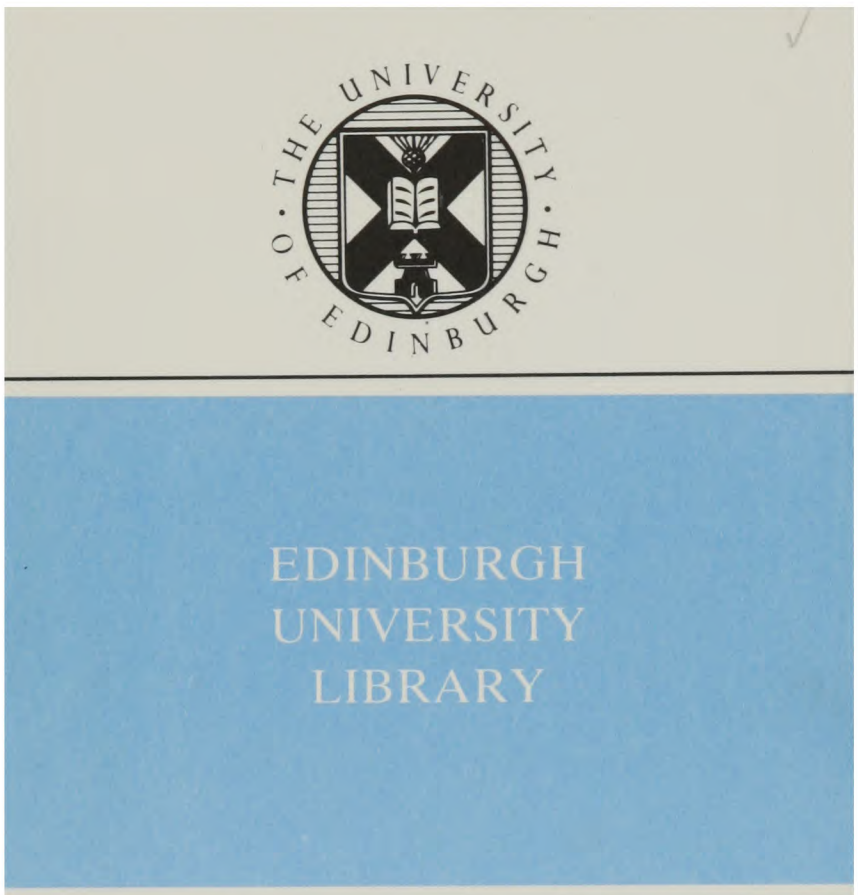

The GENETICS of SILAGE BACTERIA

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December 1991

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ABSTRACT

The conversion of raw forage crops such as grass and cereals into silage is brought about by a fermentation carried out by the indigenous lactic acid bacteria. The most important of the lactic acid bacteria in terms of silage fermentations are members of the genera *Lactobacillus* and *Pediococcus*. Due to recent advances in the genetics of the lactic acid bacteria, there is now significant potential to modify strains by genetic manipulation and hence create a new range of strains with additional desirable characteristics such as cellulase production which can be used as silage additives.

The strains used in this study were all natural silage isolates and were identified as either *Lactobacillus plantarum* or *Pediococcus pentosaceus* using a range of characteristics including homo/heterofermentative activity, Gram staining, the API 50CH system and growth temperature. Six strains of *L. plantarum* and twelve strains of *P. pentosaceus* were isolated. All were screened for the presence of native plasmids, with a range of sizes from two kilobases (kb) to eleven kb identified within the *L. plantarum* strains and a more limited size range identified within the *P. pentosaceus* strains.

None of the isolates were naturally transformable so electroporation was used as a means to facilitate the uptake of exogenous DNA. The transformation protocol was optimised by examining the effects of electroporation buffer, growth conditions of the bacteria, DNA concentration and various electrical parameters. The optimised procedure used exponential phase cells, 0.5M sucrose as the electroporation solution, one μg plasmid DNA, electrical parameters of 2.5 kilovolts, 100 ohms resistance and 25 microFarads capacitance followed by an expression period of two hours.

A range of vectors, viz pTG262, pIL253, pIL277 and pMTL500E, was used in an attempt to introduce heterologous genes into the *Lactobacillus* and *Pediococcus* strains. The genes chosen for study were two ß-1,4-endoglucanase (cellulase) encoding genes from *Cellulomonas flavigena* and the *cel*C gene (also a ß-1,4-endoglucanase) from *Clostridium thermocellum*. The expression of these genes was indicated by a zone of clearing in a

congo red plate assay.

None of the plasmids constructed which contained the *Cellulomonas flavigena* genes resulted in detectable cellulase activity from any of the experimental strains but cellulase-producing isolates from some of the *Lactobacillus* strains containing the *Clostridium thermocellum celC* gene carried on plasmid pCC1 were identified.

INTRODUCTION

Silage is the material produced by the controlled fermentation of a crop of high moisture content (McDonald, 1981). Ensilage is the name given to this conservation process and it involves a bacterial fermentation. The objective of ensilage is to preserve the crop at its optimum growth stage and with minimum loss of nutrients for use as an animal feed in the seasons when it does not grow. Basically, ensilage involves harvesting the crop, storing in a large container (called a silo), compacting the crop at various times and finally sealing the silo to exclude as much air as possible.

During the first hours in the silo, respiration will still occur within the plant tissues and the energy used in the intact plant for biosynthetic reactions will be dissipated as heat leading to an increase in temperature in the silo. Plant respiration will eventually cease due to a combination of denaturation of enzymes by heat and the pH drop due to acid production by the bacteria indigenous to the crop. The release of plant juices by plasmolysis is required for the initial bacterial stages of the fermentation. Under anaerobic conditions, the fermentation will continue until the preservation pH of 4.0 is achieved.

Knowledge of the ensilage process has been traced back to Ancient Greece and it has been used in Britain as a major conservation process for the last century. Despite this long history, the control of silage making is not as detailed as the other natural fermentation process known to man *eg* brewing and baking.

For a more detailed review of the ensilage process, the reader is referred to McDonald, 1981; Woolford, 1984.

CROPS for ENSILAGE

The ideal crop for ensilage has the following characteristics (McDonald, 1981):

a) A good level of fermentable substrate in the form of water-soluble carbohydrate.

b) Low buffering capacity *ie* not very resistant to pH change.

c) High dry matter content in the fresh crop.

In Western Europe the main ensilage crop is grass, of which there are many types *eg* Italian ryegrass, perennial ryegrass, timothy grass. Other crops which can be ensiled include maize, legumes (lucerne) and sugar beet tops & leaves.

SILAGE BACTERIA

The fermentation is carried out under anaerobic conditions by the indigenous bacteria. Surprisingly, the initial bacterial numbers on fresh forage tend to be very low (10^1-10^2/ml macerated plant material) and it has been proposed that the harvesting equipment and the silo itself act as additional sources of bacteria (Stirling & Whittenbury, 1963). The principal group of bacteria involved, the lactic acid bacteria (LAB), use the water-soluble carbohydrate (WSC) from the crop as an energy source to produce lactic acid (LA) which serves to reduce the pH and preserve the crop.

The LAB form one of a number of groups of micro-organisms that occur within silage. The various groups are listed below:

1) LAB: This is a complex group of bacteria all of which produce lactic acid as a principal fermentation product. Members are microaerophilic, Gram-positive, non-spore forming and can be classified into two groups according to their fermentation products:-

1) Homofermentative: produce only lactic acid from hexoses

2) Heterofermentative: produce acetic acid, ethanol and carbon dioxide as well as lactic acid.

Members of the following genera are lactic acid bacteria: *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Streptococcus* (some members of this genus have recently been redefined as *Enterococcus* and *Lactococcus*).

