 0.95
4	DCU101 + 4-1-3	1 : 0.94
5	DCU101 + AF2	1 : 1.04
6	DCU101 + B2	1 : 0.70
7	DCU101 + 1-3-33	1 : 1.27
8	DCU101 + B4	1 : 1.56
9	DCU101 + 1-1-49	1 : 0.96

L. plantarum DCU101 in each of the co-inoculations was added at a rate of $0.45x10^6$ cfu/g of grass.

Table 4.9. Composition of inoculants in the first co-inoculation assay. The inoculants were prepared to give a total inoculation rate of $1x10^6$ cfu/g grass and a ratio of 1:1 (test : indicator strain). The actual strain ratios enumerated on MRS prior to inoculation are listed. *L. plantarum* DCU101 in each of the co-inoculations was added at a rate of $0.45x10^6$ cfu/g grass.

The initial ratio of DCU101 to the test strain (ideally 1:1), the ratios after 2 and 14 days were determined for each replication of the treatments using the *L. plantarum* DCU101 specific DNA probe, pGB100. This allowed the dominance or paucity of strain *L. plantarum* DCU101 to be assessed and by extension, the performance of the second strain in the mixture. LP medium containing sorbitol was used to selectively enumerate *L. plantarum* and samples of 300 yellow colonies (100 from each treatment replication) were

screened by colony hybridisation. Plate 4.3 shows a typical colony hybridisation result. To ensure that the LP medium was selective for *L. plantarum*, a total of 3000 yellow colonies were replica plated onto LP medium in which raffinose or ribose replaced sorbitol as the sugar source. All were found to utilise both sugars indicating that they were *L. plantarum* and that other *Lactobacillus* species were not present in detectable numbers. The number of *L. plantarum* colonies on the uninoculated control silage was 10,000 fold lower than the inoculated silages and so did not affect the population in the inoculant treated silages. The results of the hybridisation experiments are summarised in Table 4.10 and figure 4.5.

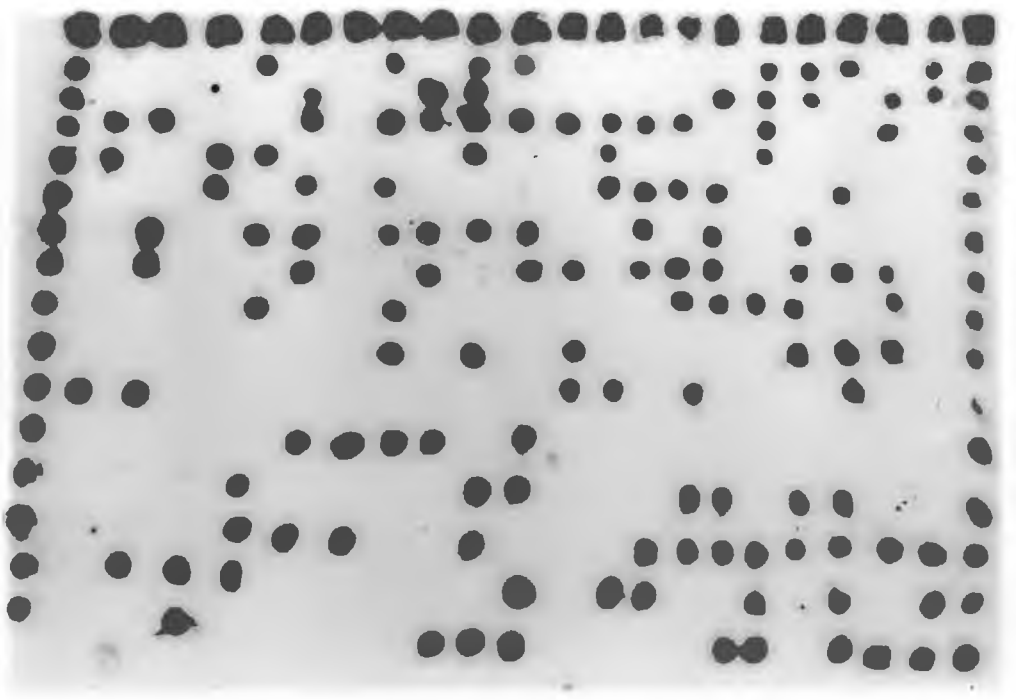


Plate 4.3. A typical colony hybridisation result. *L. plantarum* DCU101 was spotted at the beginning and end of every row and the top of each column to facilitate orientation and to act as an internal lysis control.

	Percentage of *L. plantarum* DCU101				pH, day 15
	based on cell counts		**based on hybridization results**		
Treat.	**day 0**	**day 0**	**day 2**	**day 14**	
1	-	-	-	-	4.19 ± 0.05
2	-	99.67 ± 0.58	98.99 ± 1.01	98.33 ± 2.89	4.04 ± 0.01
3	51.28	42.67 ± 2.52	46.67 ± 1.53	45.17 ± 3.34	4.03 ± 0.01
4	51.55	75.67 ± 7.37	95.29[(a)] ± 3.82	100.0 ± 0.0	4.11 ± 0.02
5	49.02	38.00 ± 4.36	55.33 ± 4.16	70.33 ± 8.39	4.04 ± 0.02
6	58.82	51.33 ± 5.51	46.00 ± 1.73	21.33 ± 4.93	4.01 ± 0.01
7	44.05	47.00 ± 4.58	44.93 ± 6.18	56.00 ± 8.89	3.98 ± 0.01
8	39.06	42.67 ± 2.52	36.67 ± 3.06	50.69 ± 10.69	4.07 ± 0.02
9	51.02	41.37 ± 1.36	40.17 ± 0.79	45.00 ± 10.4	4.03 ± 0.01

(a) number of colonies probed in this case was 154.

Table 4.10 Percentage *L. plantarum* DCU101 over the co-inoculation ensilage period (n=3, standard deviation printed small). The initial pH of the grass was 6.3 and the final pH's are shown. No *L. plantarum* ($< 10^2$/g grass) were detected for treatment 1 over the 14 days. Treatments were as described in Table 4.9.

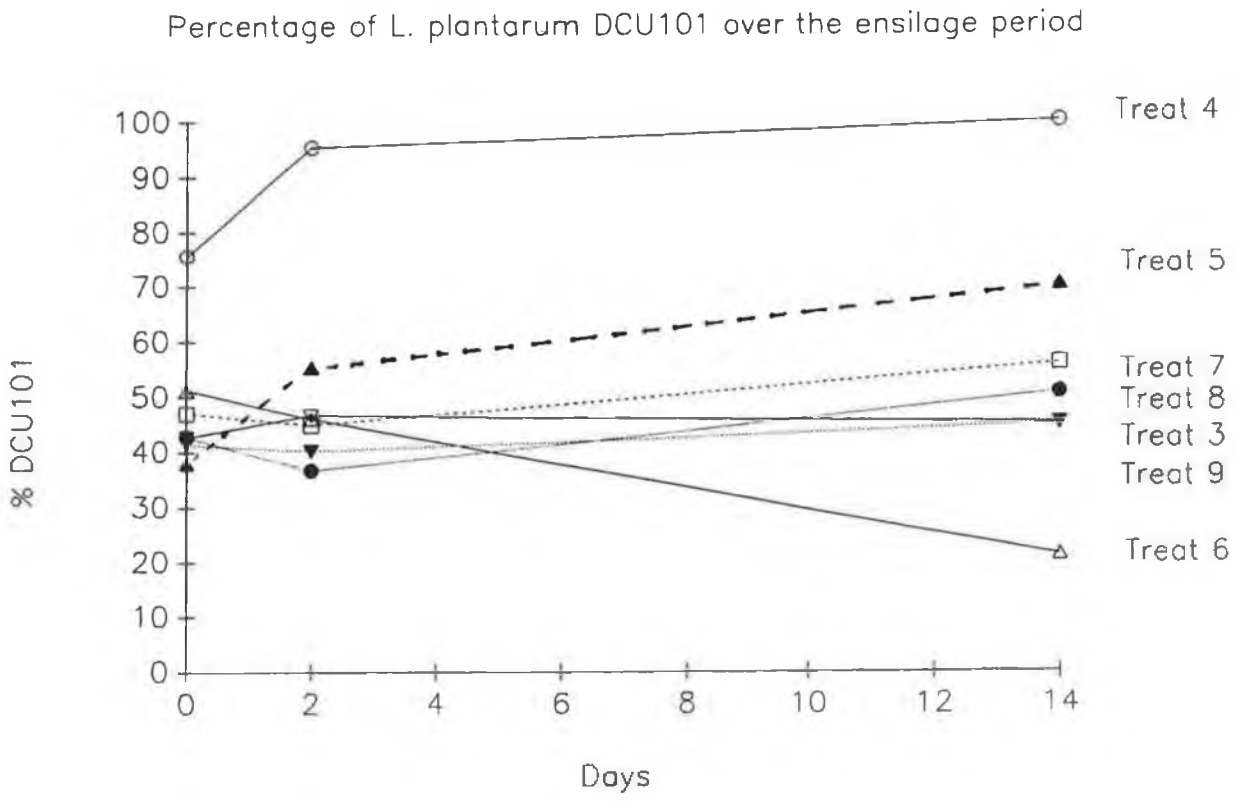


Figure 4.5. Percentage of *L. plantarum* DCU101 in mixed inoculations based on the colony hybridisation results over the fermentation period. The standard deviations are listed in Table 4.10.

The results indicated that in treatment 2, where *L. plantarum* DCU101 was inoculated alone at $1x10^6$ cfu/g, no significant loss of the plasmid p101a over the 14 day ensilage period was observed (98.33 $\pm$ 2.89% retained the plasmid). Treatment 6 resulted in the superior establishment of the test strain *L. plantarum* B2 (21.33 $\pm$ 4.93% DCU101) after 14 days, while in treatment 5, *L. plantarum* DCU101 was assuming dominance on the final day of sampling (70.33 $\pm$ 8.39% DCU101). The remaining treatments indicated varying abilities of the test strains to compete with *L. plantarum* DCU101.

To eliminate the possibility of plasmid loss or transfer to the test strain during fermentation and subsequent abberant hybridisation signals, a subsample of colonies which were both positive and negative in the hybridisation were further analysed. Chromosomal restriction endonuclease patterns confirmed that all the samples which were homologous to the pGB100 probe had a *L. plantarum* DCU101 restriction pattern, while those that showed no homology to the probe had the pattern of the co-inoculated test strain. Total DNA restriction patterns from a subsample of 30 isolates from treatments 3, 4 and 6 after 14 days were examined but no loss or transfer of plasmid p101a was detected (Plate 4.4).

The discrepancy between cell counts (51.55% DCU101) and the hybridisation results (75.67 $\pm$ 7.37% DCU101) in treatment 4 and the virtual elimination of strain 4-1-3 on day 2 (95.29 $\pm$ 3.82% DCU101) suggested that this strain had a poor viability at 10°C, the ambient temperature in the inoculating shed. Under laboratory conditions, it was subsequently confirmed that 4-1-3 was cold sensitive decreasing in number from $1x10^6$ cfu/ml to $3.6x10^5$ cfu/ml after a 27 hour incubation at 10°C.

The pH of the silage was monitored over the 14 days and the results are presented in Table 4.11. The cold conditions (10°C) experienced in April 1992 also resulted in the retardation of fermentation and no reduction in pH was detected on day 2 for any of the treatments. In all treatments, the final pH was lower for the inoculated silos when compared to the uninoculated controls.

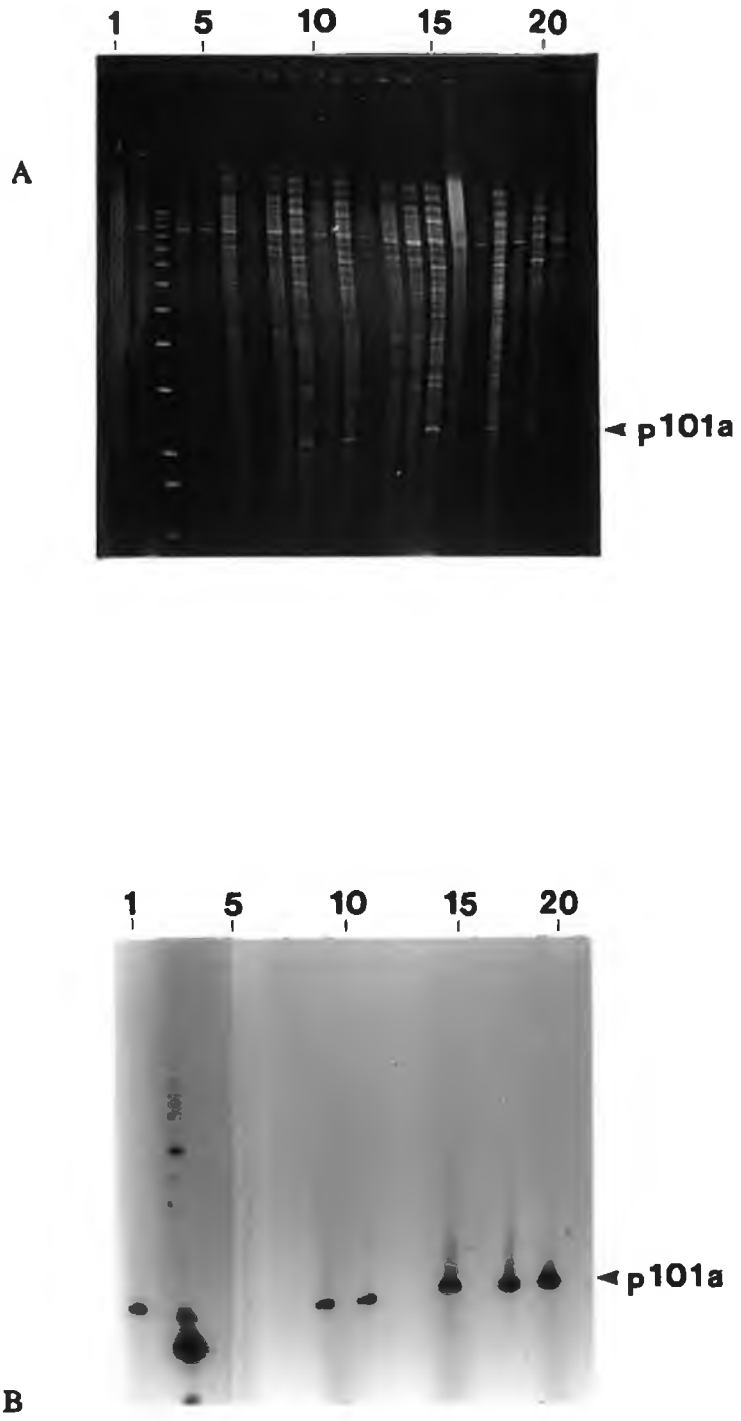


Plate 4.4. Chromosomal restriction (*Eco*RI) patterns (A) of a subsample of isolates from treatment 7 after 14 days fermentation and the resulting autoradiograph following hybridisation with pGB100 (B). The plasmid p101a is indicated. Lanes: (1) *L. plantarum* DCU101, (2) *L. plantarum* B2, (3) 1kb ladder, (4-22) *L. plantarum* isolates from treatment 7.

Treatment	Day 2	Day 7	Day 14
1	6.39 ± 0.01	5.13 ± 0.05	4.19 ± 0.05
2	6.49 ± 0.02	5.33 ± 0.13	4.04 ± 0.01
3	6.58 ± 0.02	5.07 ± 0.13	4.03 ± 0.01
4	6.44 ± 0.10	5.16 ± 0.05	4.11 ± 0.02
5	6.70 ± 0.08	5.03 ± 0.05	4.04 ± 0.02
6	6.63 ± 0.02	5.10 ± 0.14	4.01 ± 0.01
7	6.66 ± 0.04	4.93 ± 0.17	3.98 ± 0.01
8	6.59 ± 0.02	5.10 ± 0.08	4.07 ± 0.02
9	6.67 ± 0.07	4.93 ± 0.09	4.03 ± 0.01

Table 4.11. pH of the silage in the co-inoculation experiment over the 14 day fermentation period. Standard deviations (±) are in small print.

4.4.3 Silo trial of the selected *L. plantarum* strain B2.

Arising from its dominance in the first co-inoculation experiment, the *L. plantarum* strain B2 was selected for a further trial. Italian ryegrass was inoculated with *L. plantarum* B2 at a rate of $1x10^6$cfu/g grass. *L. plantarum* numbers and pH measured after 1, 3, and 8 days ensilage are shown in Tables 4.12 and 4.13 respectively. Inoculation with *L. plantarum* B2 brought about a more rapid pH reduction than the control silos which correlates with the increase in *L. plantarum* numbers.

Treatment	Day 1 (cfu/g)	Day 3 (cfu/g)	Day 7 (cfu/g)
uninoculated	$4.10x10^3 \pm 1.9$	$1.62x10^5 \pm 0.5$	$9.80x10^5 \pm 4.0$
B2	$2.25x10^6 \pm 0.6$	$1.19x10^8 \pm 0.4$	$3.28x10^8 \pm 1.0$

Table 4.12. *L. plantarum* numbers over seven day ensiling period following inoculation with *L. plantarum* B2. The initial *L. plantarum* numbers were about 47 cfu/g of grass for the uninoculated silos and $1.06x10^6$cfu/g of grass for the inoculated silos. The standard deviations ($\pm$) are in small print.

Treatment	Day 1	Day 3	Day 7
uninoculated	5.60 ± 0.07	4.51 ± 0.02	4.20 ± 0.01
B2	5.60 ± 0.03	4.45 ± 0.02	4.13 ± 0.01

Table 4.13. pH of silage following inoculation with *L. plantarum* B2 over the seven day fermentation (n=3, standard deviation printed small). The initial pH of the grass was 6.2 ± 0.01

This result indicated that *L. plantarum* B2 was capable of effecting preservation when inoculated alone and that the competition assay provided valuable information on its dominating ability.

4.4.4 Assessment of a further six *L. plantarum* strains over a 56 day ensilage period using the competition assay.

A second co-inoculation experiment was carried out to assess the performance of six more *L. plantarum* strains. The performance of freeze dried *L. plantarum* DCU101 in the silo in comparison to a fresh overnight MRS broth culture and the performance of the inoculants over a longer ensilage period of 56 days were also examined in this experiment.

The *L. plantarum* strains all originated from baled silage. *L. plantarum* B2 was included to repeat the evaluation of its performance in the competition assay. Six freeze dried vials of *L. plantarum* DCU101 ($7x10^9$ cfu in each) were obtained from V. Laffitte, Texel, Dange Saint Romain, France and were each resuspended in 10ml of sterile Ringers diluent. Fifty μl ($3.5x10^7$ cfu) was removed from one vial and serially diluted to confirm cell numbers. The treatments are listed in Table 4.14 and the ratios and final inoculation rates are listed in Table 4.15.

Unfortunately, the cell counts and subsequent hybridisation results indicated that the ratios of *L. plantarum* DCU101 to each test strain were not ideal as the culture of *L. plantarum* DCU101 had not grown optimally. The experiment was continued to examine the effect of applying cultures at different ratios and possibly inocula at different growth stages and to examine the long term stability of the inoculated silage.

Treatment	*L. plantarum* strain
1	sterile diluent
2	DCU101 (freeze dried)
3	DCU101 (fresh culture)
4	DCU101 + B2
5	DCU101 + A1
6	DCU101 + A2
7	DCU101 + A4
8	DCU101 + AF3
9	DCU101 + B18
10	DCU101 + B12

Table 4.14. Composition of inoculants in the second co-inoculation experiment.