2) Clostridia: These bacteria are obligate anaerobes which form spores and are the main spoilage organisms within silage. The main source of clostridia is soil and hence soil contamination of the grass should be kept to a minimum during silage production. The optimum pH for clostridial growth is between pH 7 to 7.4 and they cannot tolerate the

acid conditions of pH 4 and below (McDonald, 1981). Clostridia reduce the nutritional value of the silage by catabolism of amino acids leading to ammonia production and some of the products of amino acid fermentation may be toxic. The fermentation product from sugars and LA by clostridia is butyric acid which is not as strong an acid as lactic acid and hence the remaining silage components are not well preserved. Clostridial silages are generally characterised by a foul odour from the butyric acid which is distinct from the sweet smell of lactate silages and this will generally lead to decreased voluntary intake by cattle and sheep.

3) Bacilli: These bacteria are facultative anaerobes which can produce LA and acetic acid from the silage WSC although they do not appear to contribute to a reduction in pH. Bacilli compete with the LAB for the substrate thereby reducing the efficiency of the fermentation and hence their presence is considered disadvantageous (Woolford, 1977).

4) Enterobacteria: Strains found in silage tend to be non-pathogenic, have an optimum pH of 7.0 and are probably only active in the early, aerobic stages of the fermentation before the pH is lowered.

5) Moulds: Stable silage conditions,*ie* low pH and anaerobiosis, are unfavourable for the growth of moulds, hence they are generally only associated with those areas of the silage which are exposed to air and may be involved in aerobic deterioration. The presence of moulds in silage can be detrimental due to the catabolism of nutritional silage components and in some cases the formation of potentially lethal toxins.

6) Yeasts: Yeasts are not inhibited by acid conditions and hence high numbers can be observed in silage. Their presence is undesirable because of their association with aerobic deterioration and also because they compete with the LAB for the WSC producing ethanol which has little preservative effect (Woolford, 1976).

BACTERIA-INDUCED CHANGES WITHIN SILAGE

During the stages before air is excluded, aerobic organisms are the major silage microbes but they will be quickly dominated by the anaerobes. Residual enterobacteria are

outnumbered by the streptococci and the leuconostocs which are aerobically fast-growing. The pH is lowered by the production of lactic acid and finally the lactobacilli and pediococci take over to maintain a pH level of 4.0 and preserve the crop. Silage LAB are non-proteolytic so the majority of the silage nitrogen is maintained in a protein or amino acid form (processing of protein to amino acids is carried out by plant enzymes in the initial ensilage stages). In a well-preserved silage the LAB should dominate within three to four days of sealing the silo.

Clostridia are also active in the early days of ensilage. Suppression of clostridia is achieved by:

1) Rapid establishment and maintenance of pH 4.0

2) High dry matter content. Clostridia are present in the silage as spores and hence require a high water level to germinate. The moisture content of a crop can be decreased by in-field wilting *ie* water will evaporate if the crop is allowed to lie after cutting. Obviously, this is climate-dependent.

Preservation of the nutritional value of the crop is important because ensilage is a long term process. The silo may remain sealed for several months, *eg* June-December, before the silage is required for feeding. During this time the high lactic acid level should maintain the silage in a steady state if no air enters. At the end of the fermentation little WSC will remain, the majority of the protein is present as amino acids and the acids have converted chlorophyll into a brown pigment characteristic of silage. The voluntary intake of silage tends to be lower than that of fresh forage (McDonald, 1981) but this does not seem to be due to any single chemical factor and silage remains one of the most economic means of producing winter feed.

SILAGE ADDITIVES

Silage making is very dependent on climate. Ideally, grass for ensilage should be cut around the time of early ear ripening when the dry matter (DM) content and digestibility

of the plant is high. Low DM crops should also be wilted to reduce water content. Obviously, both of these stages are themselves dependent on climate: it may not always be possible to cut at the ideal time or possible to allow wilting at all. Crops with a low DM content will require higher levels of lactic acid production to achieve the critical pH of clostridial inhibition and hence are more likely to succumb to a clostridial fermentation. A wide range of silage additives are available which can increase the likelihood of a lactic fermentation occurring and thus aid in the preservation of the crop. The majority of additives are added to the crop as it is unloaded into the silo.

TYPES OF SILAGE ADDITIVES:

1) ACIDS

Acids act as fermentation inhibitors, the aim being to reduce the pH to a level where plant and microbial enzymes are inhibited. Generally, a pH of 3.6 is considered a target level. Some plant respiration will occur at this level until all the oxygen disappears but breakdown of protein is prevented and hence nutritional value is retained.

The original acids used were mineral acids,*eg* hydrochloric, sulphuric, phosphoric acids. The main problems associated with their use was that they were corrosive to machinery and dangerous to handle.

Less corrosive fatty acids, *eg* formic acid, can also be used and because of its almost guaranteed success at producing a well preserved silage, formic acid is now probably one of the most commonly used silage additives. It is a much weaker acid than the mineral acids and it is not practical to attempt to lower the silage pH to below 4.0. At the commercial application-rate a pH of 4.9 to 4.6 generally results. The antibacterial action is a combination of hydrogen ion concentration and selective antibacterial action of undissolved acid, although not all lactobacilli are inhibited by this and a certain degree of lactate fermentation may still occur. In addition to protein, the WSC is also preserved and an increase in WSC levels may even be detected due to hydrolysis of polysaccharides.

Other acids such as propionic and acetic acids may also be used as fermentation

inhibitors but are expensive because they are weak acids. Another obvious choice would be lactic acid but again its use is not economic.

2)CARBOHYDRATES

The fermentation may be stimulated by the addition of carbohydrate as a readily available source of energy for the LAB. This is probably the oldest and most traditional type of silage additive and is particularly important with crops of low natural WSC such as legumes.

Direct applications of sugars, *eg* glucose and sucrose, although effective, are generally prohibited by cost. Much more common is the addition of molasses, a by-product of the sugar beet and sugar cane industries. Molasses addition results in an increase in LA content, pH decrease and decrease in loss of nitrogen as ammonia. As the WSC level in molasses-treated silage is much greater than in control silages, higher residual WSC will be present when the fermentation is complete. Cereals, *eg* maize, barley, oats, may also be used and results tend to be similar to those with molasses.

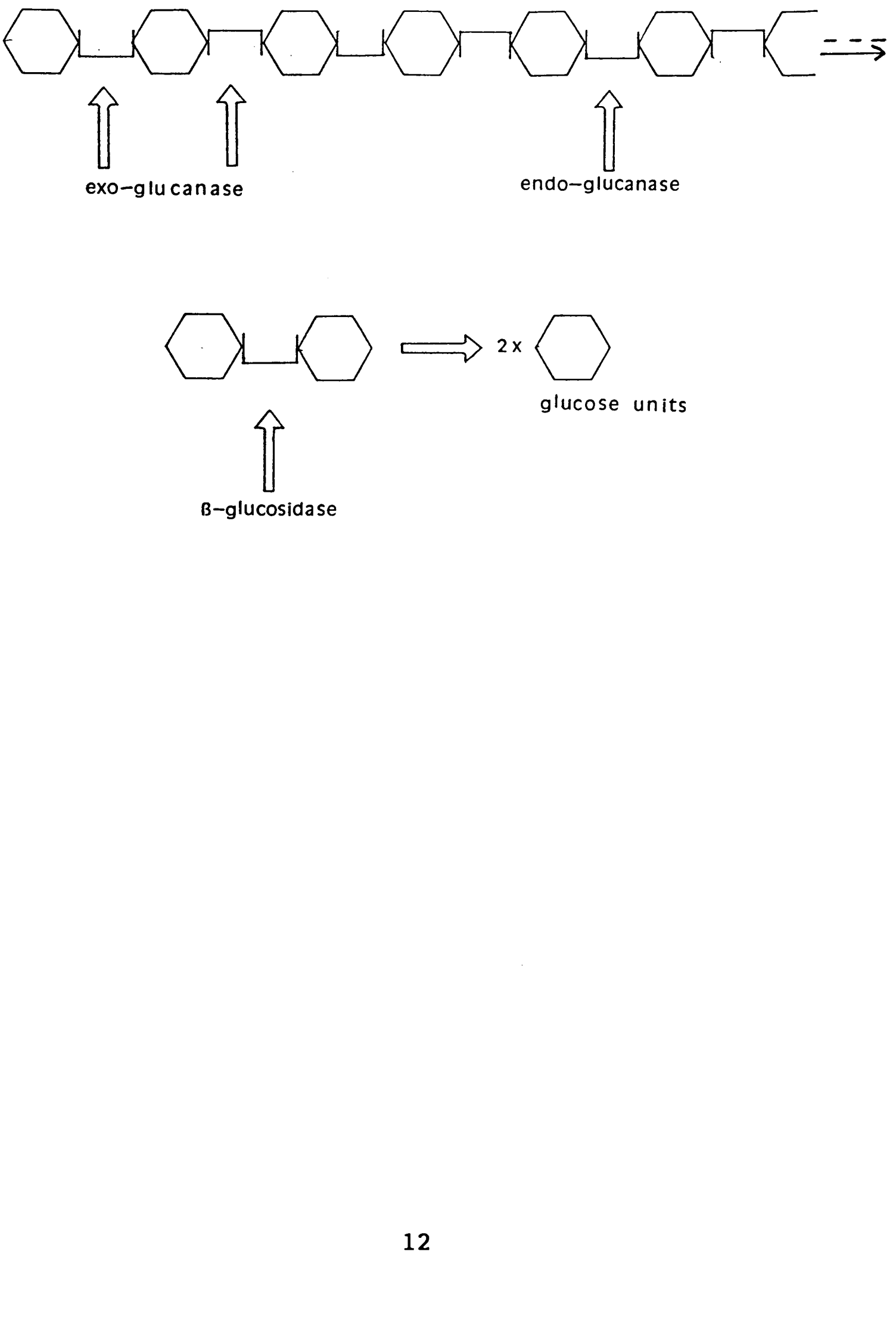
3) ENZYMES

The cellulose component of plants is not available as a substrate for LAB nor is it successfully digested when eaten by livestock, so a substantial amount of the crop is not utilised to its full potential in silage.

Many cellulase enzyme preparations are effective within pH range 4-6 and hence are suited to use in silage. Increased cellulose hydrolysis has been demonstrated by using an enzyme as an additive, which gives the added advantage of increasing the available WSC, but a quantitative increase in digestibility has been more problematic to show (Whittermore & Henderson, 1977).

Cellulose is a very complex molecule consisting of ß1-4 linked glucose polymers *eg* Figure 1.1.

Figure 1.1. Diagrammatic representation of cellulose structure and sites of action of cellulase enzymes.



Enzymes which cleave this polymer, known as cellulolytic enzymes or cellulases, all cleave this ß1-4 glycosidic bond but vary with respect to the microenvironment in which this bond occurs. There are three main types of cellulase enzymes:

i) ß1-4 exoglucanases (or cellobiohydrolases) which cleave off external glucose units from the polymer.

ii) ß1-4 endoglucanases which cleave internal bonds.

iii) ß-glucosidases which break up glucose dimers produced by the other enzymes to give monomer units.

Cellulolytic bacteria and fungi generally produce a range of cellulase enzymes, each varying in its specificity. Cellulose may contain up to 10,000 glucose units (Knowles *et al.*, 1987) and the same polysaccharide molecule can contain regions of ordered crystalline structure as well as more randomly bonded areas. This, in addition to the common association between cellulose and other polymers such as lignin and hemi-cellulose, may help to explain why a family of enzymes is necessary for degradation.

Some bacterial cellulases form a tight multi-enzyme complex known as a cellulosome *eg* those in *Clostridium thermocellum* (Lamed *et al.*, 1983).

Most of the bacterial cellulases which have so far been cloned have been detected by expression in *Escherichia coli* grown on carboxymethyl-cellulose (CMC), a soluble form of cellulose (Knowles *et al.*, 1987). The derived amino acid sequences of many of these enzymes are quite dissimilar; even the same class of enzyme (*eg* endoglucanases) from the same bacterial species can show distinct differences. This may be an indication that the present classification of these enzymes is over simplified.

Given the complexity of cellulose and indeed of cellulases, it is unlikely that one enzyme alone used as a silage additive would produce a significant increase in WSC. In most cases enzyme additives are contained in a preparation which also contains a bacterial inoculum and/or carbohydrates.

Other enzymes are also used as silage additives and are similarly used in an attempt to

increase the available energy source for the LAB. These include:

i) Amylases: These bring about the random cleavage of alpha-1,4 glycosidic bonds.

ii) Cellobiohydrolases: Cellobiose is the disaccharide subunit of cellulose and cleavage by these enzymes produces glucose monomers.

iii) Xylanases: These enzymes cleave the ß1-4 linked xylopyranosyl residues in xylose, another polysaccharide polymer.

iv) Hemi-cellulases: Hemi-cellulose is a heterogeneous group of polysaccharides which is found in plant cell walls *eg* Hemicellulose B which is a polymer of xylose and glucuronic acid, arabinose and galacturonic acid and hemi-cellulases can be used to release arabinose and xylose.

4) NUTRIENTS

While silage presents a relatively easy and cheap way of feeding livestock in winter, it does not necessarily meet all dietary requirements. As an alternative to giving dietary supplements at feeding, deficiencies can be overcome by adding nutrients to the silage; some will also act as fermentation stimulants eg molasses.

Nitrogenous compounds, *eg* urea to overcome any nitrogen deficiencies of the silage; and minerals such as limestone to overcome deficiencies in calcium, are the main nutrients which do not affect the fermentation. Addition of both is particularly important with maize silage.

5) ABSORBENTS

During the silage fermentation, effluent will be produced. This contains WSC, LA, acetic acid and minerals *ie* many of the nutritional elements of the silage. Silages with higher moisture contents will produce more effluent than dryer ones. The biological oxygen demand of silage effluent is huge and hence it is a severe pollutant of waterways should it be allowed to leak.

The easiest and cheapest method of disposing of effluent is collecting it in a tank for use

as a grass fertiliser or as a feed supplement. Recently, a new type of additive has come on to the market designed to combat the effluent problem. These absorbents reduce effluent production by effectively mopping up excess moisture. Most are based on straw or sugar beet pulp (*ie* common, and hence cheap, waste products) and are spread over the bottom of the silo to act as sealant and/or mixed in with the silage.

6) STERILANTS

Some compounds inhibit the silage fermentation by preventing any or most bacterial activity.

For example:

i) Formaldehyde: This is used as formalin, a 40% aqueous solution of formaldehyde. It is a bacteriostatic agent which also protects plant proteins from microbial degradation in the rumen hence the nutritional value of the silage is kept intact and may even be increased due to better utilisation in the animal. A chemical reaction occurs between the formalin and the plant proteins so the amount added is critical; too much can inhibit the animal's gut flora, too little may promote clostridia (Wilkins *et al.*, 1974).

ii) Propionic acid: This inhibits most of the silage organisms and is especially useful in delaying the onset of aerobic deterioration.

7) INOCULANTS

The addition of bacterial cultures to a silage also acts as a fermentation stimulant: the strategy is to ensure lactic fermentation by addition of large amounts of LAB to the silage. Several authors have described the benefits of inoculated silages *eg* Carpintero *et al*, 1979; Lindgren *et al*, 1983; Weinberg *et al*, 1988 and it can be argued that inocula are the most desirable of the silage additives since they are safe to handle and act by aiding the natural fermentation. Whittenbury (1961) defined the characteristics of the ideal biological silage additive as follows:

i) Vigorous growth & a good competitor (preferably to the level of dominance) against

other organisms

ii) Homofermentative

iii) Acid tolerant & able to produce a final pH of 4.0 rapidly

iv) Able to ferment hexoses and pentoses

v) No dextran or mannitol production (products of heterofermentative organisms)

vi) No hydrolytic action on organic acids

vii) Growth temperature range up to 50°C

viii) Able to grow in low moisture content

Other criteria have also been added to this list *eg* no proteolytic activity, genetic stability. It is unlikely that one single organism will fulfil all the criteria and most commercial inocula contain two or more strains of LAB, generally a *Lactobacillus* species with either a *Pediococcus* or a *Streptococcus*. A possible exception to this is a pure culture of *Lactobacillus plantarum* and even here the early stages of the fermentation are probably carried out by the LAB naturally present since *L. plantarum* is slow to produce acid above pH 5.0 (McDonald, 1981).