Three hundred yellow colonies from treatment 2 (freeze dried DCU101) on the day of ensilage were hybridised with pGB100 to confirm the stability of the cryptic plasmid, p101a, over the larger scale industrial fermentation and freeze drying procedure. One hundred percent of the tested colonies yielded a positive signal indicating the presence of p101a, the cryptic plasmid upon which the probe was based. Chromosomal restriction analysis confirmed that the strain was not a contaminant with homology to the probe.

Treatment	Ratio of DCU101 to test strain	Inoculation rate
2	na	0.99 x10^6 cfu/g
3	na	0.61 x10^6 cfu/g
4	1 : 2.22	0.97 x10^6 cfu/g
5	1 : 2.20	1.13 x10^6 cfu/g
6	1 : 1.77	0.85 x10^6 cfu/g
7	1 : 1.55	0.97 x10^6 cfu/g
8	1 : 2.25	0.99 x10^6 cfu/g
9	1 : 1.75	1.04 x10^6 cfu/g
10	1 : 2.10	0.92 x10^6 cfu/g

Table 4.15. The ratios of *L. plantarum* DCU101 to the test strain in the second co-inoculation experiment. The treatments are listed in Table 4.14.

The pH of the silage and the microbial analysis over the fermentation period are listed in Tables 4.16 and 4.17 respectively. As is evident, the *L. plantarum* inoculants have effected a more homolactic fermentation resulting in a lower final pH. This is reflected in the microbial analysis where the *L. plantarum* numbers are more or less equal to the LAB numbers for the inoculated silages. The highest microbial counts were for treatment 2 (freeze dried DCU101) which was applied at the correct rate of 1x10^6 cfu/g grass and this treatment resulted in the lowest overall final pH (3.98). Treatment 3 (DCU101 from overnight MRS broth culture) was unfortunately inoculated at the lower level of 6.10x10^5 cfu/g grass and so no direct comparison between the two forms of inoculum preparation could be made. The reconstitution of the freeze dried DCU101 inoculum only a few hours before inoculation did not seem to have any deleterious effect on its preservation potential.

Treatment	Day 2	Day 56
1	6.01 ± 0.02	4.19 ± 0.02
2	6.03 ± 0.05	3.98 ± 0.02
3	6.09 ± 0.02	4.07 ± 0.02
4	6.12 ± 0.03	4.07 ± 0.01
5	6.14 ± 0.01	4.07 ± 0.02
6	6.16 ± 0.02	4.07 ± 0.01
7	6.16 ± 0.04	4.07 ± 0.01
8	6.16 ± 0.04	4.07 ± 0.02
9	6.20 ± 0.04	4.07 ± 0.01
10	6.17 ± 0.01	4.07 ± 0.02

Table 4.16. pH of the silage in the second co-inoculation experiment over the 56 day fermentation period. The standard deviations (±) are in small print. Treatments are described in Table 4.14.

Treatment	*L. plantarum* (x10^8 cfu/g)	LAB (x10^8 cfu/g)
1	< 10 cfu/g	0.99 ± 0.01
2	3.87 ± 1.98	3.04 ± 1.06
3	1.69 ± 0.09	1.95 ± 0.30
4	1.96 ± 0.53	1.39 ± 0.21
5	1.40 ± 0.31	1.60 ± 0.46
6	1.57 ± 0.61	1.57 ± 0.55
7	2.64 ± 0.29	1.18 ± 0.07
8	1.68 ± 0.71	1.54 ± 0.78
9	1.79 ± 0.13	1.40 ± 0.05
10	1.62 ± 0.45	1.65 ± 0.12

Table 4.17. Microbial analysis on the silage in the second co-inoculation experiment following 56 days fermentation. The standard deviations (±) are in small print. Treatments are described in Table 4.14.

Due to the incorrect inoculation rates, the number of colonies hybridised with the probe pGB100 was reduced to 100, with the exception of treatment 4 (*L. plantarum* B2) to rapidly determine if there was a pattern emerging from the incorrect *L. plantarum* ratios. The results are listed in Table 4.18.

Treatment	Day 0, (% based on cell counts)	Day 0	Day 56
3	nd	100.0	99.00
4	31.06	33.00	45.77[a]
5	33.33	30.00	66.00
6	36.10	34.00	48.00
7	39.22	19.00	41.00
8	30.77	34.00	52.00
9	36.36	39.00	65.00
10	33.22	32.00	46.00

[a] Number of colonies tested was 225

Table 4.18. Percentage of *L. plantarum* DCU101 over the 56 day fermentation period. Treatments are described in Table 4.14.

In all treatments, *L. plantarum* DCU101 appeared to increase from between 30.77 - 39.22% to 41 - 66%. This increase may be due to the higher competitive ability of the strain or due to the growth phase at which it was inoculated. The optical density (OD_{600nm}) of the *L. plantarum* DCU101 culture from which the inocula were prepared was 2.065 which corresponded to a cell count of about $6.1x10^8$ cfu/ml compared to the test strains which had reached

stationary phase, with cell counts of about $2x10^9$ cfu/ml. This difference in growth stage may have enabled *L. plantarum* DCU101 to recover rapidly and therefore compete more effectively than the co-inoculated strains during the initial stages of fermentation and subsequently predominate towards the latter stages of ensilage. Alternatively *L. plantarum* DCU101 may be more competitive than some test strains. The treatments which deviated from this domination by *L. plantarum* DCU101 included the inoculant *L. plantarum* A2, A4, B12 and B2 - a strain which had previously demonstrated its competitive ability when presented with a challenging *L. plantarum* DCU101 population. These results suggest that under correct co-inoculation levels, these four strains may be of interest for further analysis and that perhaps the growth stage of the inoculum is of importance.

4.5 Discussion

In selecting successful bacterial silage inoculants, the most important characteristics are dominance over the epiphytic microbial population and promotion of a rapid reduction in pH. All of the trials described in this chapter were carried out in laboratory silos described by O'Kiely and Wilson (1991) that had been previously shown to reflect farm scale silo conditions. For statistical purposes each treatment was carried out in triplicate and each silo opened only once which resulted in the utilisation of about 60 silos for each trial. In each experiment the *L. plantarum* inoculants effected a lower final pH than the uninoculated controls with a subsequent reduction in enterobacterial contamination. Where LAB numbers were estimated, the inoculants appeared to dominate the indigenous population. The application rate of $1x10^6$ cfu/g grass yielded a 2-10 fold excess of inoculant over the epiphytic LAB population which was found in one experiment to be $2.7 \pm 2.3x10^5$ cfu/g grass at ensiling. This reinforces the views of Rooke (1990) and Pahlow and Ruser (1988) who suggested an inoculation rate of $1x10^6$ cfu/g for grass silage.

In the selection of silage inoculants, it is vital that strains from well preserved silage, of a type at which the final inoculant is directed, are obtained. Grass isolates are unlikely to effect the best preservation in legume or maize silage. All of the *L. plantarum* strains described were obtained from well preserved Irish grass silages with the exception of *L. plantarum* LP115. Strains from well preserved silages are appropriate for consideration as the most suited to the unique microenvironment within the silo. Previous trials reported in the literature using lactobacilli isolated from environments other than silage were unsuccessful [Moon *et al.*, 1981; Ely *et al.*, 1982] and this was attributed to the inability of the strains to adapt to ensiling conditions and to outcompete the indigenous microbial population. The spontaneous lactic acid fermentation is a very complex, microbial process in which a very small population of LAB becomes the predominating microbial flora [Daeschel *et al.*, 1987] and so selection of the most competitive homofermentative inoculants is required. Preliminary laboratory trials incorporating measurement

of growth rate, rate of pH decrease, pH and temperature optima are usually carried out before the selection of strains for ensilage. Preliminary laboratory results (not reported here) indicated in this case that there were no significant differences using these criteria between the silage isolates. Indeed Woolford and Sawczyc (1984 a,b) following lengthy laboratory experiments, selected three strains including *L. plantarum* but reported no notable influence on grass silage preservation when inoculated at between 10^3 and 10^6 cfu/g grass. The most successful bacterial inoculant trials reported in the literature have included LAB isolated from silage [Gordon, 1989; Mayne, 1990; Kung *et al.*, 1991; Nesbakken & Broch-Due, 1991]. These reports prompted the development of a competition assay to evaluate potential silage inoculants.

To demonstrate the dominating potential of a silage inoculant strain, the following criteria must be fulfilled:

(1) A suitable silo model must be used, as optimum growth conditions provided in *in vitro* culture do not necessarily reflect those experienced in the silo [Woolford & Sawczyc, 1984a; Woolford & Sawczyc, 1984b].

(2) The inoculant strain must face a rigorous challenge from the indigenous population which therefore should be present in equal numbers to the inoculant strain. Trials with inoculants have reported improved crop preservation when the indigenous microbial population was low [Cocconcelli *et al.*, 1991]. However, this provides no assessment of strain performance when placed in a more competitive environment.

(3) The inoculant strain must increase in number or proportion in comparison to the indigenous population over the fermentation period. This would conclusively demonstrate that the inoculant is outcompeting the challenging population by virtue of its superior adaptation to silo conditions.

(4) The pH of the silage must be reduced, due directly to the addition of the inoculant strain, as preservation of the herbage is the desired ultimate effect.

Previous reports suggesting dominance of an inoculant based on biochemical results, microbial enumeration or strain tracing using DNA probes did not consider all of these criteria.

An assay based on the above requirements where the competitive

ability of each test strain was assessed against that of *L. plantarum* DCU101 was developed. The use of *L. plantarum* DCU101 as an internal control allows results from experiments carried out under different ensiling conditions to be compared. The four criteria mentioned above were fulfilled as follows. (1) Laboratory silos previously shown by O'Kiely and Wilson (1991) to provide conditions reflecting those experienced in farm scale silos were used. (2) The inoculant faced the challenge of competing for substrate with equal numbers of the co-inoculated *L. plantarum* strain. While LAB numbers can vary in the ensiled grass, it is generally agreed that numbers between 10^5 and 10^6/g grass exist at ensilage [Pahlow & Ruser, 1988; Fenton, 1987] and so the *L. plantarum* strains were co-inoculated at the equal rate of $5x10^5$/g grass. (3) The proportion of test strain to standard strain over the ensiling period was determined using DNA probe techniques. To identify a specific strain, a characteristic unique to that strain must be found. In the past, traits such as antibiotic resistance [Sharpe *et al.*, 1992; Torriani *et al.*, 1987] metabolic selectable markers [Drahos *et al.*, 1986; Dauenhauer *et al.*, 1984; Shaw & Kado, 1986], immunofluorescence [Trevors *et al.*, 1990; Postma *et al.*, 1988] plasmid profiling [Hill & Hill, 1986] and DNA probe hybridization [Cocconcelli *et al.*, 1991; Sayler *et al.*, 1985; Van Elsas *et al.*, 1989; Trevors *et al.*, 1990] have been used. Strain monitoring using an indigenous plasmid DNA probe provides the advantage that large numbers of colonies can be identified rapidly without the need to genetically manipulate the strain in any way. The use of plasmid profiling of *L. plantarum* in silage fermentations reported by Hill and Hill (1986) demonstrated the stability of plasmids over the fermentation period. Plasmid profiling, while simple and rapid, is not practical for screening thousands of isolates. The application of a strain specific plasmid probe and large-scale colony hybridisations overcame this limitation. The DNA probe was based on a small cryptic plasmid shown to be stable and non-transferable over the ensiling period. The stability of the *L. plantarum* DCU101 plasmid as demonstrated by chromosmal restriction analysis and colony hybridisation has also been demonstrated for *P. pentosaceous* and *L. plantarum* strains isolated from maize silage [Cocconcelli

et al., 1991]. In combination with LP medium, selective for *L. plantarum*, the ratios of test strain to indicator strain were monitored. Due to the low numbers of indigenous *L. plantarum*, the *L. plantarum* isolates screened were assumed to have all originated in the inoculum. The occurrence of dominance within this population was thereby detected. (4) A reduction in pH compared to uninoculated controls was detected over the ensiling period.

This strategy ensured that in each case the test strain was challenged in a rigorous and reproducible manner for dominance within the silo regardless of the epiphytic LAB numbers which vary considerably and the assay was carried out in conditions resembling those encountered by a commercial inoculant under farm conditions.

Care must be taken when selecting strains from silage for use as silage inoculants as the present work demonstrates that, even within the subpopulation of *L. plantarum* isolated from well preserved silages, the ability to compete varies, indicating the need to assess competitiveness of potential inoculants. The results from chapter 3 suggested that competitive strains could be identified by analysing strain heterogeneity among silage isolates. Both dominant and non-dominant strains were identified and included in the co-inoculation competition assay. However, no correlation between dominance at the latter stages of the fermentation and competitive ability during the initial stages of ensilage could be made. The isolate, *L. plantarum* B2 which appeared to be a unique strain (non-dominant) among the isolates from silage B was the most competitive strain in the first silo trial while *L. plantarum* AF2, the second most dominant strain type in silage A performed badly in the competition assay (resulting in about 70.33% *L. plantarum* DCU101 on day 14). The strains used in this study were isolated from well preserved silages which were at the latter stages of the fermentation and so a low pH and high levels of acetic acid are likely to have been present. This would have resulted in the survival and dominance of strains most suited to these conditions. However, these strains would not necessarily be the most suited to conditions at the onsest of fermentation. Perhaps *L. plantarum* B2 was more dominant in silage B during the early stages of ensiling and only a few isolates of this

strain type remained. This would seem to explain the results from the competition experiment which was carried out at the onset of fermentation. Perhaps *L. plantarum* AF2 would have become more dominant if left for a longer period. This suggests that to identify competitive inoculants, strain heterogeneity should be examined in silages at the early stages of the fermentation, the most dominant strains selected, tested in a competition assay such as that described in this chapter and reintroduced as a single inoculant.

The results of the first co-inoculation experiment indicated that *L. plantarum* B2 was the most competitive strain tested, and it was subsequently shown to be capable of improving silage preservation when used as a single inoculant. This is the first report to my knowledge of the demonstration of dominance between *L. plantarum* strains co-inoculated at equal rates. Co-inoculation ensures that under variable ensiling conditions, regardless of the epiphytic bacterial population, competition is always provided at the same rate by the internal control strain *L. plantarum* DCU101, thus facilitating an assessment of the capability of the test strain to dominate in the silo.

Chapter 5

The analysis and evaluation of PCR amplified variable regions of 16S rRNA for use as genus and species specific DNA probes

5.1 The Polymerase Chain reaction

5.1.1 Introduction

The polymerase chain reaction (PCR) permits the *in vitro* amplification of selected nucleic acid sequences. It consists of repetitive cycles of DNA denaturation, primer annealing and then extension by a DNA polymerase. Two oligonucleotide primers bind to sequences flanking the sequence to be amplified and are repeatedly heat denatured, hybridised to their complementary sequences and extended with *Taq* DNA polymerase - the most useful of the DNA polymerases for this technique. The primers are chosen so that when each is extended the newly synthesised strands will overlap the binding site of the opposite oligonucleotide resulting in the exponential increase in the number of target sequences.

Many applications for PCR have been reported since the first description of the method, including its use in the identification of bacteria. Barry *et al.* (1990) reported a method that permitted the rapid generation of DNA probes for eubacteria based on PCR amplification of the variable regions of 16S rRNA. The method was shown to be applicable to *Salmonella typhimurium, Staphylococcus aureus, Clostridium perfringens, Klebsiella pneumoniae, Pseudomonas fluorescens, Aeromonas salmonicida* and *Mycobacterium bovis*. Using primers based on conserved regions flanking the variable regions, V1 and V6, fragments of about 100bp were generated. These fragments were sequenced and genus and species specific oligonucleotides identified.

The construction of genus and species specific probes using this method for the identification of vagococci [Williams & Collins, 1992] and the mesophilic lactic acid bacteria [Klijn *et al.*, 1991] has also been reported.

The success of the PCR reaction is dependent on a number of factors which are described in detail below.

5.1.2 Temperature Cycling Parameters

The selection of times, temperatures and number of cycles depends on the DNA being amplified and the primers chosen. PCR is performed by incubating the samples at three temperatures corresponding to denaturation, annealing and extension. In a typical PCR cycle, the DNA is denatured by briefly heating the sample to 90°C - 95°C for one minute, cooling to 40°C - 60°C for 30 seconds to allow primer annealing, followed by primer extension at 72°C for one minute. The first cycle is preceded by an initial denaturation step at 94°C - 95°C for 3-5 minutes.

The most critical temperatures are the denaturation and annealing temperatures. Inadequate heating at the denaturation stage leads to failure of DNA melting and no amplification. The annealing step determines the specificity of the PCR. Using too low a temperature results in mispriming and amplification of non-target sequences. Too high a temperature causes a lack of primer annealing with no DNA amplification. In the case of amplification of a short target sequence (< 100-300bp), the extension step can be eliminated as *Taq* DNA polymerase retains significant activity at lower temperatures and so will complete extension during the transition from annealing to denaturation. Extension times which allow one minute per 1kb target DNA should be enough and this step can be between one and seven minutes depending on the length of target DNA to be amplified.

The temperature at which annealing is done depends on the length and G+C content of the primers. A temperature of 55°C is a good starting point for typical 20 base oligonucleotides with about 50% G+C content; even higher temperatures may be necessary to increase primer specificity. Because of the very large molar excess of primers present in the reaction, hybridisation occurs almost instantaneously and long incubation at the annealing temperature is not required.

The number of cycles required depends on the abundance of target DNA and efficiency of the PCR. A common mistake is to execute too many cycles which increases the amount and complexity of nonspecific backround products. For the amplification of plasmid inserts from tooth-picked colonies,

20 cycles is sufficient, amplification from genomes usually requires a minimum of 30 cycles although typically, 40 cycles are performed.

Due to the tremendous amplification power of the PCR, great care should be taken to guard against accidental contamination of solutions and samples with exogenous DNA. Negative controls should always be included to monitor the purity of solutions used in the procedure. Because of its amplification power, there is concern over the fidelity of DNA replication by PCR. The reports on error rate during PCR amplification vary but as a general rule base substitutions occur about one in every 9000bp and frameshifts occur about one in every 40,000bp [Tindall & Kunkel, 1988]. The most common changes reported by Dunning *et al.* (1988) were A to G and T to C. Seventy seven percent of the changes noted were associated with run off bases of the same sequence. While one error in 9000bp may seem insignificant, after 30 cycles of PCR it leads to an error in every 300bp of product. If amplification is performed from single DNA molecules, or PCR products are cloned prior to sequencing, this high error rate will be encountered.

5.1.3 Primer selection

PCR primers are oligonucleotides typically 15-30 bases long and are complementary to sequences defining the 5' ends of the complementary template strands. Sequences not complementary to the template can be added to the 5' end of the primers to allow a variety of useful post amplification operations on the PCR product, such as restriction, without disrupting the PCR itself. Where possible primers should have an average G+C content of 40-60% with no long stretches of any one base and pairs of primers should have the same melting temperature (T_m). It is important that the primers do not contain any complementary structures longer than 2 bases, especially at the 3' ends, as this may promote the formation of an artifactual product called a primer-dimer. DNA polymerase adds nucleotides to the 3' end of its target sequence. When the 3' ends of two primers hybridise, they form a primed template complex and primer extension results in this short duplex product

usually about twice the length of the primers; the primer-dimer. This reduces the availability of dNTP's for the correct amplification product and may have a role in product inhibition (figure 5.1).

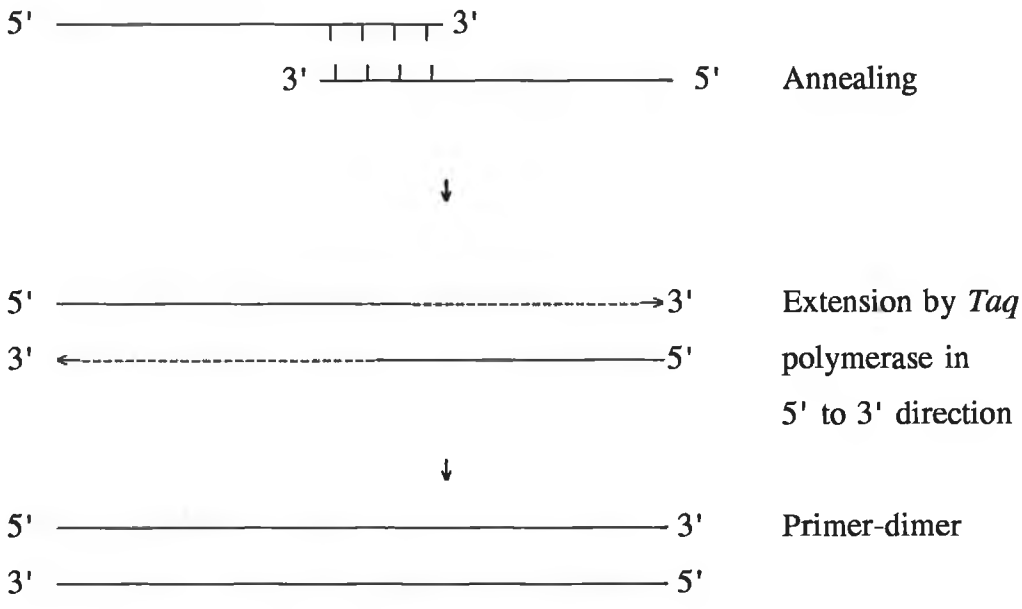


Figure 5.1. Primer-dimer formation resulting from base complementarity at the 3' ends of primers. The primer-dimers can act as templates for the following annealing and extension steps.

Internal secondary structure should be avoided in primers particularly at the 3' ends of each. Usually each primer is used in the 0.1 - 1μM range for symmetric PCR amplification. Higher concentrations may promote mispriming and accumulation of nonspecific products. In asymmetric PCR, the ratio of primers may be 50:1 or 100:1 and absolute primer amounts may range from 100 - 0.5 pmol in a 100μl reaction.

It is generally advisable to use purified oligonucleotides of the highest chemical integrity. Using lower primer concentration reduces cost and may help to reduce the primer-dimer artifact. The primers should be stored at -20°C when not in use and the shelf life of oligonucleotides is at least 6 months when stored in liquid and 12 - 24 months when stored after lyophilisation [Bej

et al., 1991].

The annealing temperature (T_a) of the primers should be kept between 60°C and 65°C for specific priming and removal of unnecessary "ghost" amplified bands when genomic DNA or mRNA are used for PCR amplification. The T_a can be calculated from the melting temperature (T_m) of the primers using the equation;

$$T_a = T_m - 5°C = 2(A+T) + 4(G+C) - 5°C$$

Optimum annealing temperatures must be determined empirically and may be higher than predicted. *Taq* DNA polymerase does have activity in the 37°C - 55°C region so primer extension will occur during the annealing step and the hybrid will be stabilised [Williams, 1989].

5.1.4 Target DNA

The amount of target DNA typically used for PCR of single genomic targets is 0.05 to 1μg. There should be at least one intact DNA strand and impurities (EDTA, phenol, detergent) should be eliminated or diluted so as not to inhibit amplification. Extensive purification of the DNA is not necessary for successful DNA amplification and often boiling or lysing the cells is enough. It is essential to ensure full denaturation of the template before starting the PCR, usually by heating to 93°C - 95°C for 5 minutes. This provides the additional safeguard of inactivating endogenous enzymes.

A serious problem in PCR amplification is contamination with previously amplified DNA or other exogenous sequences which can serve as target for the primers. Enzymes themselves are often contaminated with DNA. Several methods such as UV treatment, restriction enzyme digestion or use of psoralen have been described to overcome this problem. UV treatment of the PCR reaction prior to amplification involves exposure of the target DNA to a combination of 254nm and 300nm UV for 5 - 20 minutes. Psoralen which intercalates into double stranded DNA and forms a covalent interstrand crosslink after UV treatment (320-400nm) has been used successfully to remove contaminating exogenous double stranded DNA [Bej *et al.*, 1991].

5.1.5 DNA polymerase

With a specific activity of ca. 200,000 units/mg, a broad temperature optimum of 70 - 80°C (depending on the template used) and a nucleotide incorporation rate of about 150 nucleotides/second/enzyme molecule, *Taq* DNA polymerase replaced *E. coli* DNA polymerase I as the enzyme of choice for PCR. *Taq* DNA polymerase activity is optimal over a broad pH range from 8.2 - 9.0 in 10mM Tris but declines at lower or higher pH [Ehrlich, 1989]. In a 100μl reaction, 2 - 2.5 units of *Taq* DNA polymerase are sufficient to extend 1000 - 4000 bases per minute in an average PCR cycle.

Although *Taq* DNA polymerase has a very limited ability to synthesise DNA above 90°C, the enzyme is relatively stable at and is not irreversibly denatured by exposure to high temperatures. In a PCR mix, *Taq* DNA polymerase retains about 50% of its activity after 130 minutes, 40 minutes and 5 - 6 minutes at 92.5°C, 95°C and 97.5°C repectively. A PCR reaction of 50 cycles can retain 65% polymerisation activity when the upper limit temperature is 95°C for 20 seconds in each cycle [Ehrlich, 1989].

Taq DNA polymerase activity is dependent on the concentration of free Mg^{2+} in the reaction. High concentrations are inhibitory, with 40 - 50% inhibition at 10mM $MgCl_2$. Since dNTP's can bind Mg^{2+}, the precise concentration of $MgCl_2$ that is required for optimum enzyme activity is dependent on the dNTP concentration.

Modest concentrations of KCl stimulate the synthesis rate of *Taq* DNA polymerase by 50 - 60% with an apparent optimum at 50mM. Higher KCl concentrations begin to inhibit activity. Low concentrations of urea, DMSO, Dimethlyformamide or formamide have no effect on *Taq* DNA polymerase incorporation activity. Ethanol (10%) fails to inhibit *Taq* activity and the inhibitory effects of low concentrations of SDS can be completely reversed by high concentrations of certain nonionic detergents (Tween 20 and NP40).

5.1.6 Deoxynucleoside triphosphates (dNTP's)

The dNTP's (dATP, dCTP, dGTP and dTTP) are usually present at 50 - 200μM each. It is important to keep the four dNTP concentrations balanced for the best base incorporation fidelity. Lower concentrations may promote higher fidelity and specificity with no reduction in product yield. Greater than 50mM (final concentration) dNTP in the PCR reaction inhibits *Taq* DNA polymerase activity [Bej *et al.*, 1991]. 20μm of each dNTP in a 100μl reaction is theoretically sufficient to synthesise 2.6μg of DNA or 10 pMol of a 400bp sequence.

5.1.7 Magnesium Chloride concentration

The free Mg^{2+} ion concentration must be adjusted for different PCR experiments. An optimum usually exists in the 1 - 10 mM range. *Taq* DNA polymerase requires free Mg^{2+} along with that bound by template DNA, primers and dNTP's. The presence of EDTA or other chelators may disturb the apparent Mg^{2+} optimum. The concentration of Mg^{2+} affects primer annealing, strand dissociation temperature, product specificity, formation of primer-dimer artifacts, enzyme activity and PCR fidelity. The standard PCR buffer containing 50mM KCl, 10mM Tris-HCl (pH 8.4), 1.5mM $MgCl_2$ and 100μg/ml of gelatin should be adequate for the majority of PCRs. In the presence of 0.8mM total dNTP concentration, a titration series in small increments over the 1.5 - 4mM range will locate the Mg^{2+} concentration producing the highest yield of specific product. Too little Mg^{2+} will result in no PCR product and too much will produce a variety of unwanted products. The total dNTP and Mg^{2+} concentration are related and should not be changed independently. Initially, the Mg^{2+} concentration should exceed the total dNTP concentration by 0.5 - 1mM [Williams, 1989].

5.1.8 Mineral oil

During PCR amplification, it is recommended to overlay 80 - 100μl of light, reagent grade mineral oil on top of the PCR reaction mix. This prevents evaporation and internal condensation which, particularly at the denaturation

step, can greatly affect the PCR product yield in a reaction volume of 100μl or less. It has been shown that the use of light mineral oil increased the yield of amplified product approximately 5 times [Bej *et al.*, 1991]. This may be due to the maintenance of heat stability and salt concentrations throughout the reaction mixture. The volume of oil should be minimised to achieve efficient cycling. In addition mineral oil or glycerin is sometimes used as a thermal transfer fluid in the wells of the sample block. With close fitting tubes this practice is unnecessary [Williams, 1989].

In this section, the 16S rRNA variable regions, V1 and V6 of *L. plantarum* were amplified, cloned and sequenced. The sequences were compared and analysed using the computer programme Seqaid^{TM} and access to the GenBank and BLAST facilitated homology searches. Species specific oligonucleotides were selected for *L. plantarum* based on computer alignments.

5.2 Results of PCR reactions

5.2.1 Primer selection

The primers designed to amplify the V1 and V6 regions of rRNA were as described by Barry *et al.* (1990) and the sequences are given in figure 5.2. A secondary structure model of the variable regions in procaryotic 16S rRNA is reproduced from Neefs *et al.* (1990) in figure 5.3 and the *E. coli* 16S rRNA nucleotide sequence, reproduced from Woese *et al.* (1983), in figure 5.4. The theoretical melting temperature (T_m) for each primer was calculated according to a rough estimation of allowing 2°C for an A or T and 4°C for a G or C. The sum of the bases yields the T_m [Ehrlich, 1989].

Primers

	%GC	Theoretical T_m
V1 region		
R1 5'-AATTGAAGAGTTTGATCATG-3' (position 2-21)	30	52°C
R2 5'-ACATTACTCACCCGTCCGGC-3' (position 104-123, converse complement sequence)	60	64°C
V6 region		
R3 5'-GCAACGCGAAGAACCTTACC-3' (position 966-985)	55	62°C
R4 5'-AGCCATGCAGCACCTCTCTC-3' (position 1041-1060, converse complement sequence)	60	64°C

The positions outlined are from the *E. coli* numbering system of Woese *et al.*, 1983.

Figure 5.2 Primer sequences designed to amplify the V1 and V6 variable regions of 16S rRNA.

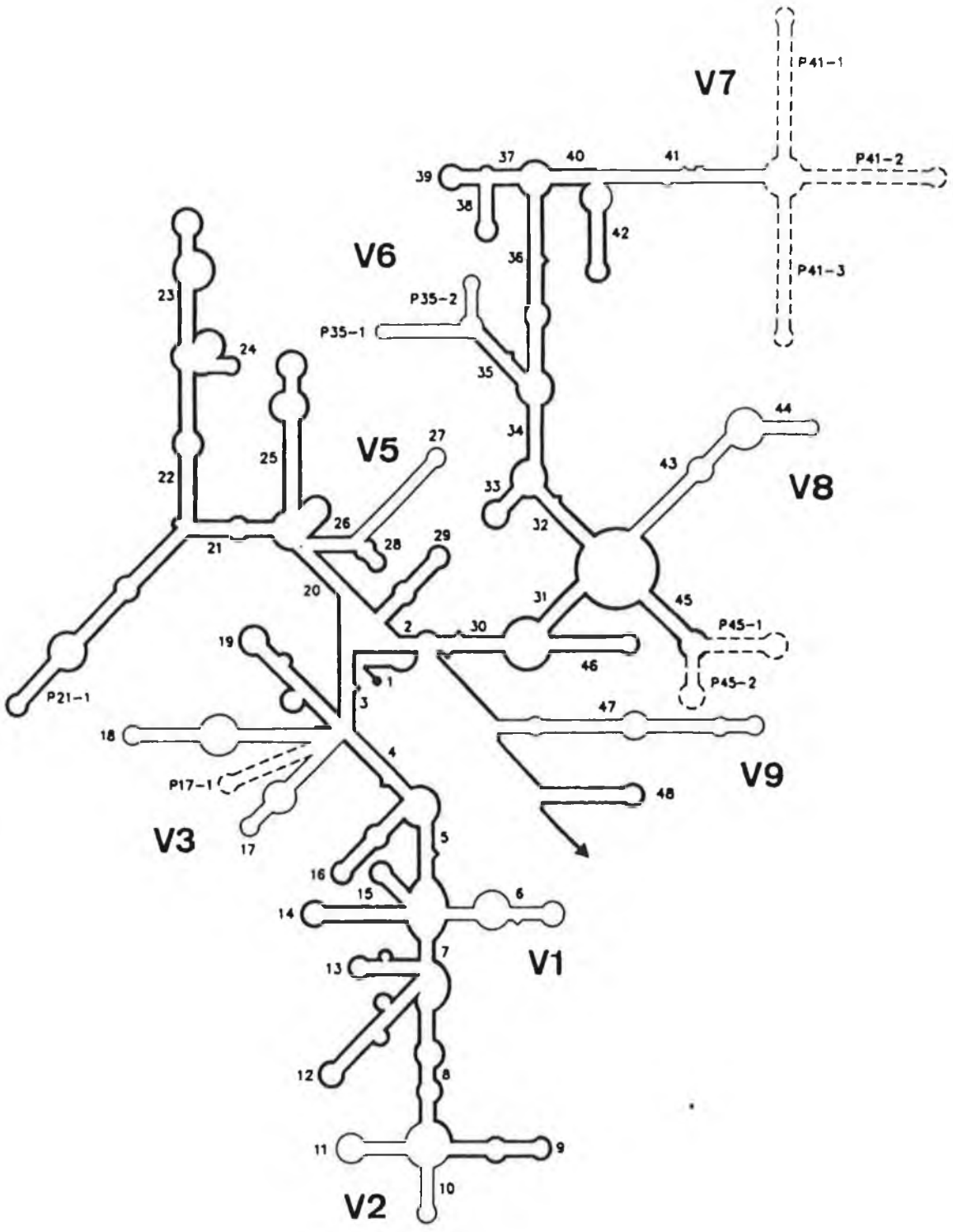


Figure 5.3. A secondary structure model of the variable regions in prokaryotic 16S rRNA, reproduced from Neefs *et al.* (1990).

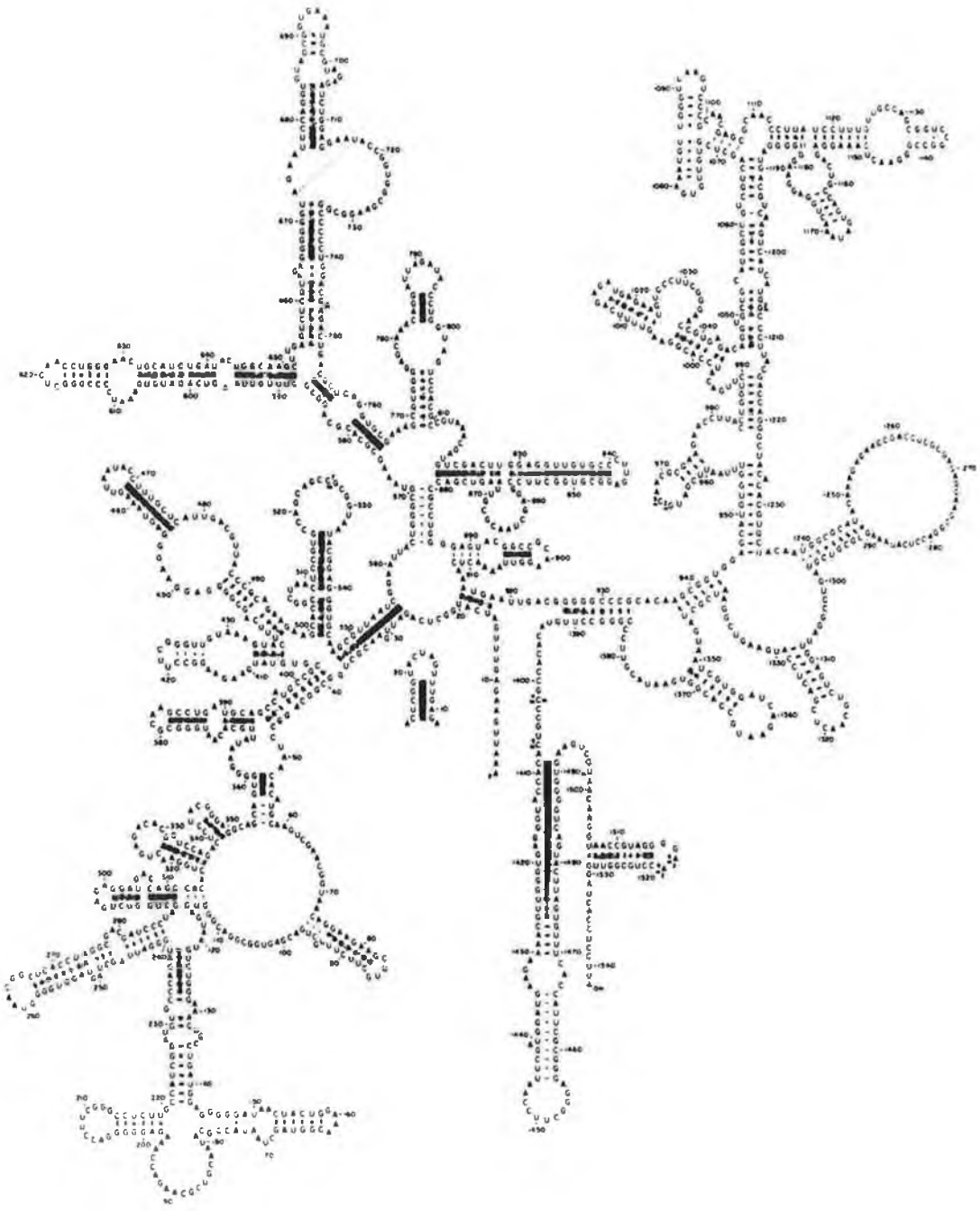


Figure 5.4. The nucleotide sequence of *E. coli* 16S rRNA reproduced from Woese *et al.*, (1983).

5.2.2 Optimisation of PCR amplification conditions

The annealing temperature was chosen as 45°C for the initial set of PCR reactions which were carried out as follows;

10x PCR buffer (Promega)	10μl
primers	2 x 3μl*
dNTP's	10μl†
H_2O	70.5μl
template	3μl‡
Taq DNA polymerase	0.5μl (2.5 units)
	100μl

The PCR cycling steps were as follows;

1.	95°C	5 minutes
2.	95°C	1 minute
3.	45°C	1 minute, 30 seconds
4.	72°C	2 minutes

Steps 2 to 4 were repeated for 30 cycles and the samples refrigerated

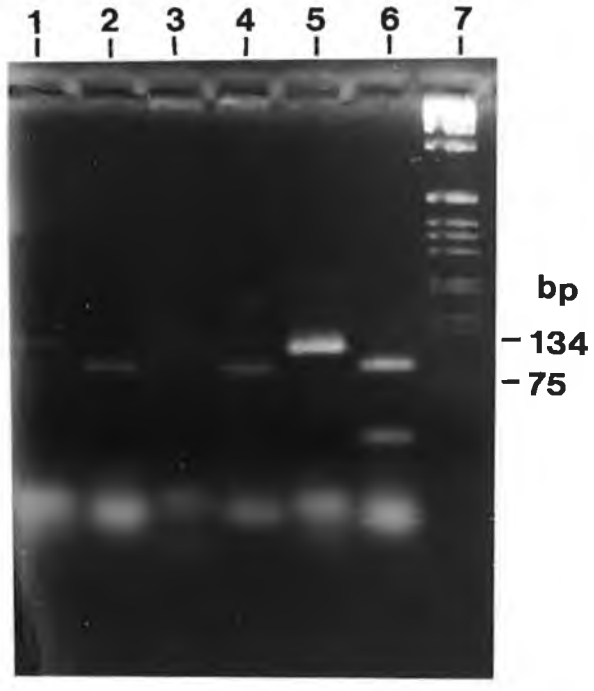
* The primer concentration was estimated using gel electrophoresis with a 300bp fragment of known concentration. Primers R1 and R2 were estimated to be 62.5ng/μl and primers R3 and R4 were approximately 12.5ng/μl. When added to a 100μl reaction as described, the final quantities were 0.6μM and 0.12μM in total.