The following are the most common LAB to occur in silage inocula:-

Lactobacillus plantarum; *L. acidophilus*; *L. bulgaricus*; *Pediococcus pentosaceus*; *P. acidilactici*; *Streptococcus salivarius* subsp. *thermophilus*; *S. faecium* (now *Enterococcus faecium*, Schleifer & Kilpper-Bälz, 1984).

According to manufacturers literature, most additive strains are natural isolates from silage (rather than culture collection strains) which have been selected for as many of the ideal criteria as possible. Most are available as freeze-dried preparations which are rehydrated prior to application. The cultures are stable in the dehydrated form and have a long shelf life. Inocula are applied in the region of 10^5-10^6 bacteria /g of crop as levels below this do not ensure dominance in the fermentation (Woolford, 1984). Silage is a very complex material with its exact composition varying with the crop used. Hence it is difficult to define units of activity for such a medium in, for example, terms of rate of pH

fall or rate of LA production in a way which is standard for other commercially available cultures (*eg* dairy starter cultures). The high number of variables between different silages, such as type of crop, climatic conditions, ensiling technique and properties of the inoculant mean that a given inoculant may not always be beneficial (Henderson & McDonald, 1984).

At present the selection of silage inocula is based mainly on biochemical properties, *ie* Whittenbury's criteria, with a plasmid profile as perhaps the only genetic data included. The alternative to a mixture of strains as a silage inoculum is a single strain which has been genetically manipulated. Recombinant DNA technology allows the possibility of the introduction of properties which silage LAB do not naturally possess but which could be beneficial. Some examples are the production of anti-microbial factors particularly those inhibitory to the clostridia; amylase and cellulase production for better utilisation of substrate; easily-assayable properties such as light emission which would allow labelling of strains to measure their action in the silage fermentation.

The dominance of inoculated strains over the indigenous flora has been demonstrated recently by Cocconcelli *et al.* (1991) who demonstrated using DNA probes that 95% of the bacteria recovered from the silage were identical to those used as inocula.

8) SPECIALISED ADDITIVES AND RECENT DEVELOPMENTS

In the last few years a revolution in silage making has occurred with the introduction of the big bale method. The silage is made in large bales, each of which is sealed separately instead of sealing in one container. The fermentation occurs just as efficiently in both methods, the main benefit of big bales being no requirement for a large, expensive silo. As big bales have become more popular, specialised additives have appeared on the market. These are mainly sterilants aimed at decreasing aerobic deterioration; with a larger surface to volume ratio big bales are more susceptible than silos.

Another new development has been the introduction of additives containing clostridial bacteriophage which aims to decrease the clostridial population and hence leave the LAB

to continue the fermentation.

CONTROL OF SILAGE ADDITIVES

At present, there is no form of registration or monitoring of silage additives within Britain and many other EC countries. On the British market there are 100 or so additives each of which claims to be the best in its own particular area but only a few have been rigorously tested and have experimental data to reinforce their claims. The European market is continuing to expand and this has led to an EC directive on the registration of additives. Three categories have been proposed:

(Farmers Weekly Silage Additive Supplement- February 1989)

1) Full Approval

Entry in this category would require detailed dossiers on exact contents, performance in both laboratory and field trials and toxicological studies. Manufacturers providing this information to the satisfaction of the commission would be allowed to market their product in all member states.

2) Marketing licence for limited time period

If initial safety evidence were available, a product may be given a temporary licence to allow a full dossier to be prepared. Failure to provide full details in the time scale stated would lead to product withdrawal.

3) Additives currently in use

Additives which are already marketed and in use within member states would be registered under the same conditions as Category Two. The requirement for group Three should be eliminated after the introductory phase of the registration.

As yet, these proposals have not been finalised or passed into law. While, in theory, the registration of additives would allow farmers to make a balanced decision on the usefulness of a particular additive under certain conditions, in practise the correlation of trial results poses vast problems (Seale, 1986). For example, even within one country there is no standard crop for ensilage: an additive which is effective with grass may not

have a beneficial effect when used with maize. The effect of a particular additive can easily be compared with a negative control (*ie* silage with no additive) but what of a positive control? Due to its widespread use, a formic acid treatment is often used as a positive control but this in turn can lead to problems when comparing two additives with different modes of action *eg* an acid with an inoculant.

Laboratory scale trials are an obvious first step in testing. If an additive is not effective under the ideal conditions of a laboratory silo, then it will be of no use on a farm scale. Farm trials are also an essential part of the documentation but are hugely expensive. The requirement of two or more silos to be set up in as near as possible to equivalent conditions limits trials to those farms with more than one silo available. This tends to be available only on farms associated with research institutes and companies rather than those which are privately owned and hence a finite number of trials can be performed at any one time. The need for results from more than one trial, one area and one set of seasonal conditions further compounds the cost.

In addition to chemical and microbial analysis of silages under different additive treatments, animal studies must also be carried out. As well as statistics on voluntary intake, changes in milk production and live weight gain are of prime importance to the farmer.

Hence, while it is clear that the registration of additives is vital for their continued use, the standardisation and implementation of trials pose important problems which must be overcome before the legislation is complete.

GENETICS OF LACTIC ACID BACTERIA

The work reported in this thesis is based on the study of certain genetical aspects of silage strains of *Lactobacillus* and *Pediococcus*. To set this report into its proper context, it is necessary to give some background into the current status of genetics of the Lactic Acid Bacteria.

Within members of the LAB, the genetics of the genus *Lactococcus* is the most advanced and hence will be discussed first.

LACTOCOCCUS

Members of this group were originally part of the genus *Streptococcus*; the group initially known as the lactic streptococci have now been reclassified as *Lactococcus* (Schleifer *et al.*, 1985).

Lactococci are food grade organisms *ie* they and their metabolic products are considered fit to consume. The manufacture of dairy products eg cheese, yoghurt, traditionally utilises lactococci as selected starter cultures. One important feature of these organisms is that they can be grown in large scale fermentations and hence large scale production is possible. Due to their economic importance, and the less stringent safety considerations involved in their use as compared with other, potentially pathogenic micro-organisms, the lactococci have been, and continue to be, the focus of much attention for genetic manipulation. Recombinant DNA technology has an important role to play in the development of new starter cultures, along with more traditional methods of selection *eg* biochemical characteristics.

MODES OF GENETIC TRANSFER

In order to develop strains with new and desirable characteristics, it is first necessary to establish means of genetic transfer between bacteria. Naturally-occurring lactococci have

their own complement of plasmids, many of which are still cryptic. Most genetic studies have been carried out on plasmid-free strains *ie* natural strains that have been cured of their plasmids. While this removes problems of incompatibility and some restriction-modification systems, it means that these strains are not suitable for production unless the necessary phenotypes are re-introduced.

The transfer of genes by natural means can elucidate important information about the genetic characteristics of an organism. With the lactococci, conjugation has been a particularly useful tool in this respect (see below).

In addition to natural transfer methods, it is also possible to genetically manipulate members of the LAB by using other methods to create gene transfer which may not occur naturally. This requires vector molecules to carry DNA into and out of strains as necessary.

There are two main classes of vectors that are used in the lactococci:

1) Derivatives of the broad host-range plasmids pAMß1 (Leblanc & Lee, 1984) and pIP501 (Evans & Macrina, 1983). (See below for further details).