† The dNTP's were stored separately as 10mM stock solutions. For the PCR, 2.5μl of each were mixed and 10μl added to the reaction. The final concentration of dNTP's was 1mM (250μM each).

‡ The template DNA was isolated from *L. plantarum* according to the method described in section 2.11 and the concentration varied between 0.2 and 1μg/μl.

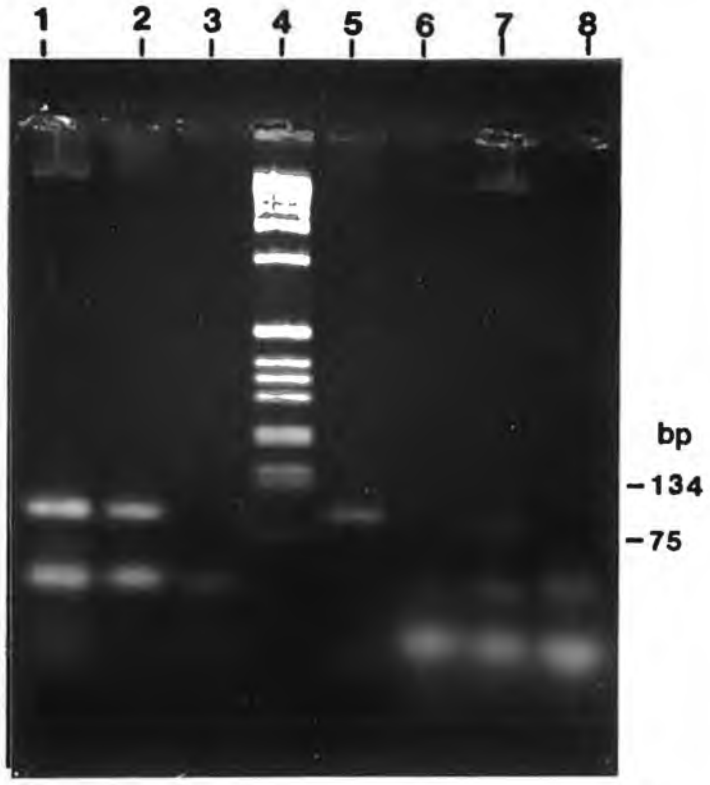
Both the V1 and V6 regions for *L. plantarum* strains DCU101, LP115 and *L. amylovorus* NRRLB4540 were amplified and analysed using 4% Nusieve™ agarose (FMC). Plate 5.1 shows an example of the number of PCR products resulting from the initial amplification conditions. A lot of non-specific bands occurred above the expected 100bp fragment and primer-dimers can be seen weakly for the V6 region of *L. amylovorus* (PLate 5.1, lane 4) and very strongly for the V6 region of *L. plantarum* DCU101 (Plate 5.1, lane 6).

An increase in annealing temperature to 50°C, a reduction in time at 72°C to 30 seconds and an increase in cycle number to 35, for the V6 region helped decrease the number of non-specific bands. However, primer-dimers were still very strong (plate 5.2). To try and reduce the non-specific amplification, a number of control experiments were carried out for PCR optimisation.



Lane	PCR product
1	V1 of *L. plantarum* LP115
2	V6 of *L. plantarum* LP115
3	V1 of *L. amylovorus*
4	V6 of *L. amylovorus*
5	V1 of *L. plantarum* DCU101
6	V6 of *L. plantarum* DCU101
7	1kb ladder

Plate 5.1. PCR amplification of the V1 and V6 regions of 16S rRNA using an annealing temperature of 45°C.



Lane	PCR product
1	V6 of *L. plantarum* LP115 at 50°C
2	V6 of *L. plantarum* DCU101 at 50°C
3	V6 negative control at 50°C
4	1 kb ladder
5	V6 of *L. amylovorus* NRRLB 4540 at 45°C
6	V1 of *L. plantarum* LP115 at 45°C
7	V1 of *L. plantarum* DCU101 at 45°C
8	V1 of *L. plantarum* LP115 at 45°C

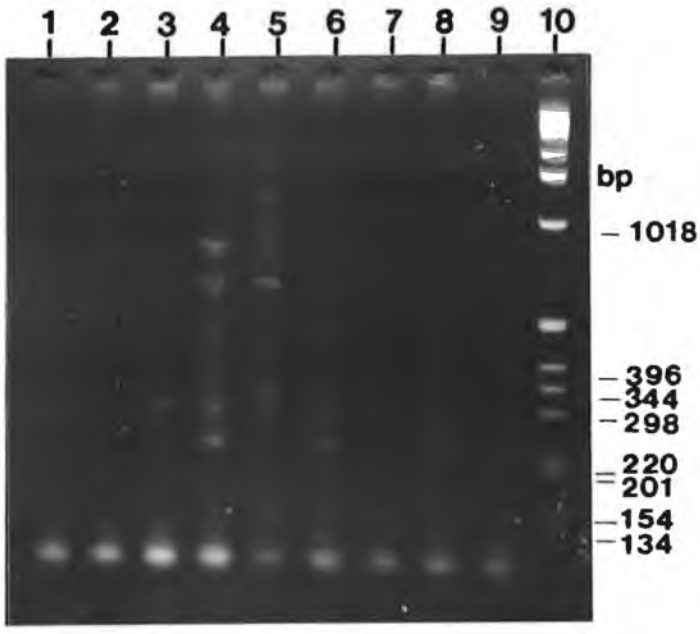
Plate 5.2. PCR amplification of the V1 and V6 regions of *L. plantarum* LP115 and *L. plantarum* DCU101 at an annealing temperature of 50°C and 45°C. The negative control PCR reaction contained no target DNA.

$MgCl_2$ concentration

The $MgCl_2$ concentration was changed in 1mM increments between 1.5mM (normal buffer) and 4.5mM for the V6 region at the annealing temperature of 50°C for the target DNA of *L. plantarum* DCU101 and *L. plantarum* LP115. Sterile mineral oil (40μl) was overlaid on the 100μl reactions to prevent evaporation or condensation and the results are shown in plate 5.3. It clearly demonstrated that with an increase in $MgCl_2$ concentration, the desired PCR product was reduced, with an increase in the number of non-specific products. The optimum was concluded to be 1.5mM $MgCl_2$.

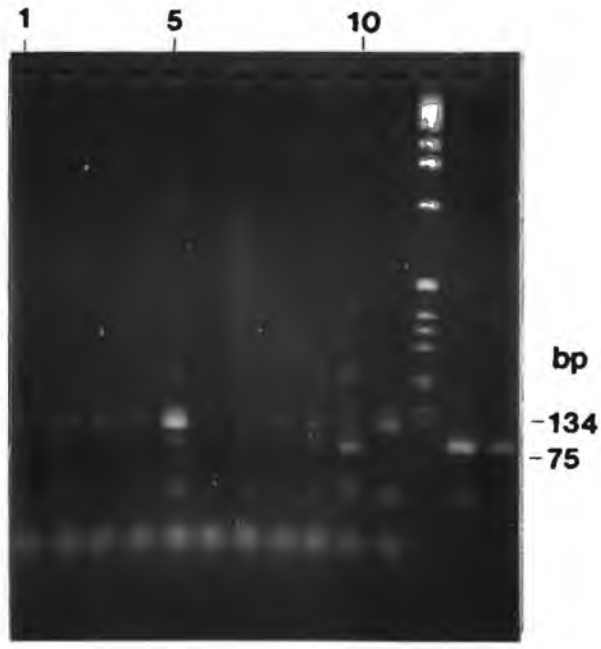
Taq DNA polymerase concentration.

A titration of *Taq* DNA polymerase from 0.5 units to 2.5 units in the 100μl reaction volume was carried out using the V1 region of *L. plantarum* LP115 and *L. plantarum* DCU101. The annealing temperature was 50°C and the $MgCl_2$ concentration was 1.5mM however no mineral oil was added to the samples. This may have resulted in the negative control reaction yielding a 100bp band along with a primer-dimer (plate 5.4), due to contaminated condensation falling from the lid back into the reaction, or one of the reaction components may have been contaminated. With the increase in the *Taq* DNA polymerase concentration, the number of non-specific amplification products also increased, but the overall product yield was very low in comparison to lanes 13 and 14. These contained the V6 regions of *L. plantarum* DCU101 and *L. plantarum* LP115 amplified at the same annealing temperature (50°C) but with an overlay of mineral oil. Because R1 has a theoretical melting temperature of 52°C, it was thought that an annealing temperature of 50°C may have caused the lower product yield but the effect of mineral oil was also investigated.



Lane	PCR product
1	1.5 mM $MgCl_2$, *L. plantarum* LP115
2	1.5 mM $MgCl_2$, *L. plantarum* DCU101
3	2.5 mM $MgCl_2$, *L. plantarum* LP115
4	2.5 mM $MgCl_2$, *L. plantarum* DCU101
5	3.5 mM $MgCl_2$, *L. plantarum* LP115
6	3.5 mM $MgCl_2$, *L. plantarum* DCU101
7	4.5 mM $MgCl_2$, *L. plantarum* LP115
8	4.5 mM $MgCl_2$, *L. plantarum* DCU101
9	1.5 mM $MgCl_2$, negative control
10	1kb ladder

Plate 5.3 The result of varying $MgCl_2$ concentration on the PCR of the V6 region, amplified at an annealing temperature of 50°C for *L. plantarum* DCU101 and *L. plantarum* LP115.



Lane	PCR product
1	0.5 units, *L. plantarum* LP115
2	1.0 units, *L. plantarum* LP115
3	1.5 units, *L. plantarum* LP115
4	2.0 units, *L. plantarum* LP115
5	2.5 units, *L. plantarum* LP115
6	0.5 units, *L. plantarum* DCU101
7	1.0 units, *L. plantarum* DCU101
8	1.5 units, *L. plantarum* DCU101
9	2.0 units, *L. plantarum* DCU101
10	2.5 units, *L. plantarum* DCU101
11	2.5 units, negative control
12	1kb ladder
13	2.5 units, V6 LP115 with mineral oil
14	2.5 units, V6 DCU101 with mineral oil

Plate 5.4. The effect of varying Taq DNA polymerase concentration on the amplification of the V1 regions of *L. plantarum* LP115 and *L. plantarum* DCU101. The annealing temperature used was 50°C.

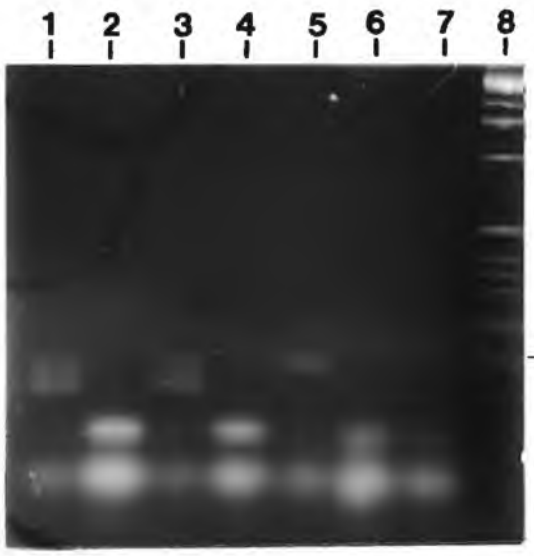
Effect of mineral oil addition

A series of PCR reactions were carried out on V1 at an annealing temperature of 42°C with and without a mineral oil overlay and the results were quite startling (plate 5.5). It was very obvious that without mineral oil the predominant PCR product was a primer-dimer which was completely eliminated when oil was added. This was repeated using 2.5 and 5 units of *Taq* DNA polymerase and a total of 0.4μM and 0.6μM of the primers. In each case the addition of oil eliminated or severely reduced primer-dimer formation. The second band seen just below the desired fragment in lanes 1 and 3 may have been a single stranded amplification product resulting from a partially denatured product.

The PCR cycle used to amplify the V1 and V6 regions for cloning was as follows. Mineral oil was overlaid, the *Taq* DNA polymerase concentration was 0.5 units and the $MgCl_2$ concentration was 1.5mM.

1. 95°C	10 minutes
2. 95°C	30 seconds
3. 50°C	30 seconds
4. 72°C	30 seconds

Steps 2-4 were repeated for 30 cycles and a refrigeration step included at the end.



Lane	PCR product
1, 3	DCU101 with oil
2, 4	DCU101 without oil
5	LP115 with oil
6	LP115 without oil
7	negative control
8	1kb ladder

Plate 5.5. The effect of mineral oil overlay on the V1 PCR products of *L. plantarum* DCU101 and *L. plantarum* LP115. The annealing temperature was 42°C.

5.2.3 Preliminary analysis of PCR products

When carrying out PCR reactions, two types of negative controls are always included. These are controls where all the components of the reaction except the template DNA are added and put through the amplification cycle and negative DNA controls where DNA sequences not containing the target sequence are amplified. Due to the ubiquity of rRNA and the degeneracy of the primers, bacterial DNA could not be included for the second type of negative control.

To verify that the PCR product did indeed represent the variable region of 16S rRNA and not some artifact or contaminating sequence, two experiments were carried out. Initially pKK3535, the recombinant plasmid containing the entire *E. coli* rRNA operon was subjected to PCR amplification alongside *Lactobacillus* DNA and this yielded a strong band of the same molecular weight (approximately 100bp). Since there were only vector and rDNA sequences in the sample, it was assumed that the PCR fragment was indeed rDNA (plate 5.6).

However, chromosomal DNA may have been present in minute undetectable quantities in the plasmid preparation which may also have resulted in the amplification of the rDNA regions and so the amplified regions were used in a hybridisation experiment with *L. plantarum* total DNA. Both the V1 and V6 regions amplified from *L. plantarum* LP115, cleaned using the Magic PCR preps™ columns, were labelled with ^{32}P using random priming and hybridised at 64°C overnight with DNA from DCU101 and *L. plantarum* LP115. Following stringent washing (final wash at 68°C for 1½ hours) the resulting signal demonstrated that V6 hybridised with the L. plantarum strains yielding a number of bands (plate 5.7). Unfortunately the V1 region yielded no signal which may have been due to inadequate denaturation prior to labelling or hybridisation, or degradation due to contaminating nucleases. Sequence analysis and further hybridisation experiments have confirmed that the amplified V1 region is indeed homologous to 16S rRNA. This was also the first indication that these regions were not strain specific which had been suspected.

Plate 5.6. PCR amplification of 16S rRNA V6 sequences from pKK3535 (lane 1) and *L. amylovorus* (lane 2). The 1kb ladder is in lane 3.

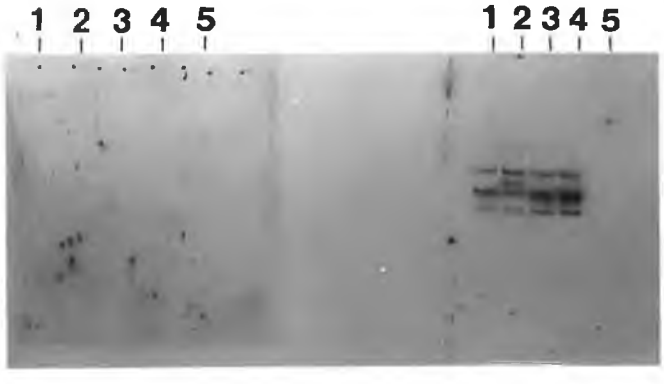


Plate 5.7. Hybridisation of V1 (A) and V6 (B) regions amplified from *L. plantarum* LP115 to *L. plantarum* chromosomal DNA restricted with *Eco*RI. Lanes: (1) *L. plantarum* DCU101, (2) *L. plantarum* LP115, (3) *L. plantarum* DCU101, (4) *L. plantarum* DCU101 (5) 1kb ladder.
The 1kb ladder acted as a negative control in the hybridisation.

5.3 Cloning of PCR fragments

5.3.1 Introduction

PCR amplified DNA can be directly cloned into a plasmid or M13 vector. To aid ligations or to directionally clone the fragment, restriction sites can be incorporated into the 5' end of each primer provided that the sequence of the target DNA is known. A GG or CTC clamp is also added to prevent "breathing" of the DNA during digestion [Bej et al., 1991]. Great care should be taken in choosing the restriction enzyme recognition site added to the 5' end of the primer as some enzymes fail to cleave at sequences located near the extreme ends of DNA fragments. Kaufman and Evans (1990) demonstrated that several common restriction enzymes including *Sal*I, *Hind*III and *Xba*I are unable to digest at sites located close (2 nucleotides from the distal end of the primer) to the ends of PCR fragments. This limitation can be overcome by ligation of the PCR product to itself, prior to restriction with the enzyme.

The ligation of PCR amplified fragments without the incorporation of restriction enzyme sites into the primers can be done using blunt end cloning or by a novel method described by Mead *et al.* (1991). The observation that *Taq* DNA polymerase catalyses the non-templated addition of a single dAMP residue to the 3' termini of a blunt ended duplex [Clarke, 1988] led to the hypothesis that PCR amplified products could be directly ligated to a DNA molecule containing a 3' dTMP extension. The construction of a vector yielding a single 3' dTMP extension on both ends of the plasmid on restriction with *Xcm*I or *Hph*I permits the universal cloning of PCR amplified DNA. The plasmid restriction sites have been engineered in phase with a truncated *lac*Z gene to select recombinant clones and cloning is reported to be approximately 50 times more efficient than with blunt ended ligations.

The rapid and reliable cloning of PCR products using blunt ended ligation was described by Lorens (1991) who combined a DNA polymerase I (Klenow fragment) and kinase reaction with the ligation. It was observed that previously recalcitrant PCR products could be cloned using the concurrent incubation of Klenow, T4 polynucleotide kinase and T4 DNA ligase with the PCR product to repair, phosphorylate and ligate the PCR termini to the vector.

5.3.2 Cloning of PCR amplified fragments from *L. plantarum*

The PCR fragments generated from *L. plantarum* strains DCU101, LP115 and B2 were purified using either the Magic PCR preps™ DNA purification system or the Qiagen >PCR purification< spin kit to aid cloning and direct sequencing. Both methods removed contaminating primers, primer-dimers, nucleotides and Taq DNA polymerase from the PCR reactions but the Magic PCR preps™ proved to be simple, effective, reliable and very rapid on a routine basis. The entire procedure can be completed in less than 15 minutes with no organic extractions or ethanol precipitations. The DNA is eluted from the Magic PCR preps™ resin with water or TE buffer, free of any salt or macromolecular contaminants. The recovery efficiencies depend on the size of the PCR fragment and are outlined in figure 5.5.

DNA size	% recovery (average of 6 samples)
ds DNA	
1500bp	95.9%
1000bp	107.9%
500bp	98.4%
300bp	99.1%
200bp	68.6%
100bp	8.1%
75bp	3.2%
50bp	1.9%
ss DNA	
73 bases	1.1%
45 bases	1.5%
29 bases	1.0%

ds double stranded

ss single stranded

Figure 5.5. Recovery efficiencies of Magic PCR preps™ DNA purification system (reproduced from Promega Notes; Mezei, L.M., December 1991).

Numerous attempts were made at cloning the PCR fragments either directly from the reaction mix, or purified using the above methods. Cloning involved blunt end ligation with pUC19 linearised with *Sma*I and both dephosphorylated and untreated vector were used. Transformations of these ligations into *E. coli* JM83 competent cells ($CaCl_2$ treated) were unsuccessful.

Eventually successful cloning was achieved using a modified procedure derived from Lorens (1991) where the PCR fragments were treated with the Klenow fragment of DNA polymerase and T4 polynucleotide kinase (T4 pnk) to repair the ends. Ligation with *Sma*I restricted pUC19 and electroporation into competent *E. coli* TG1 cells (section 2.14.4) yielded positive transformants. Details of the procedure are outlined below;

1. Four 100μl PCR reactions were pooled and extracted twice with chloroform/isoamylalcohol (24:1 v/v) to remove the mineral oil overlay.

↓

2. The PCR fragment was cleaned using the Magic PCR preps™ DNA purification protocol.

↓

3. The repair reaction using Klenow and T4 pnk was as follows;

fragment (cleaned)	18μl
10x buffer*	3μl
dNTP's (2 mM)	6μl
Klenow fragment	2μl (6 units)
T4 polynucleotide kinase	1μl (8 units)
	30μl

* 10x Nick translation buffer/ Klenow reaction buffer as described in section 2.7.

↓

4. Following incubation at 37°C for 30 minutes, the reaction was heat inactivated at 70°C for 20 minutes.

↓

5. The ligation reaction was carried out as described in section 2.23.5.

6. The ligation was column cleaned (section 2.19) and 5μl used for electroporation into TG1 (section 2.14.4). Selection of the transformants exploited the blue/white *lac* system. A control transformation with pBR322 yielded 7.5 x 10^8 transformants/μg DNA.

5.3.2 Analysis of PCR transformants

To analyse the putative transformants, plasmids were isolated from 12 *E. coli* transformants carrying the *L. plantarum* DCU101 V1 region, 9 transformants carrying the *L. plantarum* B2 V1 region and 7 transformants carrying the *L. plantarum* LP115 V1 region. There was little or no size difference between the isolated plasmids and pUC19 observed on electrophoresis. None of the transformants were restrictable with SmaI demonstrating the loss of restriction site. Three of the DCU101 transformants were subjected to 30 cycles of PCR amplification and one yielded a strong band at 100bp. To screen all of the V1 transformants for correct inserts, the V1 region from DCU101 was labelled with digoxygenin (section 2.21.1) and hybridised to a dot blot of the isolated plasmids. As positive controls, total DNA and the amplified V1 regions from *L. plantarum* B2, *L. plantarum* LP115 and *L. plantarum* DCU101 were included and pUC19 acted as a negative control. A Southern blot of L. plantarum chromosomal DNA restricted with *Eco*RI was also included as a positive control. Following hybridisation overnight at 68°C, stringent washing and two hours in the colour reaction, positive signals were observed. The transformants yielding the strongest hybridisation signal after two hours were noted, as after 24 hours the negative controls also yielded a weak signal (plate 5.8). Eight transformants were analysed further by sequencing with T7 polymerase. The cloning procedure was repeated for the V6 region.

Because the dot blot hybridisation signal may have resulted from cloned PCR primers having weak homology with the probe, the transformants had to be sequenced, before use in hybridisation experiments, to confirm the presence of the 16S rRNA variable regions.

1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	
24	25	26	27		
28					

1. tf4-V1 (DCU101)
2. tf7-V1 (DCU101)
3. tf11-V1 (DCU101)
4. PCR-V1 (DCU101)
5. PCR-V1 (LP115)
6. PCR-V1 (B2)
7. tf13-V1 (B2)
8. tf14-V1 (B2)
9. tf15-V1 (B2)
10. tf16-V1 (B2)
11. tf17-V1 (B2)
12. tf18-V1 (B2)
13. tf19-V1 (B2)
14. tf20-V1 (B2)
15. tf21-V1 (B2)
16. tf29-V1 (LP115)
17. tf30-V1 (LP115)
18. tf31-V1 (LP115)
19. tf32-V1 (LP115)
20. tf33-V1 (LP115)
21. tf34-V1 (LP115)
22. tf35-V1 (LP115)
23. tf36-V1 (LP115)
24. C/somal (DCU101)
25. C/somal (LP115)
26. C/somal (B2)
27. pUC19 plasmid
28. C/somal (DCU101)

Legend for Plate 5.8A. Transformants have been assigned a number (tf4).

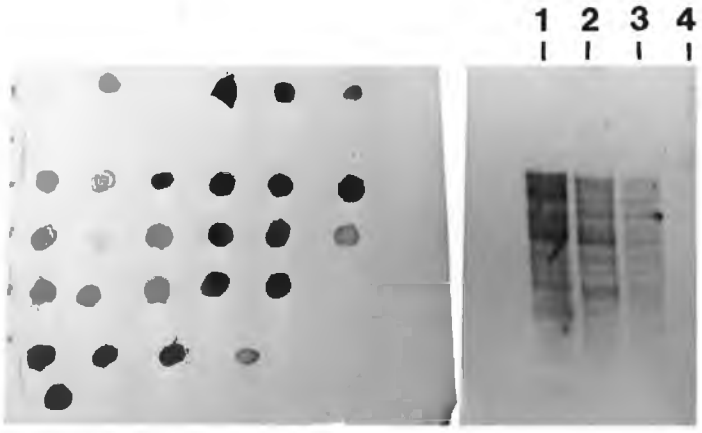


Plate 5.8. Identification of clones containing the PCR amplified V1 regions from *L. plantarum* strains DCU101, LP115 and B2. The V1 region from DCU101 was labelled with digoxygenin and hybridised to a dot blot of plasmid DNA (A) and a southern blot of total DNA (*Eco*RI) (B). Lanes: (1) *L. plantarum* DCU101, (2) *L. plantarum* LP115, (3) *L. plantarum* B2, (4) 1kb ladder.

5.4 Sequencing of PCR amplified products

5.4.1 Introduction

PCR products can either be cloned prior to sequencing or directly sequenced. Cloning and sequencing allows the use of standard sequencing protocols but is sensitive to the error rate of Taq DNA polymerase, necessitating the sequencing of a number of independent clones to determine the correct sequence.

The ease with which clear and reliable sequences can be obtained directly without resorting to cloning is determined by the ability of the PCR primers to amplify only the target sequence (specificity) and the method used to obtain a template suitable for sequencing. Where non-specific bands result from PCR, the desired fragment can be purified using gel electrophoresis but the large quantities of DNA required for Sanger sequencing may limit this possibility.

PCR reactions contain a significant amount of primers used for amplification. Annealing and subsequent extension of these primers can cause a high backround during incorporation dideoxy sequencing because extension products from both primers are superimposed on each other. Sequencing with an end-labelled primer ensures that the only ladder detected after exposure to film is the one generated from the desired annealing site. However, the unlabelled primer in the amplification competes for the annealing site reducing the signal strength. Consequently, it is essential to remove the amplification primers or else use a third labelled sequencing primer that anneals to sequences internal to the original amplification primers.

Another problem is that small, linear DNA molecules (< 500bp) rapidly reanneal preventing efficient annealing of the sequencing primer and these small molecules exhibit regions of strong secondary structure such as hairpin loops. These hairpin loops impede the progress of the polymerase along the template. Elevated temperatures may be used to destabilise the secondary structure but are limited by the thermostability of the DNA polymerase and the melting temperature of the primer.

Direct sequencing of double stranded PCR amplified DNA has been

described by Böttger (1989), Rogall *et al.* (1990) and Both *et al.* (1991) who purified PCR fragments and used the Sequenase™ DNA polymerase with α-^{32}P labelled nucleotides in the standard Sanger sequencing protocol. All of these fragments were larger than 880bp and so purification protocols and secondary structure would not have caused problems.

The advantages of using PCR sequencing are the reduction of *Taq* DNA polymerase errors because the sequencing ladder is representative of a population of fragments as opposed to individual clones and elimination of extensive and time consuming cloning protocols.

Sequencing of PCR amplified V1 and V6 regions

Two approaches to sequencing the PCR amplified V1 and V6 regions from the *L. plantarum* strains B2, LP115 and DCU101 were taken.

5.4.2 Direct sequencing from PCR

Initially, sequencing direct from the PCR reaction was investigated. To rapidly determine the sequence of the PCR amplified fragments, two DNA purification procedures were used for the isolation of clean template DNA. The desired PCR product has to be separated from the primers, dNTP's, primer-dimers, Taq DNA polymerase and salts prior to sequencing. To this end the Magic PCR preps™ and Qiagen >PCR purification< spin kit, mentioned previously, were tested. The V1 region from *L. plantarum* LP115 was purified using both protocols and directly sequenced using the *fmol*™ DNA sequencing system. The sequencing primer used was R1 and the following amplification protocol was carried out according to the manufacturers instructions.

1. 95°C	2 minutes
2. 95°C	30 seconds
3. 42°C	30 seconds
4. 70°C	1 minute

Steps 2 to 4 were repeated for 30 cycles and refrigerated.;

This was unsuccessful (plate 5.9) as the *Taq* DNA polymerase seemed to be pausing which resulted in heavy lines accross all the lanes. Since both the "Magic" and "Qiagen" purified DNA yielded the same pattern and the *fmol*™ control DNA yielded a clean sequence (results not shown), the problems were attributed to the amplified DNA and not to the purification protocol.

A B

Plate 5.9. *fmol*™ sequencing of the V1 region from *L. plantarum* LP115 purified using the Qiagen (A) and Magic PCR preps™ (B) procedures. The annealing temperature for the sequencing reactions was 42°C.

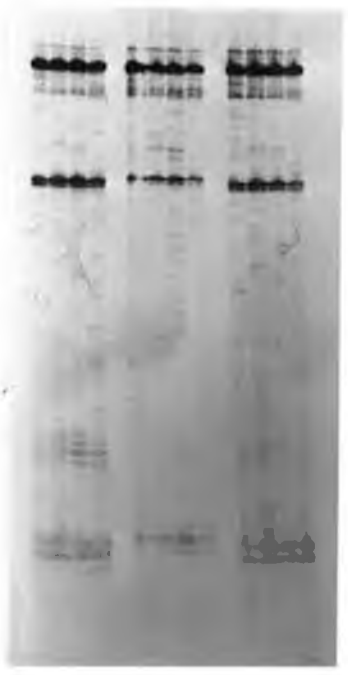
The R1 and R2 primers which were estimated, using the primer included in the kit, to be ten times more concentrated than that recommended were reduced accordingly and the annealing temperature was increased to 60°C. The fmol™ sequencing reactions were carried out using these conditions for the Magic purified V1 regions of *L. plantarum* DCU101 and *L. plantarum* LP115 in both directions (primers R1 and R2). The sequencing programme was as follows;

1. 95°C	2 minutes
2. 95°C	30 seconds
3. 60°C	30 seconds
4. 72°C	30 seconds

Steps 2 to 4 were repeated for 30 cycles and then the samples were refrigerated at 4°C.

The resulting sequenceing gel is shown in Plate 5.10. The bands appear diffuse because the filter paper backing on the gel was exposed instead of the gel, which had disintegrated in the drier. There were very heavy bands crossing all the lanes but the intermediate sequence was becoming a little clearer.

One of the transformants known to carry the V1 region from *L. plantarum* B2 (number 15) by dot blot hybridisation was subjected to the fmol™ sequencing reaction to see if the presence of pUC19 (2.2kb) sequences could stabilise the PCR amplified fragment. The pUC19 forward primer was used (T_m of 78°C) and the programme was that outlined in the manufacturers instructions with an annealing temperature of 70°C. The resulting sequence ladder is shown in plate 5.11 and demonstrated complete homology with the T7 DNA polymerase generated sequence. It can be seen that the heavy banding pattern had disappeared completely. At this stage it was decided to continue with the T7 DNA polymerase sequencing as it was more reliable and did not require a thermal cycler.



A B C

Plate 5.10. Sequence resulting from the *fmol*™ reaction on the V1 regions of *L. plantarum* DCU101 using sequencing primer R1 (A), *L. plantarum* LP115 using sequencing primer R2 (B) and *L. plantarum* LP115 using sequencing primer R1 (C). The annealing temperature of the reaction was 60°C.

Plate 5.11. Sequence resulting from the *fmol*™ reaction on the V1 region from *L. plantarum* B2 cloned into pUC19. The annealing temperature was 70°C.

5.4.3 Sequencing of the cloned PCR fragments

The transformants demonstrating homology with the digoxygenin labelled PCR fragments were sequenced using T7 DNA polymerase (Promega). The DNA was isolated using a modified STET plasmid mini preparation procedure as described in section 2.15.1. No problems with heavy banding patterns were encountered on sequencing the cloned PCR fragments but this may be because of the increase in size and stability due the presence of pUC19 sequences. An example of a typical sequencing gel is shown in plate 5.12.

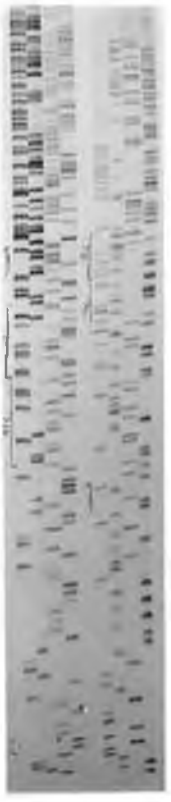


Plate 5.12. A typical sequence resulting from the T7 DNA polymerase reaction of the cloned PCR amplified fragments. The sequence shown is that of transformant 15 harbouring pUC19-V1 (B2).

5.5 Sequence analysis of cloned PCR fragments

All of the sequences were entered into the Seqaid™ programme where primer site and multiple cloning site identifications were carried out. Restriction site analysis and alignment between the transformants were also facilitated by this programme. The complete nucleotide sequence of the transformants could often be read following one loading of the sequencing gel (until the bromophenol blue reached the end of the gel) however two and sometimes three loadings were carried out to ensure accurate sequencing. The transformants were sequenced in both directions using the pUC19 primers flanking the multiple cloning site. Due to the short size of the PCR fragments there was no need to construct deletions of each transformant.

A schematic of each transformant is shown in figure 5.6. As is evident, the blunt ended cloning procedure allowed the primers to ligate to pUC19 and often more than the PCR amplified insert was cloned. This complicated the sequencing a little bit as often the multiple cloning site wasn't situated directly adjacent to the PCR fragment.

All of the transformants containing the V1 amplified DNA contained regions of about 75bp (between the primers) while the transformants containing the V6 amplified DNA contained regions of 54bp (between the primers).

The legend is for figure 5.6 is given below;

═══ multiple cloning site of pUC19

▬▬ PCR primer

——— PCR amplified insert

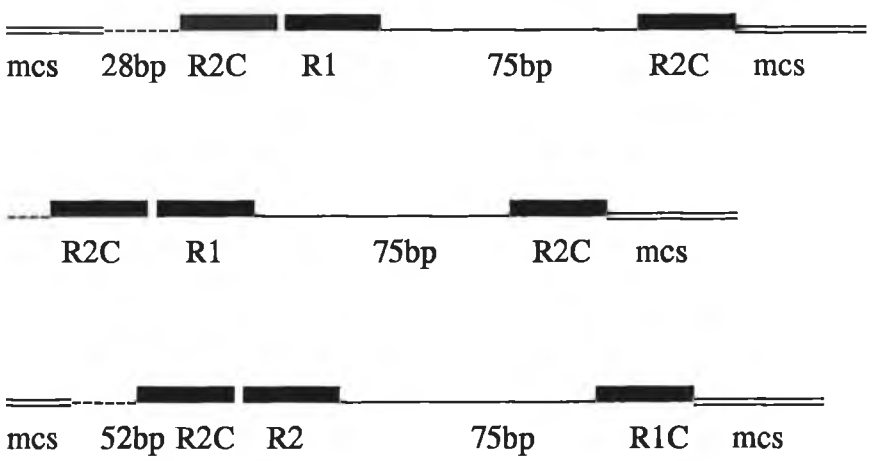
C converse complement sequence

--- small insert of unknown origin

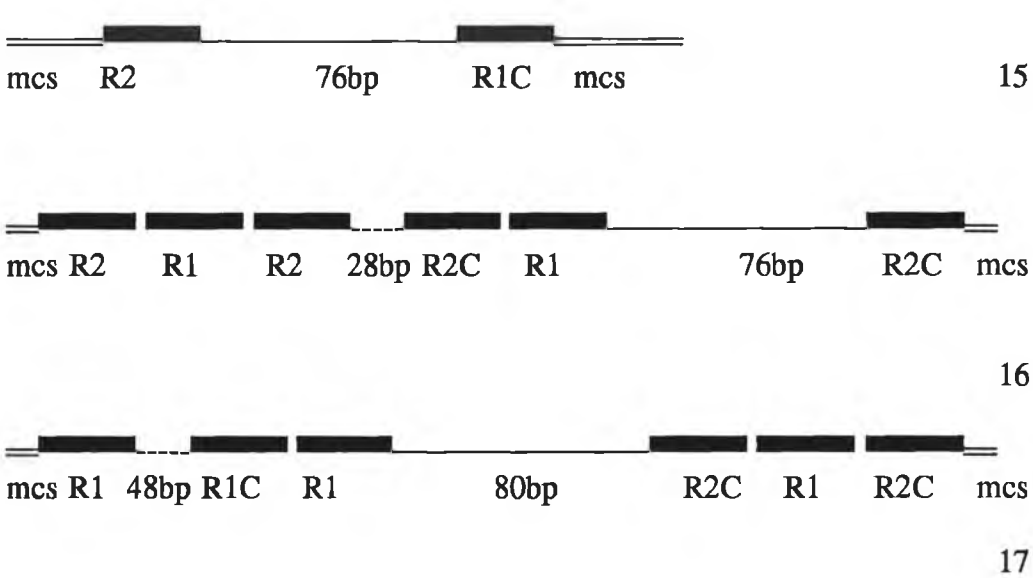
The following diagrams represent the cloned insert reading in the 5' to 3' direction (*Eco*RI to *Hind*III) in the multiple cloning site of pUC19 starting from the M13/pUC19 sequencing primer; GTAAAACGACGGCCAGT

V1 region of *L. plantarum* DCU101

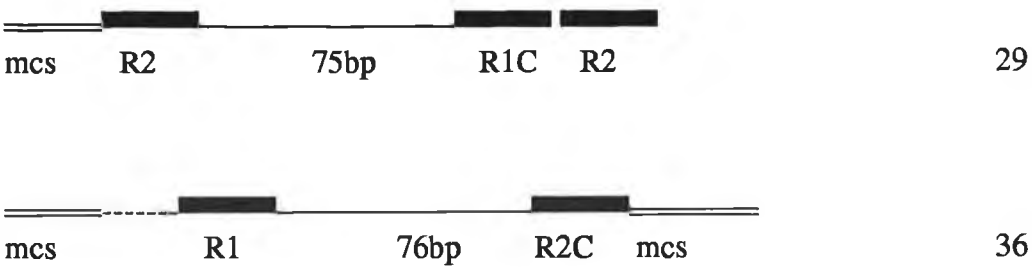
transformant



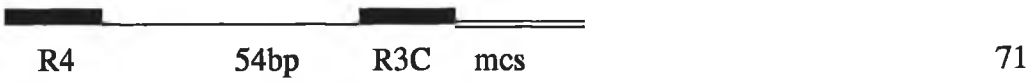
V1 region of *L. plantarum* B2



V1 region of *L. plantarum* LP115



V6 region of *L. plantarum* DCU101



V6 region of *L. plantarum* LP115

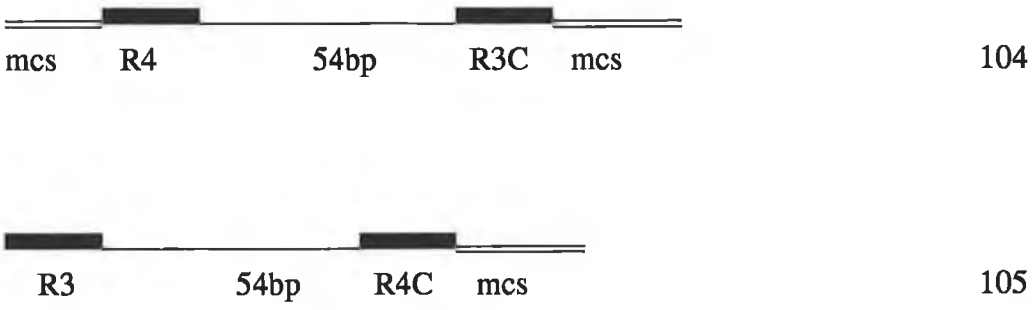


Figure 5.6. A schematic representation of the V1 and V6 transformants.