2) Vectors derived from lactococcal plasmids.

In general, lactococcal plasmids do not contain selectable markers (*eg* genes for antibiotic resistance) and these must be introduced from other plasmids *eg* pGK12, which carries the chloramphenicol-resistance marker from pC194, the erythromycin-resistance marker from pE194 and the replication functions from pWV01 (Kok *et al.*, 1984); pNZ12 which carries the chloramphenicol-resistance determinant from pC194 and the kanamycin-resistance determinant from pUB110 (de Vos, 1986).

The majority of these vectors are also capable of replication in heterologous hosts *eg* *Bacillus subtilis*, *E. coli* (especially important because it is Gram-negative), *Streptococcus sanguis* (which is naturally transformable), thus facilitating cloning strategies.

In most cases the antibiotic-resistance gene is expressed via the inherent lactococcal promoters.

Other foreign genes have also been cloned in lactococci using lactococcal expression signals. For example, the *LacZ* gene from *E. coli* (de Vos & Simons,1988) and the bovine prochymosin gene (Simons & de Vos, 1987).

Expression Signals

The consensus lactococcal promoter which has been elucidated from randomly selected DNA fragments using promoter probe vectors (de Vos, 1987) and the sequences of cloned genes, conforms to the consensus derived from other Gram-positive organisms (Graves & Rabinowitz, 1986), *ie* the lactococcal RNA polymerase seems to be a typical Gram-positive polymerase. Differences do still exist, however. For example, strong *B. subtilis* promoters can be weak in *L. lactis* (van der Vossen *et al.*, 1985), suggesting that not all the controls are universal.

For a much more detailed background of lactococcal genetics than the overview presented the reader is referred to: de Vos, 1986; Abstracts and reviews of the second symposium on lactic acid bacteria, 1987; Fitzgerald & Gasson, 1988; de Vos & Simons, 1988; van der Lelie, 1989; Abstracts and reviews of the third symposium on lactic acid bacteria, 1990.

NATURAL METHODS OF DNA TRANSFER IN LACTOCOCCI:

1) Transduction

Transduction involves the transfer of bacterial genes from one bacterium (the donor) to another (the recipient) by a phage. During the infection of the donor cell by the phage, the donor's DNA may be erroneously packaged into a phage particle and introduced into the recipient cell during infection. Recombination between this foreign DNA and the recipient's own DNA can lead to stable inheritance of the new genetical traits.

In the case of temperate phage where its own genome has been integrated into the DNA of the host bacterium *eg* phage and is replicated as part of the host's chromosome, incorrect excision of the phage can lead to the bacterial genes immediately adjacent to the

integration site being packaged and transferred along with part of the phage DNA.

Transduction was the first example of genetic transfer to be reported for *Lactococcus* (Sandine *et al.*, 1962) and its occurrence seems to be more frequent in these bacteria than other members of the LAB.

Evidence for plasmid-encoded lactose metabolism genes in *L. lactis* subsp. *lactis* C2 was first obtained during a transduction experiment using a temperate phage (McKay *et al.*, 1976). The transfer of plasmid material by this phage gave lactose-positive (able to metabolise lactose) transductants which contained a smaller plasmid than the parent strain. The reason for the size difference is considered to be the result of transductional shortening *ie* the original 30 Md lactose-encoding plasmid was too large to fit into the phage head and was deleted to the size of the phage genome during packaging (McKay *et al.*, 1976). With *L. lactis* subsp. *lactis* 712 the parent plasmid was discovered to be unstable (Gasson, 1983[a]) and spontaneously-deleted mutants of the correct size were then simply packaged by the phage.

The possibility of using transduction as a major method of genetic transfer in the laboratory is limited by the narrow host range of currently available phage and the relatively small amount of DNA which can be transferred.

2) Conjugation

Conjugation is a process in which genetic material is transferred from one bacterial cell to another via a physical connection between the two cells. The element which codes for the transfer functions commonly occurs as a plasmid (or part of a plasmid) but chromosomal genes can also be conjugally transferred.

Conjugation appears to be widespread among the lactococci and both intra-species and inter-species transfer occur naturally.

An early conjugation experiment confirmed that lactose-fermenting ability is plasmid coded (Kempler & McKay, 1979). Some lactose-positive transconjugants from strains of *L. lactis* subsp. *lactis*, subsp. *cremoris*, and subsp. *diacetylactis* have been shown to

contain larger lactose-encoding plasmids than the parent strain (Scherwitz *et al.*, 1983; Snook & McKay, 1981; Walsh & McKay, 1981). With *L. lactis* subsp. *lactis* strains ML3, 712 and C2, *Lac+* transconjugants exhibited a "clumping phenotype" (Gasson & Davies, 1979; Walsh & McKay, 1981) in broth culture, and were also able to transfer lactose-fermenting ability in secondary matings at higher frequencies than the original donor. This high frequency transfer (HFT) ability was further investigated in strains ML3 and 712.

With ML3 *Lac+* transconjugants, the new plasmid was identified as a hybrid of pSK08, the original lactose-coding plasmid, and a 27Md low copy number plasmid, pRS01. pRS01 contains the HFT ability and the clumping functions (Anderson & McKay, 1984). Integration of the two plasmids occurred at a specific site on pSK08 but at various sites on pRS01. The clumping phenotype (*clu*) is unstable with spontaneous reversion to Clu^- from Clu^+ transconjugants by precise or imprecise excision of pRS01. A model proposed to explain these events suggested the involvement of a transposable element which mediated in co-integrate formation (Anderson & McKay, 1984). This was subsequently proved by the identification of an insertion sequence on pSK08 (Polzin & Shimizu-Kadota, 1987).

With 712, similar HFT ability was recorded in transconjugants (Gasson & Davies, 1979). Again, DNA fragments were shown to be integrated into the lactose-encoding plasmid pMG820, although in this case the insert size varied (Gasson, 1983[a]). In some cases, inserts of identical sequence but different sizes were identified; in each case one end of the insert seemed to be fixed while the other end varied (Fitzgerald & Gasson,1988). These data are also consistent with the involvement of an insertion sequence.

Some lactococci produce substances known as bacteriocins which are antagonistic to the growth of other, generally related, organisms. Bacteriocin-producing activity can be transferred by conjugation events in several species (Scherwitz *et al.*, 1983; Neve *et al.*, 1984).

The production of nisin, a polypeptide antibiotic, and sucrose-fermenting ability are

plasmid linked and can also be conjugally transferred (Gasson, 1984).

Large scale dairy fermentation involving lactococci can be particularly susceptible to bacteriophage infection and hence selection of phage resistance is important. Several different mechanisms of phage resistance can be conjugally transferred in the lactococci (Klaenhammer & Sanosky, 1985; Sanders *et al.*, 1986; Steele & McKay, 1986).

The broad range plasmids pAMß1 and pIP501, which were originally isolated from *Streptococcus* spp., can also be conjugated into and between species of *Lactococcus* (*eg* Gasson & Davies, 1980). Both pAMß1 and pIP501 and also derivatives of them can be used to mobilise other non-self-transmissible plasmids (Fitzgerald & Gasson, 1988; van der Lelie, 1989).

pAMß1, pIP501 and their derivatives are the main sources of conjugative vectors within the lactococci. These plasmids can also be used to render non-transformable strains susceptible to genetic manipulation. Using the method originally developed for *E. faecalis* (Smith & Clewell, 1984), cloning is carried out first in an easily transformable strain followed by conjugative mobilisation to a non-transformable host. Langella & Chopin (1987) and Romero *et al.* (1987) have successfully used this method for entry of recombinant DNA into non-transformable lactococci.

Conjugative Transposons

Insertion sequences (IS) are small (1-2 kb) regions of DNA which are capable of moving from one location to another. This process is known as transposition and IS elements generally code only for the functions required for their transposition.