Figure 5.7. Alignment of the V1 sequences (between the primers R1 and R2C) resulting from the cloned PCR amplified V1 regions of 16S rRNA from *L. plantarum*.

The dots (.) represent identical sequence, dashes (-) where the sequence had to be shifted to facilitate alignment and the bases which differed between sequences are written. The positions are indicated above each group of sequences and don't include the gaps required for correct alignment.

V1 region of *L. plantarum* DCU101 transformants (7-10), *L. plantarum* B2 (15-17) and *L. plantarum* LP115 (29 and 36).


```
                                                                    Transformant
          10        20        30        40        50        60        70
          '         '         '         '         '         '         '
GCTCAGATTGAACGCTGGCGGCAGGC-TAACACATGCAAGTCGAGCGGTAGAGAGAAGCTTGCTTCT-----CTTGA-G-AGC     7
.........................-.....................................-----.....-.-...     9
.........................=.....................................C-----.....-.-...    10

.........................T.....................GG....TT-....CGG.AA-----T.GACCT-...    15(fmol)
.........................T.....................GG....TT-....CGG.AA-----T.GACCT-...    15
.........................T.....................GG....TT-....CGG.AA-----T.GACCT-...    16
.........................-.....................ACAG.G.CTAGCT.G.ATCCTG..-..C.-...    17

....................-.....C.....................................-----.....-.-...    29
.........................TC....................GG....TT-....CGG.AA-----T.GACCT-...    36
```

Figure 5.8 Alignment of V6 sequences (between the primers R3 and R4C) resulting from the cloned PCR amplified V6 regions of *L. plantarum*. The dots (.) represent identical sequence.

V6 region of *L. plantarum* DCU101 (71) and *L. plantarum* LP115 (104 and 105) transformants.

```
         10        20        30        40        50              Transformant
          '         '         '         '         '
TGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGT            71

..................................................              104
A..C...........AT...C...CT......AGATTG.G...........AT.             105
```

In analysing the transformant sequences, a few problems ensued. Despite each amplified region containing the correct primer sequences, significant sequence variation between transformant and strains resulted.

Each of the three transformant sequences for the V1 region of L. plantarum DCU101 and one of the transformant sequences for *L. plantarum* LP115 were identical with the exception of a cytosine in place of a thymine which could be attributed to the error rate of Taq DNA polymerase (1/300). However, two of the transformant sequences for the V1 region for *L. plantarum* B2 and one from *L. plantarum* LP115 while being practically identical to each other differed significantly to that of the V1 region from *L. plantarum* DCU101. Also, one of the transformants (number 17) yielded a unique sequence and was assumed to be a contaminant. Similarly, for the V6 region one transformant sequence (number 105) differed to the others. To analyse the specificity of the amplified sequences to a wide range of species, transformants number 15 and 104 (which seemed to be representative) were used in the DNA hybridisation experiments.

5.6 Use of V1 and V6 as cloned Genus and Species specific DNA probes

5.6.1 Introduction

The use of cloned rRNA gene fragments as hybridisation probes has been investigated by a number of researchers for two main reasons;
(1) determination of relatedness and (2) species identification.

Schleifer *et al.* (1985) used cloned 5S, 16S and 23S rRNA genes to study distant phylogenetic relationships between *Pseudomonas* and other species. They reported that compared with oligonucleotide sequence analysis of 16S rRNA, a good correlation was found between DNA-rDNA homology values and relatedness which they expressed in terms of percent homology.

Regensburger *et al.* (1988) cloned different sections of the 23S rRNA gene of the sizes 472bp, 182bp, 127bp and 99bp and under stringent conditions (T_m-20°C) used these regions as a universal probe, a eubacterial probe, *Arthrobacter* and *Micrococcus* specific probe and a *Micrococcus lylae/ Micrococcus luteus* specific probe respectively.

Kraus *et al.* (1986) carried out hybridisation experiments with a cloned fragment (2kb) of the 23S rRNA from *Bacillus subtilis* under varying conditions. Under stringent hybridisation conditions (T_m-10°C in their report), the four *Bacillus* species could be differentiated from the other Gram-positive and Gram-negative species examined.

The cloned V1 and V6 regions of *L. plantarum* 16S rRNA were then investigated under varying hybridisation conditions to determine their usefulness as genus or species specific probes.

5.6.2 Hybridisation with the cloned V1 and V6 regions

To evaluate the potential of cloned V1 and V6 regions as group specific DNA probes, a variety of genera, species and strains were obtained and are listed in Tables 5.2. Total DNA was isolated and quantified (using absorbance at A_{260nm} and A_{260nm} / A_{280nm}) for each strain and 2μg were applied to nitrocellulose using the dot blot procedure described in section 2.18.5. Hybridisation with clones containing the V1 and V6 regions were carried out under varying stringencies as outlined in Table 5.1. The resulting signals were examined to estimate the hybridisation efficiencies for each strain in an attempt to differentiate groups of organisms under specific hybridisation conditions.

	Hybridisation and wash temperature (°C)			
Probe	37°C	48°C	58°C	68°C
V1	✓	✓	✓	✓
V6	✓	✓	✓	✓

Table 5.1 Hybridisation conditions of dot blots containing 22 strains as listed in Table 5.2. Each blot was hybridised with the V1 and V6 clones (transformants number 15 and 104 respectively) labelled with ^{32}P and washed twice with 2xSSC/0.1% SDS for 20 minutes each and twice with 0.2xSSC/0.1% SDS for 20 minutes each at the above temperature.

No.	Genus	Species	Strain	Gram
A1	*Pediococcus*	*acidilactici*	PLL03	+
A2	*Pediococcus*	*acidlactici*	PLL04	+
A3	*Pediococcus*	*acidilactici*	PLL08	+
A4	*Lactobacillus*	*plantarum*	LP80	+
A5	*Enterococcus*	*faecalis*		+
B1	*Pediococcus*	*pentosaceus*	FBB61	+
B2	*Lactobacillus*	*plantarum*	LMG 10755	+
B3	*Lactobacillus*	*plantarum*	NCDO type L	+
B4	*Pediococcus*	*acidilactici*	PAC1.0	+
B5	*Lactobacillus*	*reuteri*	20016	+
C1	*Lactobacillus*	*plantarum*	DCU101	+
C2	*Saccharomyces*	*cerevisiae*	DBY746	na
C3	*Lactobacillus*	*amylovorus*	NRRLB 4540	+
C4	*Cellulomonas*	*flavigena*	ATCC 482	+
C5	*Lactobacillus*	*reuteri*		+
D1	*Lactobacillus*	*hilgardii*		+
D2	*Lactobacillus*	*helveticus*		+
D3	*Lactobacillus*	*delbrueckii*		+
D4	*Lactococcus*	*lactis*	CH919	+
D5	*Lactobacillus*	*plantarum*	LP115	+
E1	*Lactobacillus*	*plantarum*	B2	+
E2	*Escherichia*	*coli*	JM83	-

Table 5.2 Strains hybridised with V1 and V6 in dot blot experiment. The numbers in column 1 refer to the position of the strain Plate 5.13.

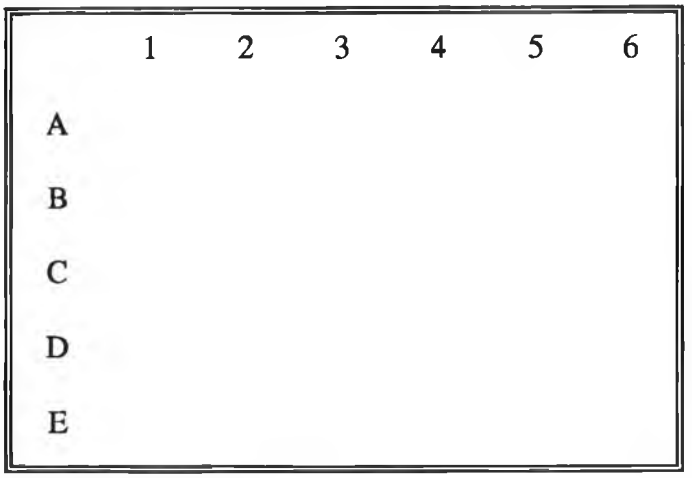


Figure 5.9 Position of strains as listed in Table 5.2 (column 1) on each dot blot shown in Plate 5.13. Positive controls were in the following positions:

Position	Plasmid DNA
E3	tf15-V1 (B2)
E4	tf15-V1 (B2)
E5	tf104-V6 (LP115)
E6	tf104-V6 (LP115).

The hybridisation with the cloned V1 and V6 regions demonstrated that at each temperature (37°C, 48°C, 58°C and 68°C), a signal was obtained from the positive controls. Hybridisation with the V6 clone (Plate 5.13B) resulted in signals from the positive controls only, while hybridisation with the V1 clone yielded faint signals from all (at 37°C) of the strains tested. Aside from the positive controls, the strongest signals were obtained from *E. coli* total DNA (position E2 on each blot) and to a lesser extent *E. faecalis* total DNA (position A5 on each blot). There were no significant signals from the two *L. plantarum* strains included in this experiment (positions D5 and E1 on each blot).

The V1 and V6 amplified sequences were then examined further using computer alignments with sequences extracted from the GenBank.

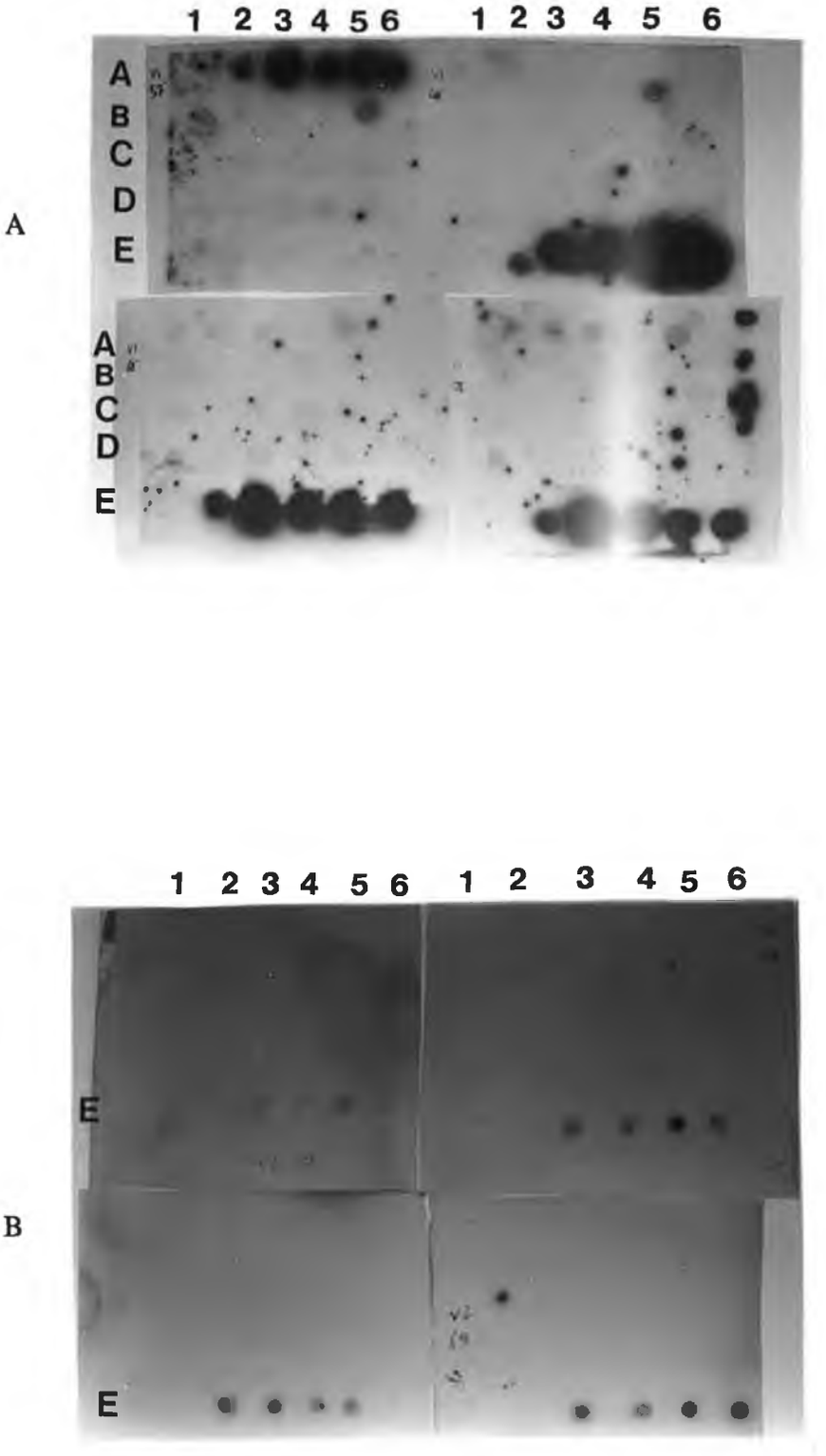


Plate 5.13 Result of hybridisation of cloned V1 (A) and V6 (B) regions with dot blots of the strains listed in Table 5.2. The position of the strains is indicated in Figure 5.9.

5.7 Selection of group specific oligonucleotide probes based on computer alignment of the V1 and V6 regions.

5.7.1 Introduction

Due to the lack of specificity of the cloned V1 and V6 regions as described in section 5.6, attempts were made to identify oligonucleotides that would enable genus, species and perhaps strain differentiation among the LAB to be carried out. There are numerous reports in the literature of oligonucleotides complementary to 16S or 23S rRNA allowing genus or species identification of a wide variety of eubacteria [Amann *et al.*, 1990; Barry *et al.*, 1990; De Long *et al.*, 1989; Giovannoni *et al.*, 1988; Göbel *et al.*, 1987; Rossau *et al.*, 1989; Stahl *et al.*, 1988; Williams & Collins, 1992; Wilson *et al.*, 1988].

Of the LAB, Betzl *et al.* (1990) used oligonucleotides targeted to 23S rRNA to differentiate between *Lactococcus lactis, Enterococcus faecalis, Enterococcus faecium* and *Enterococcus malodoratus/Enterococcus avium* by dot and colony hybridisation. However of the LAB, no pediococci or leuconostocs and only one *Lactobacillus* species (*Lactobacillus casei* susp. *rhamnosus*) were tested. Hertel *et al.* (1991) carried out a more extensive examination of the usefulness of 23S rRNA targeted oligonucleotides which were specific for *Lactobacillus curvatus, Lactobacillus sake* and *Lactobacillus pentosus/L. plantarum* by hybridisation to a wide range of LAB. The probes for *L. curvatus* and *L. sake* differed by only 2 base changes but hybridisation at 38°C for 4 hours and washes at room temperature were stringent enough to allow differentiation. Due to the close phylogenetic relationship between *L. pentosus* and *L. plantarum*, these two species could not be differentiated from each other.

Klijn *et al.* (1991) designed oligonucleotide probes for the identification of *Lactococcus* and *Leuconostoc* species based on V1 and V3 regions of 16S rRNA respectively. They observed that the sequences of the V1 region appeared to be identical in all species of the genus *Leuconostoc* analysed (however they only appear to have analysed *L. mesenteroides* and *L. lactis*) while it contained sufficient variation to differentiate between

Lactococcus lactis, *Lactococcus garvieae*, *Lactococcus plantarum* and *Lactococcus raffinolactis* when targeted to PCR amplified rDNA regions.

5.7.2 Selection of group specific oligonucleotides

To identify genus and species specific oligonucleotides extensive sequence alignment using a combination of sequences extracted from the literature and those extracted from the GenBank, was carried out. The GenBank sequences were imported into Wordperfect™, the V1 and V6 regions identified by searching for conserved sequences and edited to suit the alignment programme, Multalin™. The results from this programme were once again imported into Wordperfect™ and edited to simplify presentation.

Initially, the question of sequence variation between strains of the same species arose and so the available two *Lactobacillus plantarum* V1 and V6 regions and four *Lactococcus lactis* 16S V1 regions were aligned as presented in Figures 5.10 and 5.11 As is evident there were no definite sequence differences between the two *L. plantarum* strains in the V1 region (differences were nucleotides represented as N) but two changes in the V6 region. In contrast there were between 1 and 7 base changes in the V1 region of the *L. lactis* strains. Unfortunately no V6 regions were available for these strains in the GenBank. With an increasing number of sequence entries to data bases, strain differences might arise more frequently, but this may be due to incorrect classification of a species, or a species which is actually more diverse than previously thought.

To examine the possibility of obtaining a species specific oligonucleotide probe for *L. plantarum*, the V1 and V6 regions of a wide variety of *Lactobacillus* species were aligned (Figures 5.12 and 5.13). The most variation exhibited was between the positions 64-130 (*E. coli* numbering 65-131) for V1 and the positions 33-57 (*E. coli* numbering 999-1023) and 70-77 (*E. coli* numbering 1036-1043) for V6. Four oligonucleotides were designed for these areas and sent via electronic mail to the BLAST alignment programme (see section 2.24.8) to investigate their suitability as species specific probes.

Figure 5.10a Alignment using the Multalin™ programme of part of the V1 region (between the PCR primers R1 and R2C, position 2-123, *E.coli* numbering) from two *L. plantarum* sequences extracted from the GenBank to examine strain variation. The dots (.) represent conserved bases between the second sequence and the top *L. plantarum* sequence, dashes (-) represent gaps inserted for optimum alignment.

```
                        10         20         30         40         50         60         70         80
                         '          '          '          '          '          '          '          '
L.plantarum a AATTTGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACTCTGGTATTG
L.plantarum b ............N.......................N..............................N....N.....N

          90         100        110        120
           '          '          '          '
ATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGA
.........................................
```


Figure 5.10b Alignment of part of the V6 region (between the PCR primers R3 and R4C, position 966-1060) from two *L. plantarum* sequences extracted from the GenBank.

```
                        10         20         30         40         50         60
                         '          '          '          '          '          '
L.plantarum a  GCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTAGACGTT
L.plantarum b  A.....................................................

          70         80         90
           '          '          '
CCCTTCGGGGACACGGATACAGGTGGTGCATGGTN
.--.........T...................C
```

Figure 5.11 Alignment of four different *Lactococcus lactis* V1 regions (between the PCR primers R1 and R2, *E. coli* numbering 22-103) extracted from the GenBank using the Multalin™ programme. The dots (.) represent conserved bases between each sequence and the top *L. lactis* sequence.

```
          1         10         20         30         40         50         60         70
          '         '          '          '          '          '          '          '
llactoa   GCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTTGAGCGCTGAAGGTTGGTACTTGTACCAACTGGAT
llactob   ..................................................A.....A.....G....C.....T.T..A
llactoc   ...........................................................................G.......
llactod   ...........................................................................G.......
```

Figure legend

llactoa	First *Lactococcus lactis* V1 region extracted from the GenBank
llactob	Second *L. lactis* V1 region extracted from the GenBank
llactoc	Third *L. lactis* V1 region extracted from the GenBank
llactod	Fourth *L. lactis* V1 region extracted from the GenBank

Figure 5.12 Alignment using the Multalin™ programme of some *Lactobacillus* V1 regions (*E. coli* no. 2-131) extracted from the GenBank.

```
                        10        20        30        40        50        60        70        80
                        '         '         '         '         '         '         '         '
L.plantarum     AATTTGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACTCTGGTATTG
L.alimentarius  ...A.....................................A.............................T.CC....N
L.farciminis    ...A.....................................A.............................CA.CC.GAA.
L.brevis        ..GA.....................................A.............................G..TCC..TGAA
L.casei         -..A.....................T.....................................GT.T....CGAT
L.bifermentans  -..A.....................................................C...GAC..CGAC
L.sharpeae      .........................T.....................................TAAC.TAAT
L.amylophilus   ..A.....................TN.....................................GT......TAA.
L.sake          -.A.C...................................................C.....CN.T...
L.salivarius    ..AA.....................................................ACT.TC.TA.C
L.aviarius      ..A......................................................GAAT.TC.TA.C
L.animalis      -.A.....................TN.....................................ACT.CT.TA.C
L.agilis        ..AA.....................................................---CT.TT.TCAA
L.fructivorans  ..AA.....................................A.............................G..GC.CCTAAT
L.fermentum     -..A.....................T...........T.............T.........CGT-GGCCC.A-T
L.vermiforme    ..GA.....................................................CGTCT....CAAT
L.buchneri      ..GA.....................................................CGTCTCC..TRAT
L.kandleri      ..A......................T.....................................C...G....TGAA
L.viridescens   ..AA.....................T.....................................CTTTG....CCAA
L.confusus      ..A......................T.....................................CTTTG....TCAA
L.minor         ..AA.....................TN.....................N.........CTTTG....TCAA
L.divergens     -..A.....................................A.....................C..GAA.T.GAAA
L.acetolerans   ..C.....................................................G...G..GAAC..A.T
L.acidophilus   ..AAC...................................................G...G..GAACC.ACA
L.amylovorus    .NAA....................................................G...G.GGAACC.ACA
L.vitulinus     -.A.G...................T.....................................GGAATCT-.CGGA
L.catenaforme   .GA.G...................T.....................................GGN.GCCA.TAG.
L.thermophilus  ------..................................................TG..G..CT.TTA.AA.
primer R1       ....GA..........A..
```

Figure 5.12 continued

```
                          100        110        120        130
                           '          '          '          '
L.plantarum       ATTG-GTGCTTGCATCATGATTTACATTTGAGTGAGTGGCG
L.alimentarius    ....-A..................G..C.A..........
L.farciminis      ....-AA......T........C.G.CC.TG.........
L.brevis          TGAC-.........CTGATT.CA...A.GA..CT.......
L.casei           GAACG.........CTGAG...CGAC..AA.AC........
L.bifermentans    .GAAG.........CTGGA.G..GA-..GAC..C.......
L.sharveae        GAATGAR.TGCTTGCAC...A.GAYT.AA...C.GTGAGT.
L.amylophilus     .G.RGCG.TGCTTGCACCA.AGCRAT.AAC.A..
L.sake            ....AAG.AGCTTGCTCCTCA..GATAAACAT.TGAGTGA.
L.salivarius      ACC.AA.........TCACTCG..AGAAGTTGA.T.GC.GA
L.aviarius        .CC.A.........CTCACCG.A.GNNA.TC.AGT.
L.animalis        ACC.A.........CTCACCGAT.A.GAGTT.A.T.
L.agilis          TCATC..AGC.TGC.ACACCGA.TG.AAATT.A.T.GCGAA
L.fructivorans    GA.AGTT.A.GCTTG...T.GC.TG.C..A...
L.fermentum       GA.TGA..G.GCTTG..CCTGA.TG....TG..CGCNTACN
L.vermiforme      GAA.TTGAG.GCTTG...TTAACTN......CATTAA.AC.
L.buchneri        GAYTTTARG.GCTTG..YTTGAA.G....A.CATT.A.A..
L.kandleri        ..GAGA..AGAAGC.TGCTTCAAGTCAAATGCCA.CATTGC
L.viridescens     C.GATTT.AAGAGC.TGCTCAGATATGAC..TG..CAT
L.confusus        C.GATTT.AAGAGC.TGCTCAGATATGAC..TG..CAT
L.minor           N.GATA..AAGAGC.TGCNCGGATTTGAA..TGA.CAT
L.divergens       .GCTT.CTT..CG..NNAAG.AAGTGGCG..CG.GTGA.TA
L.acetolerans     GA.TAC.T.GG.T..G.A.TNAGGG.RGCNA.C.GCG.AT.
L.acidophilus     GA.TCAC.TCG.TGATGACG..GGGNAAC.CTAG
L.amylovorus      GA.TTACTTCGGT.ATGACG..GNA.ACNAGCG.C.GATG.
L.vitulinus       T.CCA...GCGAACGGG...GGA.T
L.catenaforme     CACTA...GCGAACGGG...GGAG..CA..
L.thermophilus    C...CT.TTAAAAGNNNA.CGGCGG.CGG.T.AGTAA
Primer R2C                                GC.GGAC.G......AATGT
```

Figure 5.13 Alignment of some *Lactobacillus* partial V6 sequences (*E. coli* numbering 967-1056) extracted from the GenBank.

```
                  10        20        30        40        50        60        70        80        90
                  '         '         '         '         '         '         '         '         '
PLANTA  CTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACACGGATACAGGTGGTGCAT
ALIMEN  -A.............................C...A...G...........T.T.............T.................
FARCIM  -A.............................C...AC..A...........T.T.............T.................
VIRIDE  .A.............................C..T..ACC.CT.C......G.AG.T.............AA.TG.............
MINOR   -A.....................C.........C..T..ACC.CT.C......G.AG.T.............AA.TG........N....
HALOTO  -A.....................N.........C..T..ACC.C..C......G..G.T.............AA.TG........N....
SALIVA  -A.............................C..T..ACC.C............G.T.............AA.TG........N....
SAKE    -A.............................C..T..ACC.CTCT......AGAG.T.............AA.TG.............
CARNIS  -A.............................C..T..ACC.CTCT......AGAG.T.............AA.TG........N....
CORYNI  -A.............................C..T..ACC.CTGT......ACAG.T.............AA.TG........N....
KANDLE  -A.............................C..T..ACC.CTCC......GGAGNT...........T..AA.TG........N....
CONFUS  -A.............................C.CT..AC..CTCC......GGAGN.............A..TG........N....
RUMINU  .A.............................CT.C..AC...TCC......GGA.............GAATG.............
MURINU  -A.............................CT.T..AC....CT......AG...T.............AAATG.............
ANIMAL  -A.....................N.........CT.C..AC....CT......AG...T....N.........GAATG........N....
BREVIS  .............................CT.C...C....T......A.................GAATG.............
SANFRA  .....................A.........CT.C...C.............................GAATG........N....
HILGAR  .............................CT.C...T.............G.............GGAATG.............
VERMIF  --.....................N.........CT.C...T.............NA...........GGAATG.............
MALI    -A.............................CT.C..AC.............TN.............GAATG.............
AVIARI  -A.............................CT.T..ACC.............NN.............AAATG........N....
AGILIS  -A.............................CT.T..ACC....T......A...TT...........N..AAATG........N....
GASSER  .A.............................C.AG......C...........GNN.............G.T..G.............
ACETOL  -A.............................CTAG...C..C...........G.................TA.G.............
ACIDOP  .A.............................CTAG.....TC.GT......AC.GN.............TA.G.............
AMYLOV  --.....................N.........CTAG...N...TGT......AT.GA...........N.G.TA.G........N....
PHILUS  -A.....................N.........CTAGC..C....CC......GG..N...TN.......A..G.TA.G.............
BIFERM  .....................N.........CTAGC..C.....T......AG.................GCTA.G.............
LACTIS  .A.............................C..GC..T.CA..T......AG.C.............GCA..G.............
DELBRU  -A.............................C..GC..T.CA..T......AG.TN............GCA..G.............
R3      G.A.................                                  primer R4C      ..G.G.....C.....
```

Legend for Figure 5.13

PLANTA	*L. plantarum*	BREVIS	*L. brevis*
ALIMEN	*L. alimentarius*	SANFRA	*L. sanfrancisco*
FARCIM	*L. farciminis*	HILGAR	*L. hilgardii*
VIRIDE	*L. viridescens*	VERMIF	*L. vermiforme*
MINOR	*L. minor*	MALI	*L. mali*
HALOTO	*L. halotolerans*	AVIARI	*L. aviari*
SALIVA	*L. salivarius salicinius*	AGILIS	*L. agilis*
		GASSER	*L. gasseri*
SAKE	*L. sake*	ACETOL	*L. acetolerans*
CARNIS	*L. carnis*	ACIDOP	*L. acidophilus*
CORYNI	*L. coryniformis*	AMYLOV	*L. amylovorus*
KANDLE	*L. kandleri*	PHILUS	*L. amylophilus*
CONFUS	*L. confusus*	BIFERM	*L. bifermentans*
RUMIN	*L. ruminus*	LACTIS	*L. lactis*
MURINU	*L. murinus*	DELBRU	*L. delbrueckii*
ANIMAL	*L. animalis*		

5.7.3 *L. plantarum* specific oligonucleotides

The following 20 base oligonucleotides, V1LP1, V1LP2, V6LP3 and V6LP4 were selected and sent via electronic mail to the BLAST programme in the US to analyse their specificity and the results are presented below.

V1 region

V1LP1

(*E. coli* position 69-88)	5' AACTCTGGTATTGATTGGTG 3'
probe sequence	5' CACCAATCAATACCAGAGTT 3'

When aligned to the databases included in the BLAST search (GenBank, EMBL, EMBL update, GBupdate, non-redundant PDB) containing 124,001 sequences (June, 1993) only one *L. plantarum* sequence was completely homologous (N's excluded the second sequence). To ensure that a weaker homology didn't exist, the stringency of the alignment was lowered and the sequence with the closest homology (15 bases) that resulted was from Hepatitis C China virus;

```
   1  CACCAATCAATACCA 15
7982  CACCAATCAATACCA 7996
```

V1LP2

(*E. coli* position 105-124)	5' TCATGATTTACATTTGAGTG 3'
probe sequence	5' CACTCAAATGTAAATCATGA 3'

There was complete homology with the two *L. plantarum* sequences in the GenBank and lowering the stringency of alignment yielded the following sequence of the CHUM salmon growth hormone gene with the closest homology (15 bases);

```
  16  GATTTACATTTGAGT  2
1119  GATTTACATTTGAGT  1133
```

V6 region

V6LP3

(*E. coli* position 998-117) 5' ACTATGCAAATCTAAGAGAT 3'

probe sequence 5' ATCTCTTAGATTTGCATAGT 3'

There was complete homology with the two *L. plantarum* sequences and lowering the alignment stringency yielded a homologous sequence from the Germiston Bunyavirus M segment of the envelope protein as follows;

```
 20  ACTATGCAAATCTAAGAGAT  1
979  ACTATGCAAATCTAAGAGAT  998
```

V6LP4

(*E. coli* position 1023-1042) 5' GTTCCCTTCGGGGACACGGA 3'

probe sequence 5' TCCGTGTCCCCGAAGGGAAC 3'

This oligonucleotide while being absolutely homologous with *L. plantarum* 16S rRNA, also had homology to other sequences which are listed below. The star (*) indicates non-homologous bases.

Flavobacterium capsulatum

```
 20  GTTCCCTTCGGGACACGG   2
968  GTTCCCTTCGGGACGCGG 986
                  *
```

Lactobacillus alimentarius

```
 19  TTCCCTTCGGGGACACGGA 1
1049 TTCCCTTCGGGGACATGGA 1067
                    *
```

Lactobacillus farciminis

```
 19  TTCCCTTCGGGGACACGGA  1
1050 TTCCCTTCGGGGACATGGA 1068
```

Leuconostoc paramesenteroides

```
 20  GTTCCCTTCGGGGACACGG 2
1058 GTTCCCTTCGGGGACAAGG 1076
                    *
```

Lactobacillus confusus

```
 20  GTTCCCTTCGGGGACACGG 2
1061 GTTCCCTTCGGGGACAAGG 1079
                    *
```

Lactobacillus acetolerans	20	GTTCCCTTCGGGGACAC	4
	1048	GTTCCCTTCGGGGACAC	1064
Lactobacillus acidophilus	20	GTTCCCTTCGGGGACAC	4
	1048	GTTCCCTTCGGGGACAC	1064

5.7.4 *Lactobacillus* Genus specific probes

To identify genus specific probes the V1 and V6 sequences of a wide variety of unrelated and related genera were aligned (Figures 5.14, 5.15, 5.16 and 5.17) and the variable regions examined with cross reference to the *Lactobacillus* species V1 and V6 alignments. Unfortunately the variable regions of the different genera coincide with the variable regions within the *Lactobacillus* species so a genus specific probe encompassing all of the Lactobacillus species could not be identified.

The *L. plantarum* DCU101, *L. plantarum* B2 and *L. plantarum* LP115 transformant sequences were included in this alignment in an attempt to identify the species of origin of the sequences however, while there was a close alignment with the *E.coli* V1 and V6 regions, substantial differences remained.

Figures 5.14 and 5.15. V1 and V6 regions aligned to identify genus specific probes and to determine the source of the transformant sequences. The following sequences were obtained from Barry *et al.* (1990); *E. coli, Clostridium perfringens, Mycobacterium bovis, Pseudomonas fluorescens, Klebsiella pneumoniae, Salmonella typhimurium* and *Staphylococcus aureus.* V1 regions from *Pediococcus acidilactici, Leuconostoc amelobiosum, Enterococcus columbae, Lactococcus lactis, Lactobacillus plantarum,* and the corresponding region from *Saccharomyces cerevisiae* 18S rRNA were extracted from the GenBank. The sequence from *Aerococcus viridans* was obtained from Collins *et al.* (1990).

Figure 5.14 **V1 regions between the primers R1 and R2C**

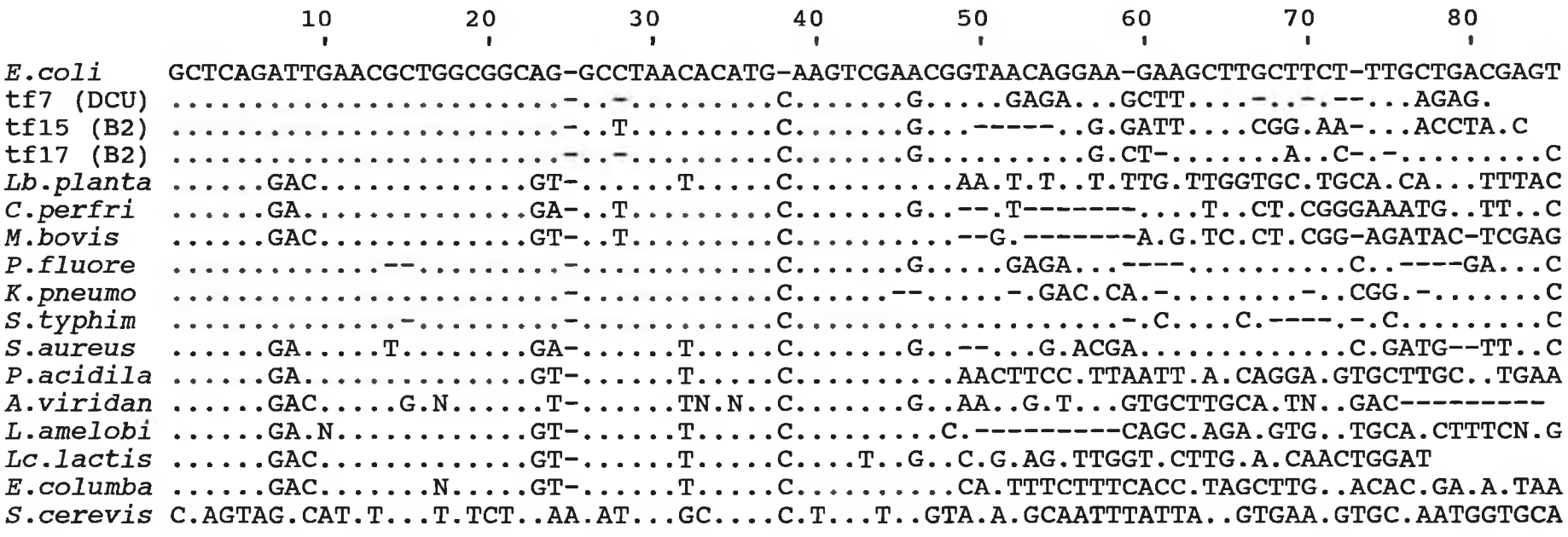


Figure 5.15 (as for 5.14) **V6 regions between the primers R3 and R4C**

There were no *Lactobacillus pentosus* or *Lactococcus lactis* V6 sequences available and the *Saccharomyces cerevisiae* corresponding region could not be identified due to extensive sequence differences.

```
                  10         20         30         40         50
                   '          '          '          '          '
E. coli    TGGTCTTGACATCCACGGAAGT-TTTCAGAGATGAGAATGTGCCTTCGGGAACCCT
tf 104     ......................-..............................G.
tf 105     A..C..........AT...C.-..CT......AGATTG.G...........AT.
L.planta   A..........A.TAT.C.AATC.AAGAGAT.-...CGT.C........G..A.G
C.perfr    .AC..........CTT.C.T.AC.C.TA.TCGAG...AT-C........G..AAG
M.bovis    ...GT.......G...A.G.CGCG.CT......AG.--C..T..C.T.T.GC.--.
P.fluor    A..C..........AT...C.-..CT......AGATTG....-.........AT.
K.pneum    ..............GA...C.-..C........GATTG..............TG.
S.typhi    ..............A...C.-..C.......CGATTG....-.........TG.
S.aureus   AAA..........TTT.-..AAC.CT......AGAGCCT.C..C.TC..G..AAA
P.acidil   A............TT.T.CCAACC.AAGAGAT.-..GNGT.C........G..AGA
P.pentos   A............TT.T..CAGTC.AAGAGAT.-...GGTCC........G..AGA
A.viride   AA...........TNT..CCA.CC.AGAGATA-G.G.NT.--........G..AAA
L.amelob   A............TTT....CT...AGAGATAG.AGTGT.CT......AG..AAA
E.columb   A............TNT..CCATCC.AGAGAT--..G.CNNN........G..AAA
```