Transposons are larger DNA sequences which carry other genes not involved in transposition. Some transposons are bounded at each end by known insertion sequences. Members of the Tn916 family of transposons (originally from *Enterococcus faecalis*) and transposon Tn919 (originally from *Streptococcus sanguis*) can be conjugally transferred (Gawron-Burke & Clewell, 1984; Fitzgerald & Clewell, 1985) Although none occur naturally in lactococci, they can be transferred into lactococci (Hill *et al.*, 1985). The

Tn916 family of transposons can be cloned from *S. sanguis* into *E. coli* and still express tetracycline resistance (*tet*^{R}). When the selective pressure is removed from *E. coli*, precise excision and loss of the transposon occurs (Gawron-Burke & Clewell, 1984). This has potential for use as a cloning strategy in which the transposon is conjugated into a recipient strain with selection for tetracycline resistance, followed by identification of a mutation created by insertional inactivation by the transposon. Shotgun cloning (*ie* placing random restriction fragments into vectors) of this DNA into *E. coli* using restriction enzymes which flank the transposon, followed by selection for tetracycline resistance should result in the mutated gene being introduced together with the transposon. Subsequent removal of the selective pressure should restore the structural integrity of the required gene (Gawron-Burke & Clewell, 1984).

Insertion of the transposon into the chromosome must be random for the technique to be of use. Technical difficulties (Hill, 1988) have suggested that this strategy may not be as widely useful as was originally hoped, although it has been used successfully to identify genes involved in malolactate, citrate, maltose, mannose and arginine metabolic pathways in a *L. lactis* subsp. *lactis* strain (Fitzgerald & Gasson ,1988).

3) Transformation

Transformation is a mode of genetic transfer in which a piece of exogenous DNA is taken up by a bacterial cell and undergoes recombination within the recipient to become stably inherited. Cells which take up such DNA naturally are known as "competent" and uptake is thought to involve receptors on the cell surface to which the DNA binds. No natural transformation has been observed in the lactococci.

Despite the lack of natural uptake systems, a range of vectors have been created for use with *Lactococcus* spp. which can be introduced via artificial means (de Vos, 1987).

Transformation experiments can be performed in vitro by utilising artificial methods of transfer:

i) Protoplast transformation

Protoplast transformation involves the removal of the cell wall, generally a barrier to DNA uptake in non-competent cells, thus allowing entry of DNA. The method developed for protoplast transformation in lactococci (Kondo & McKay, 1982) was based on the original method for *B. subtilis* (Chang & Cohen, 1979) which used polyethylene glycol (PEG) to facilitate uptake by a mechanism which remains unknown.

The cell wall is removed, generally by digestion with lysozyme, to leave a circular protoplast surrounded only by a cytoplasmic membrane. The protoplast is stabilised by the use of osmotic buffers. In the initial stages of development, the process was rather inefficient but after optimisation, transfer efficiencies of $5x10^6$ /µg DNA can be achieved using *L. lactis* subsp. *lactis* IL1403 (Simon *et al.*, 1986).

The formation of protoplasts for transformation requires the regeneration of the cell wall for subsequent multiplication of the transformed cell. This is achieved by growth on complex media and tends to be a rate-limiting step in the process; regeneration of the protoplasts can take several days. There is much strain variation with regard to the success or not of the formation and regeneration of protoplasts so it seems unlikely this method will be the one of choice for transformation of the industrially-important lactococci.

The "model" protoplast strains include four of *L. lactis* subsp. *lactis*, one of *L. lactis* subsp. *cremoris* and one of *L. lactis* subsp. *diacetylactis* (Kok, 1987).

Genes which have been successfully cloned into *L. lactis* subsp. *lactis* by protoplast transformation include phospho-ß-galactosidase from strain 712 (de Vos, 1987) and plasmid-carried UV-resistance genes (Chopin *et al.*, 1986).

ii) Protoplast fusion

Utilising the same techniques as protoplast transformation, protoplast fusion can be a useful mechanism for genetic transfer when natural transformation methods are unsuccessful. Inheritance of both plasmid and chromosomal markers can result from fusion (Gasson, 1980). Intersubspecific fusion between *L. lactis* subsp. *cremoris* and *L.*

lactis subsp.*lactis* (Okamoto *et al.*, 1985), intergeneric fusion between *L. lactis* and *B. subtilis* (van der Lelie, 1989) and between *L. lactis* and lactobacilli (Cocconcelli *et al.*, 1986; Reed, 1987) have all been achieved.

iii) Electroporation

This technique involves using an electrical charge to achieve entry of DNA into the bacterial cell. A fuller discussion of electroporation occurs later.

Table 1.1 Summary of examples of gene transfer in *Lactococcus.*

METHOD	CHARACTER TRANSFERRED	SYSTEM USED	REFERENCE
Transduction	first evidence for plasmid-coded lactose-metabolism genes	natural temperate phage	McKay *et al.*, 1976
Conjugation	transfer of lactose-metabolism genes	natural system	Kempler & Mckay, 1979
Conjugation	transfer of nisin-producing ability	natural system	Gasson 1984
Conjugation	mobilisation of native lactococcal proteinase plasmids	pAMß1 & derivatives	Hayes *et al.*, 1987

Conjugation	characterisation of lactococcal chromosomal genes in *E.coli*	conjugative transposon Tn916	Gawron-Burke Clewell, 1984
Protoplast transformation	direct cloning of a p-ß-gal in *L.lactis*	pSH71 based cloning vector	de Vos, 1986
Protoplast fusion	intra- and intergeneric transfer of markers	various plasmids	van der Lelie, 1989
Electroporation	direct cloning into lactococci	pSA3	McIntyre & Harlander, 1989b

CHARACTERISATION OF GENES FROM *LACTOCOCCUS* BY CLONING

Using some of the techniques detailed in Table 1.1 in combination with manipulations in *E. coli*, *B. subtilis* and *S. sanguis*, several genes from *Lactococcus* species have been cloned. Most of the genes so far cloned are derived from plasmids and encode readily identifiable functions.

1) LACTOSE METABOLISM GENES

Some members of the LAB metabolise lactose via a phosphoenolpyruvate-dependent phosphotransferase system (PEP:PTS). Lactose is transported into the cell by two lactose-specific enzymes from this system: Enzyme II^{lac} (transmembrane) and Factor III^{lac} (soluble). When it enters the cell, lactose is in a phosphorylated state and another enzyme in the system, 6-phospho-ß-D-galactosidase (p-ß-gal), catalyses the first stage in its metabolism. Metabolism is completed by the tagatose-6-phosphate and glycolytic pathways (Thompson, 1987).

The p-ß-gal from *L. lactis* subsp. *lactis* 712 (Maeda & Gasson, 1986; de Vos, 1986) and *L. lactis* subsp. *cremoris* H2 (Inamine *et al.*, 1986) have both been cloned in *E. coli* and, for *L. lactis* subsp. *lactis*, also in *B. subtilis*. Thus it appears that lactococcus expression signals can be recognised in other Gram-positive organisms. A further two genes from the lactose pathway, those coding for Enzyme III^{lac} and Enzyme II^{lac} have recently been cloned and it has been established that a lactose-inducible operon exsists (de Vos *et al.*, 1990). Four enzymes from the tagatose-6-phosphate pathway have been shown to preceed the lactose-specific enzyme genes within this operon (van Rooijen *et al.*, 1991).

2) PROTEINASE-ENCODING GENES

Lactococci require a proteolytic system for growth in milk as the supply of free amino acids is low. During maturation of some cheeses, flavour development involves the breakdown of casein by proteinases and peptidases from the starter bacteria.