Figure 5.16 Alignment of 16S rDNA V1 regions from *Pediococcus*, *Leuconostoc*, *Enterococcus* species, *Lactococcus lactis* (*Lc.lactis*), and *Lactobacillus plantarum* (*Lb. plantarum*), and the corresponding region from *Saccharomyces cerevisiae* 18S rRNA extracted from the GenBank. The sequence from *Aerococcus viridans* was obtained from Collins *et al.* (1990).

```
                        10        20        30        40        50        60        70        80
                         '         '         '         '         '         '         '         '
P.acidilactici AATATGAGAGTTTGATCTTGGCTCAGGATGAACGCTGGCGGCGT-GCCTAATACATGCAAGTCGAACGAACTTCCGTTAA
P.pentosaceus  ........................................-.....................................
A.viridans     NTC.........N....C..........C.....G.N.....A.-.......N.N..........G.....AGAT.AAGT
L.amelobiosum  ..AT.............C...........N.............-.....................CG---------C.
E.columbae     ----.............C..........C.......N.......-.....................C....T.T..C.
E.sulfureus    TT..............C..........C.......N.......-....N.................CTTC.TAC...T
L.fallax       -.A..........N...C...........N......N.......-......C......N.......GCT.G.CG..GACC
Lc.lactis a                        ........C.............-................T..G..CTGAAGGT.GGT
Lc.lactis b                        ........C.............-................T..G...TGAAGAT.GGT
Lb.plantarum   ...T.............C..........C.............-.........................CTG..ATT
S.cerevisiae   ----.ATCT.G......C..C.AGTA.TCAT.T...T.TCT.AAA.AT...GC......T...T..GT.TAAG.AA..T.

                        90       100       110       120
                         '         '         '         '
P.acidilactici TTGATCAGGACGTGCTTGCACTGAATGAGATTTTAAYA
P.pentosaceus  .....T.T.....A....T.....T...........C.CGAAGTGA
A.viridans     GCTTG..CTN.TGA.--------------------
L.amelobiosum  GC..GAG.TG.T...ACCTTTCN.GC.....GGCG.ACGGGTGAGTAACNCGTGGAT
E.columbae     CC.TAGCTTG.TACACC.A.AGT..GTNAG.GGCG.ACGGGTGAGTTACACGTGG
E.sulfureus    CG--AACTTCG..T.ACCA.G.A.GA.GAG.GGCG.ACGGGTGAGTAACAC
L.fallax       .GATCACTTCG...AGACGTAAAGCAT.TGGC.AG.GTGGCGAACGGGT
Lc.lactis a    ACTTGT.CC.AC..GA.
Lc.lactis b    GCTTG..CC..T.TGAA
Lb.plantarum a GATTGGT.CTT.CATCATGAT.T.CATTTGAGTG.GTGAGTGGCGA
S.cerevisiae   .AC.GTGAA..TGCGAATGG..C.T.A.ATCAG.T.TCGTTTATTTGATAGTTCCTTTACTACA
```

Figure 5.17 Alignment of V6 regions (position 968-1058, *E. coli* numbering) from *Pediococcus*, *Enterococcus*, and *Leuconostoc* species extracted from the GenBank. The *Aerococcus viridans* sequence was obtained from Collins *et al.*, (1990). There were no *Lactobacillus pentosus* or *Lactococcus lactis* V6 sequences available and the *Saccharomyces cerevisiae* corresponding region could not be identified due to extensive sequence differences.

```
                10        20        30        40        50        60        70        80        90
                '         '         '         '         '         '         '         '         '
PACID NTACGCGAAGAACCTTACCAGGTCTTGACATCTTCTGCCAACCTAAGAGATT-AGGNGTTCCCTTCGGGGACAGAATGACAGGTGGTGCAT
PPENT C.....................................A..GT.........-..AG..C.............................
AVIRA .A.............N....A...........C.N..A.C...CT......A-G...N..--...........A.................
LPLAN C.............................AC.A...A..T...........-..AC.................CGGAT.............
ECOLU .A.............................C.N..A.C.T.CT......--...ACNNN............A.G..........N....
ESULF CA............................C.T..A.C..TCT......--..AGC.TT............A.G..........N....
LAMEL CA............................C.T..AAGCTT.T......AG.A.T....T......A....A.G..............
LFALL .A............................C.NN.AAG.TG.N......NATNT.....T......A....A.G..........N....
```

Figure legend

PACID	*Pediococcus acidilactici*
PPENT	*Pediococcus pentosaceus*
AVIRA	*Aerococcus viridans*
LPLAN	*Lactobacillus plantarum*
ECOLU	*Enterococcus columbae*
ESULF	*Enterococcus sulfureus*
LAMEL	*Leuconostoc amelobiosum*
LFALL	*Leuconostoc fallax*

Summary of species specific probes identified for *L. plantarum*

Specificity	Oligonucleotide 5' to 3'	T_m (°C)
L. plantarum	CACCAATCAATACCAGAGTT	56
	CACTCAAATGTAAATCATGA	52
	ATCTCTTAGATTTGCATAGT	52

Table 5.3 *L. plantarum* specific oligonucleotides selected and their corresponding melting temperatures (T_m).

5.8 Discussion

The PCR primers chosen were complementary to the conserved regions, which flank the variable V1 and V6 regions, of DNA encoding 16S rRNA. Barry *et al.* (1990) identified these areas in the description of a general method to generate DNA probes for microorganisms. The primers for the V2 region as described in their paper were in actual fact specific for the V1 region as discovered by computer alignment and with reference to the secondary structure of prokaryotic 16S rRNA (Neefs *et al.*, 1990). The primer binding regions have been outlined in figure 5.4 and as is evident the intervening sequences contain regions of considerable secondary structure with hairpin loops prevalent in both the V1 and V6 regions. This led to problems with direct sequencing as discussed later.

The formation of primer-dimers is due to the complementarity between the 3' termini of primers and results, following PCR amplification, in the observation of fragments twice the length of the primers (figure 5.1). The primers specific for both the V1 and V6 regions resulted in primer-dimer formation and the possible combination of each primer that could have caused this phenomenon are indicated below. The primer R1 has four complementary bases (CATG) at the 3' end and would have resulted in a fragment of 36bp (see figure 5.18a), similarly R2 contains four matching bases (CCGG) which would also have resulted in primer-dimer formation (see figure 5.18d). For the V6 region however, primer-dimer formation is not so readily explained. The configurations described in figures 5.18g and 5.18h would not appear to result in stable hybrid formation especially since the homology is between the bases, adenine and thymine, which are held together by only two hydrogen bonds, positioned two nucleotides from the ends of the primers. However, under the conditions created within the PCR reaction without a surface layer of mineral oil, these primer-dimers must have been stabilised long enough to form a double stranded primer-dimer from which the next round of amplification was initiated. The stability of DNA duplexes is known to increase with increasing salt concentration [Wetmur, 1991] and this increase would have occurred due to the evaporation of liquid during the denaturation

(95°C) and extension (72°C) steps. The degree of salt concentration during the reaction is unknown but the formation of primer-dimers was eliminated upon the addition of mineral oil to the surface of the reaction. This demonstrates the caution required in designing PCR primers as primer-dimer formation results in a reduction of the substrate dNTP's for the correct product and may have a role in product inhibition.

V1 region

```
     5'  AATTGAAGAGTTTGATCATG  3'                    R1
                              ||||
(a)                          GTACTAGTTTGAGAAGTTAA   5'  R1


     5'  AATTGAAGAGTTTGATCATG  3'                    R1
                              |
(b)                             CGGCCTGCCCACTCATTAC 5'  R2
                              |  |
(c)                           CGGCCCTGCCCACTCATTACA  5'  R2

     5'  ACATTACTCACCCGTCCGGC  3'                    R2
                             ||||
(d)                        CGGCCTGCCCACTCATTACA      5'  R2
                               ||
(e)                          CGGCCCTGCCACTCATTACA 5'  R2
                           | | | |
(f)                        CGGCCTGCCCTGCCACTCATTACA  5'  R2
```

V6 region

```
     5'  GCAACGCGAAGAACCTTACC  3'                    R3
                              ||
(g)                        CCATTCCAAGAAGCGCAACG      5'  R3
                              |
(h)                          CTCTCTCCACGACGTACCGA   5'  R4
```

Figure 5.18 Possible configurations resulting in primer-dimer formation.

Negative controls should be included with every PCR reaction to detect contamination of solutions. Extra care had to be taken in the amplification of the 16S rRNA variable regions due to the highly conserved nature of the PCR primers. The primers can amplify DNA from any prokaryotic DNA. On occasion, a band was observed in the negative control reactions and in these cases all of the PCR reactions were discarded and the reaction components tested for contamination. Primers, dNTP's and buffers were aliquoted and stored in small quantities and microfuge tubes and pipette tips were kept apart from the normal DNA manipulations. PCR reactions were set up in the laminar air flow cabinet swabbed with 70% ethanol and gloves were worn at all times.

The choice between blunt and cohesive end cloning depends largely on the sequence of the PCR fragment. Blunt end cloning offers the advantages that no prior sequence knowledge and no alteration to the primers is required however, the disadvantages include reduced cloning efficiency, the possibility of cloning a number of inserts which can be in either orientation and the loss of the restriction site which complicates subcloning into another vector. Cohesive or sticky end cloning has the advantages that the insert can be cloned directionally with the regeneration of restriction sites, where non-specific amplification products occur only the target DNA with its restriction sites can be cloned and the efficiency of ligation is usually much higher. The disadvantages include the requirement for an extra restriction endonuclease step prior to ligation and if the restriction sites have been located too close to the termini of the primers, may also require a self-ligation step.

The efficiencies of blunt end cloning can be increased by repairing the PCR termini prior to ligation, by using a high efficiency transformation protocol such as electroporation and by using a phosphatased vector. Restriction of the ligation prior to transformation decreases the number of transformants to screen as the recombinant clones have lost the restriction site. The PCR fragments described in this chapter were cloned using blunt end ligations by repairing the termini with DNA polymerase (Klenow fragment) and T4 polynucleotide kinase and by using electroporation as the

transformation protocol. The cloning of both the PCR amplified insert and some PCR primers demonstrated that the Magic PCR preps™ purification procedure did not remove all of the contaminating primers. This may also have been the reason that the direct sequencing using unlabelled PCR primers was unsuccessful, as chain termination from both primers could have resulted in bands appearing in each lane of the sequencing gel.

Sequencing direct from the PCR reaction has been advocated as a method of choice to overcome the error rate of *Taq* DNA polymerase. The sequence ladder is representative of the population of fragments as opposed to individual clones and so odd mutations do not affect the resulting sequence. The *fmol*™ sequencing system used in this chapter is a linear, amplification thermal cycling system that permits rapid sequencing of femtomolar amounts of purified PCR product. Because the system uses elevated temperatures and multiple denaturation, annealing and extension cycles, signal strength is increased and secondary structure minimised. The annealing temperature is the most critical consideration for thermocycle sequencing of PCR products. High annealing temperatures inhibit strand annealing, reduce template secondary structure and improve the stringency of the primer. Strand reannealing and template secondary structure limit the ability to obtain accurate sequence data from small (<500bp) PCR products. *Taq* DNA polymerase has a reported extension rate of 90 nucleotides/minute at 37°C [Innis *et al.*, 1988], therefore primer extension is initiated during the annealing step of each cycle. As a result, mismatched primers are stabilised prior to reaching temperatures necessary for dissociation of the primer. At these reduced temperatures, the polymerase may encounter regions of strong secondary structure. This can result in *Taq* DNA polymerase dissociation and exonuclease cleavage of the template yielding bands in all four lanes [Ehrlich *et al.*, 1991]. For these reasons the highest possible annealing temperature shoud be used. In the sequencing of the V1 and V6 regions, despite an annealing temperature of 60°C, chain termination in all four lanes was observed making sequence data using this method impossible to obtain. There have been no reports to my knowledge of 100bp fragments sequenced directly from PCR. As is evident

from the secondary structure model of 16S rRNA (figure 5.4), there are hairpin loop structures at positions 73-97 (V1) and 997-1044 (V6) which would have inhibited the sequencing reaction. The PCR fragment fragment due to its small size would have rapidly reannealed preventing access to the primer annealing sites. The presence of both primers in the reaction due to inadequate purification may also have confused the results. The *fmol*™ sequencing of one of the cloned fragments resulted in a clean sequencing ladder (Plate 5.11) which was identical to the sequence obtained using T7 DNA polymerase. This demonstrated that the presence of pUC19 (2.2kb) had a stabilising effect and helped prevent rapid strand reannealing allowing a full sequence to be obtained.

The results from the T7 polymerase sequencing of the transformants demonstrated that different consensus sequences were obtained for each strain of *L. plantarum*. The three *L. plantarum* DCU101 sequences were identical to each other with the exception of a cytosine substituted for thymine in transformant number 10 (see figure 5.7). This correlates well with the reported error rate (1 in 300 bases) of *Taq* DNA polymerase, the substitution occurring within a run of cytosines. The V1 sequences of *L. plantarum* B2 and *L. plantarum* LP115 however, presented some difficulties as the *L. plantarum* B2 transformant number 17 was very different to numbers 15 and 16 and both *L. plantarum* LP115 sequences (transformants tf29 and tf36) had homology with the V1 regions from *L. plantarum* DCU101 and *L. plantarum* B2 respectively. Unfortunately due to the low ligation efficiencies, the number of V6 transformants analysed was much lower and so perhaps greater variation would have been noted for this region.

Having demonstrated homology between the V6 region amplified from *L. plantarum* LP115 and the chromosomal DNA from the *L. plantarum* strains DCU101 and LP115 in the Southern blot shown in Plate 5.7, the cloned V1 and V6 regions were used in a dot blot hybridisation experiment to a variety of species and genera. The hybridisation temperatures were varied between 37°C and 68°C to determine the conditions most suitable for species differentiation using these DNA probes. The results were quite startling in that

the strongest hybridisation signal obtained was from *E. coli* JM83 (Plate 5.13) with the *L. plantarum* strains not demonstrating any particular homology to the probes. The transformant sequences were then sent via electronic mail to the BLAST programme in the US to be aligned with sequences from the GenBank and EMBL databases. The sequences which exhibited the most homology to the transformant sequences were the V1 and V6 regions of 16S rDNA from *E. coli*. The homology between the cloned amplified V1 and V6 regions and *E. coli* rDNA explains the results obtained in the dot blot hybridisation experiment. Unfortunately, there was no *E. coli* chromosomal DNA on the Southern blot which was probed originally to the PCR amplfied V6 region and so the source of the contaminating sequences could not be defined.

As both the V1 and V6 regions amplified from *L. plantarum* did not have perfect homology with *L. plantarum* sequences extracted from the GenBank (see figures 5.14 and 5.15), it could be suggested that the original target DNA in the PCR reaction was contaminated. However, the target DNA (chromosomal DNA from *L. plantarum*) was occasionally used for the ribotyping experiments described in chapter 3. No extra rDNA bands or rDNA bands in common with the two *E. coli* strains (JM83 and JA221) tested which would indicate DNA contamination were ever observed. Other sources of contamination could have been the nucleotides, primers, water, *Taq* DNA polymerase, microfuge tubes or mineral oil but the use of different batches and the inclusion of negative controls in each PCR reaction reduced the chances of contamination going by unnoticed. Possibly concurrent with the *L. plantarum* sequences, a small quantity of contaminant DNA was amplified and cloned resulting in the transformants, but statistically this would be an extremely rare occasion and wouldn't explain all ten transformants containing non-*L. plantarum* sequences.

The possibility of having cloned non-*L. plantarum* sequences was investigated by alignment of the sequences with the V1 and V6 regions of a wide range of genera however no perfect match was found. The eubacterial sequence signatures (in this case, seven bases at particular positions in the V1

region) described by Woese (1987) demonstrated that the V1 transformant sequences were definitely eubacterial in origin. Whether the transformant sequences originated from Gram-negative or Gram-positive bacteria was also investigated. Although few in number, the signature positions characterising the Gram-positive eubacteria are strong ones [Woese, 1987] however, they were outside of the V1 and V6 regions sequenced. The possibility that very diverse *L. plantarum* exist in different environments and therefore their V1 and V6 sequences might vary could be suggested however there were major substitutions made which could warrant reclassification of the strains.

Another explanation could be the homologous recombination of the cloned genes with the host *E. coli* TG1 (which is *rec*+) as a region of similarity exists between *E. coli* and normal *L. plantarum* V1 and V6 sequences (see Figure 5.14 and 5.15) and this occurrence had been previously noted in the laboratory [personal communication with Aidan Fitzsimons]. All of the transformants with the exception of tf15 and tf104 contained unidentified short regions of between 28bp-52bp which may have been the result of a crossover event however this double recombination would be rare.

The selection of genus and species specific probes is most simply achieved by extensive computer alignment of rRNA sequences and the selection of short complementary oligonucleotides. Both the V1 and V6 regions allowed *L. plantarum* specific probes (Table 5.3) to be designed. Confirmation of their specificity was achieved using the BLAST programme but unfortunately there were no *L. pentosus* sequences submitted to the GenBank at this time. Hertel *et al.* (1990) demonstrated the construction of a *L. plantarum/ L. pentosus* specific oligonucleotide based on 23S rRNA but differentiation between the two species could not be achieved.

The heterogeneity of the *Lactobacillus* genus made the identification of genus specific probes impossible. The variable regions identified in the alignment of the different genera corresponded to the variable regions within the *Lactobacillus* genus.

Jurtshuk *et al.* (1992) combined the use of rRNA based oligonucleotides with an *in situ* microscopic technique to yield rapid

hybridisation results. The technique described offers several advantages; the detection of a specific segment of the 16S rRNA sequence within intact individual cells allowing the identification of a specific bacterium even within a mixed microbial population. *In situ* hybridisation also exhibits a greater sensitivity for the detection of bacterial cells than other nucleic acid detection methods currently in use [Jurtshuk *et al.*, 1992]. Thirdly, the *in situ* technique does not require the extraction of nucleic acids from isolated or cultured cells and the technique allows for the use of multiple probes with different labels. The hybridisation and detection protocol described can be completed in less than one hour and can be performed on site with the aid of a portable scanning fluoremeter.

Examples of some of the applications of rRNA based oligonucleotide probes in conjunction with rapid *in situ* techniques would be the identification of LAB (desirable or undesirable) in silage or other lactic acid bacterial fermentations, the estimation of populations of desirable homofermentative species present on grass prior to ensilage to determine inoculation rates and the monitoring of microbial changes in silage over the fermentation period for inoculum evaluation.

Chapter 6.
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Appendix A

Research Communications

The research carried out in chapter 4 will be published in the *Journal of Applied Bacteriology* (in press) under the title of "Dominance of *Lactobacillus plantarum* strains in grass silage as demonstrated by a novel competition assay."

Work carried out during this reseach project was presented in seminar form to;
Teagasc Agriculture and Food Development Authority, Grange research and Development Division, Dunsany, March, 1993.

The School of Biological Sciences, Dublin City University, May 1993.

In addition, poster presentations were made at the following meetings, the abstracts of which were published;
The Irish Grasslands Meeting, 1991, UCD Belfield.
(published in the *Irish Journal of Agricultural Research* **30 (1)**:78)

Fourth Symposium on Lactic Acid Bacteria, Noordwijkerhout, The Netherlands, September 5-9th, 1993.
(to be published in *FEMS Microbiology Reviews*)