A key enzyme in the proteolysis is a large cell-wall-bound serine-proteinase (Visser *et*

al.,1983). This enzyme has been cloned from *L. lactis* subsp. *cremoris* SK11 (de Vos, 1987), *L. lactis* subsp. *cremoris* Wg2 (Kok *et al.*, 1985) and *L. lactis* subsp. *lactis* NCDO 712 (Gasson *et al.*, 1987). Comparison of the sequences of these genes revealed a very high degree of homology (de Vos, 1987) despite the gene products having different specificities for different types of casein (Visser *et al.*, 1986). In addition, homology with the well characterised subtilisin, a serine-proteinase from *B. subtilis*, has led to the identification of a putative active site of the lactococcal proteinase (Chopin *et al.*, 1985). The lactococcal enzymes are produced initially as pre-protein structures with cleavage of a leader sequence and further modification occurring during maturation before the enzymes are secreted.

Recently, the stabilisation of some of the unstable, plasmid-encoded properties which are important in fermentation, *eg* ability to ferment lactose and ability to degrade caseins, has been reported by integration of these genes into the chromosome (Leenhouts *et al.*, 1991).

3) OTHER GENES

A considerable range of other genes have been cloned from *Lactococcus* species in recent years. Some examples include a gene encoding ultra-violet radiation resistance and prophage stability from *L. lactis* subsp. *lactis* IL594 (Chopin *et al.*, 1986); a gene involved in replication (*rep*A) from *L. lactis* subsp. *lactis* 712 (de Vos, 1986); the thymidylate synthase gene from *L. lactis* subsp. *lactis* (Ross *et al.*, 1990) and a peptidase, *pep*N from *L. lactis* (van Alen-Boerrigter *et al.*, 1991).

LACTOBACILLUS

Like the lactococci, lactobacilli are important commercial organisms. They are involved in the production of yogurt, fermented milk, cheeses, soy sauce, cured ham, sausage meats, distillery mashes, feed additives and silage additives. Culturing of lactobacilli is relatively simple and straightforward and their economic worth is enhanced by several

important properties:

i) Lactobacilli can produce a desired flavour in a food (*eg* Lee *et al.*, 1990).

ii) The growth of lactobacilli discourages other micro-organisms by production of LA (and hence pH drop), removal of substrates and production of bacteriocins.

iii) Vitamins, amino acids and enzymes are produced by lactobacilli.

Some lactobacilli are important commensal organisms in the gut of many mammals, including humans. Colonisation of the gut by these bacteria is profitable for the health of the individual and some urinary tract infections can be treated by direct application of a live bacterial preparation (*eg* Hughes & Hillier, 1990).

Lactobacilli are also used as starter organisms for industrial and commercial fermentations, in a similar way to the lactococci. Selection of important qualities in starter strains *eg* competitive growth, rapid production of LA, by traditional methods plays a vital role in the control of these large-scale processes. In addition genetic manipulation of starter strains has great potential for selection of desirable characteristics. To achieve this manipulation methods of gene exchange are required.

MODES OF GENETIC TRANSFER

1) Transduction

Bacteriophage that infect lactobacilli have been reported, although they appear to be less numerous than those which infect lactococci, and phage contamination of *Lactobacillus* fermentations is not often a problem.

Several bacteriophage from *Lactobacillus* species have now been characterised *eg* three from *L. casei* (Stetter *et al.*, 1978; Shimizu-Kadota & Tsuchida, 1984; Hayashida *et al.*, 1987), one from L. plantarum (Nes *et al.*, 1988), thirteen from *L. bulgaricus* (Mata *et al.*, 1986) and the genome from a *L. lactis* phage has been cloned in *E. coli* (Trautwett er *et al.*, 1986).

The first report of plasmid transduction in *Lactobacillus* involving a temperate bacteriophage from *L. casei* has recently been published (Raya *et al.*, 1989).

2) Transposons

The first putative transposon of lactobacilli was described in 1985 by Shimizu-Kadota *et al.* for *L. casei*. Two virulent mutants of a temperate phage were found to have a genome enlarged by the same piece of DNA, 1.3 kb in size. This insert had originated from the host chromosome and, by its distinctive structure (inverted repeats at both ends) and its size (codes for transfer function but probably no other genes), was termed ISL1, reflecting the fact that it is an insertion element rather than a transposon.

ISL1 does not seem to be widely distributed in nature; of a wide selection of bacterial species tested only three strains of *L. casei* contained homologous sequences (Shimizu-Kadota *et al.*, 1988). In two of these cases, ISL1 was found on plasmids associated with lactose metabolism; in nature, transposons and insertion elements are considered to contribute to adaptation of strains, so it is possible that, in some cases, ISL1 was involved in the evolution of lactose metabolism.

The "conjugative transposon" Tn919 can be transferred into *L. plantarum* from a *S.*

faecalis donor (Hill *et al.*, 1985) although the possibilities for use in shotgun cloning as in the lactococci have not yet been investigated.

3) Conjugation

There have been numerous reports of conjugation of the broad host range plasmid pAMß1 into lactobacilli (Gibson *et al.*, 1979; Vescovo *et al.*, 1983; West & Warner, 1985). Transfer of pAMß1 between lactobacilli and into other bacterial species from a *Lactobacillus* donor have also been reported (Shrago *et al.*, 1986; Morelli *et al.*, 1988). Conjugation of pIP501 into *Lactobacillus* species from *E. faecalis* (Thompson & Collins, 1988) is also possible but, for as yet undefined reasons, intra- or interspecies transfer of pIP501 between lactobacilli has not been reported*. Mobilisation of other plasmids by pIP501 and its derivatives is also possible (Shrago & Dobrogosz, 1988), although deletions occurred in the mobilised plasmids.

The only report of a naturally occurring conjugative plasmid involved a lactose plasmid from *L. casei* which codes for the lac-PTS and p-ß-gal (Chassy & Rokaw, 1981). Hence the prospects for a vector system based upon conjugative plasmids seems to be much more limited in lactobacilli than in the lactococci.

4) Transformation

There is no method of natural transformation between lactobacilli. Although there are many published papers on the presence of plasmids in *Lactobacillus* species and some have been cloned into *E. coli* (*eg* Lee-Wickner & Chassy, 1984; Damiani *et al.*, 1987; Josson *et al.*, 1989), the majority remain cryptic. Functions which appear to be plasmid encoded include lactose metabolism, n-acetyl-D-glucosamine metabolism, proteinase production and antibiotic resistance.

A number of vectors which replicate in lactococci (and in some cases also in *E. coli* and *B. subtilis*) are also maintained within lactobacilli *eg* pVA838, pSA3. Cloning vectors based on the broad host range replicons pWV01 and pSH71 from *L. lactis* subsp.

* Intraspecific transfer has been achieved (Thompson & Collins, 1988).

cremoris (eg pNZ12) are also important in lactobacilli.

There have been several recent reports on the characterisation of cryptic plasmids from *Lactobacillus* species (*eg* Takiguchi *et al.*, 1989; Bates & Gilbert, 1989; Skaugen, 1989; Vogel *et al.*, 1991; Zink *et al.*, 1991) and antibiotic resistance determinants have also been isolated (Axelsson et al., 1988; Jewell & Collins-Thompson, 1989; Rinckel & Savage, 1990). Some vectors, including shuttle vectors, have been constructed from naturally occurring *Lactobacillus* plasmids (Bringel *et al.*, 1989; Posno *et al.*, 1991).

ARTIFICIAL MEANS OF TRANSFORMATION:

1) Protoplast transformation

Formation of protoplasts involves the weakening of the cell wall, generally by incubation with lysozyme. Some strains of lactobacilli are much more resistant to lysozyme than other bacteria and in these cases mutanolysin, a N-acetyl-muramidase similar in its mode of action to lysozyme (Yokogawa *et al.*, 1974) can be used, either in combination with lysozyme or on its own, to achieve protoplast formation.

Lactobacilli are very fastidious organisms and consequently regeneration media can be expected to be very complex. The original method for protoplasting, which was designed for *L. casei* (Lee-Wickner & Chassy, 1984) has been adapted for a number of other species. Transformation by protoplasting has been achieved in *L. reuteri* and *L. acidophilus* (Morelli *et al.*, 1987) with pSA3 and in *L. plantarum* (Cosby *et al.*, 1988) with phage DNA.

2) Protoplast fusion

The first report of this method of gene transfer in lactobacilli (Iwata *et al.*, 1986) involved intragenic transfer between two strains of *L. fermentum*. The fused protoplasts contained two plasmids, each originating from one of the donor cells.

Intergenic transfer from *Lactococcus lactis* containing pAMß1 to *L. reuteri* has also been

reported (Cocconcelli *et al.*, 1986). Although fusion removes some of the problems associated with the uptake of exogenous DNA, the frequency of transfer tends to be rather low and the process is necessarily long and complex.

3) Transfection

Transfection involves the uptake of phage DNA by a transformation rather than by natural phage infection. In an experiment involving *L. casei* (Shimizu-Kadota & Kudo, 1984), spheroplasts (*ie* cells which have lost their shape due to cell wall weakening but which probably retain some of their peptidoglycan) were fused under PEG with liposomes containing the phage DNA. The liposomes help to protect the DNA from restriction and modification systems. This method does not require the regeneration step involved in protoplasting as transfected spheroplasts will form plaques in a lawn of indicator cells overnight.

The transfer frequency in the original report was rather low; 10^2 plaque forming units (pfu)/ μg DNA but a more recent report using the same technique for *L. bulgaricus* (Boizet *et al.*, 1988) achieved a frequency of $5x10^7$ pfu/ μg DNA after optimisation.

4) Electroporation

Electroporation (or electro-transformation or electro-permeabilisation) is the process of entry of DNA (or other macromolecules) into a cell by the application of an electric current. A full description of this process will occur later.

Table 1.2 Summary of examples of gene transfer in *Lactobacillus*.

METHOD	CHARACTER TRANSFERRED	SYSTEM USED	REFERENCE
Conjugation	marker transfer	pAMß1 & derivatives	West & Warner, 1985
Conjugation	cloning of a p-ß-gal gene	natural plasmid	Chassy & Rokaw, 1981
Protoplast transformation	marker transfer	pSA3	Morelli *et al.*, 1987
Protoplast fusion	marker transfer	natural plasmid	Iwata *et al.*, 1986
Electroporation	marker transfer	pNZ12	Chassy & Flickinger, 1987
Electroporation	transfer of *cel* E from *C. thermocellum*	pM25 (pSA3 derivative)	Bates *et al.*, 1989

CHARACTERISATION OF GENES FROM *LACTOBACILLUS* BY CLONING

1) LACTOSE METABOLISM GENES

The majority of genes from *Lactobacillus* spp. which have been cloned and characterised are involved in lactose metabolism. Due to a lack of a reliable Gram-positive system for cloning, all of these genes have been cloned into *E. coli.*

The same phosphoenolpyruvate dependent phosphotransferase system (PEP:PTS) for lactose transport, some components of which are plasmid coded, operates in both *Lactobacillus* and *Lactococcus*. The first *Lactobacillus* gene to be cloned was a phospho-ß-galactosidase (p-ß-gal) from *L. casei* plasmid pLZ64 which expressed in *E. coli* using its own promoter (Lee *et al.*, 1982). The sequence of this gene has since been reported (Porter & Chassy, 1988) with a putative promoter sequence and a ribosome binding site identified. This promoter sequence (TGGTCA at -35, TACATT at -10) is consistent with consensus promoters from both Gram-positive and Gram-negative species. The p-ß-gal genes from *L. casei, L. lactis* and *S. aureus* were found to be much more closely related than the organisms themselves appear to be. This may suggest a relatively recent introduction of lactose-encoding genes from a common source.

A second open reading frame from the p-ß-gal gene probably codes for another enzyme in the system, Enzyme III^{lac}, with the p-ß-gal promoter acting as an intergenic promoter between the two genes in the proposed operon organisation (Chassy, 1987). The sequences of Enzyme III^{lac} (Alpert & Chassy, 1988) and Enzyme II^{lac} (Alpert & Chassy, 1990) further indicate a common ancestry with *S. aureus* and *Lactococcus lactis.*

Another p-ß-gal gene from a different *L. casei* strain has also been cloned (Shimizu-Kadota, 1988). Using this plasmid-encoded gene as a probe, a second, chromosomal p-ß-gal gene was identified. The two genes have the same restriction map but the chromosomal gene appears to be silent in *L. casei*, as cells which have lost the plasmid, pLY101, are lactose negative. Cloning of the chromosomal gene into *E. coli* results in expression so the gene appears to be functionally intact and it may be a controller gene

which is switched off in *L. casei.*

Similarities between all three of these p-ß-gal genes occur in the coding areas but the regulatory regions differ: the genes reported by Shimizu-Kadota (1988) are not induced by glucose or galactose, whereas the other p-ß-gal reported by Lee *et al.* (1982) is.

Three ß-galactosidase genes have also been cloned; two from *L. bulgaricus* (Schmidt *et al.*, 1989 and Scheirlinck *et al.*, 1989) and one from *L. casei* (for reference see Chassy, 1987). The two *L. bulgaricus* ß-gal genes show a fair degree of homology with ß-gal genes from *E. coli* and *Klebsiella pneumoniae*; this indicates a common ancestry for these genes, perhaps between all bacteria, rather than just between Gram-positives. The putative active site which has been identified in the ß-gal coded for by the *lac*Z gene in *E. coli* is conserved in the *Lactobacillus* ß-gal genes so a common structure-function relationship is also possible.

2) OTHER GENES

One of the early genes to be cloned from *Lactobacillus* was the dihydrofolate reductase (DHFR) gene from *L. casei* (Davies & Gronenborn, 1982). DHFR plays a central role in one-carbon-unit metabolism and is the target of some important clinical drugs (*eg* trimethoprim). When cloned into *E. coli*, synthesis of the enzyme occurs and resistance to trimethoprim is conferred. This gene has also been sequenced (Andrews *et al.*, 1985).

Recent interest has been focused on the genes coding for 2-hydroxyisocaproate dehydrogenase, an enzyme important in the industrial production of amino acids. Two genes have been cloned; one from *L. casei* (Lerch *et al.*, 1989a), the other from *L. confusus* (Lerch *et al.*, 1989b) which is the only member of the lactobacilli to code for the L isomer. Both of these genes have been cloned in *E. coli* but are apparently not related to each other. One common feature between these reports and the others on cloning of *Lactobacillus* genes is the lack of the codon AGA / AGG, which codes for arginine, from

all the *Lactobacillus* genes so far investigated (Lerch *et al.*, 1989b).

Other genes which have been recently cloned include the folylpolyglutamate synthetase (Toy & Bognar, 1990), five tryptophan genes (Natori *et al.*, 1990) and a lactate deydrogenase gene (Kim *et al.*, 1991), all from *L. casei*.

PEDIOCOCCUS

There are fewer published papers on the genetics of *Pediococcus* are much fewer compared with those on *Lactococcus* and *Lactobacillus*.

Conjugal transfer of pIP501 from *S. faecalis* to *P. pentosaceus* and *P. acidilactici* has been reported (Gonzalez & Kunka, 1983) as well as intragenic transfer between the pediococci and intergenic transfer using the pediococci as donors. Under the same conditions transfer of pAMß1 was unsuccessful.

Pediococcus spp. contain a range of plasmids, some of which may be associated with bacteriocin production (Graham & McKay, 1985; Daeschel & Klaenhammer, 1985; Hoover *et al.*, 1988) although bacteriocin immunity does not always seem to be associated. Another trait which is plasmid encoded in *Pediococcus* is raffinose utilisation (Gonzalez & Kunka, 1986) which, in some cases, has associated ß-galactosidase and sucrose hydrolase activities.

One particular plasmid in *P. acidilactici* H is reported to be conjugative with a corresponding transfer of bacteriocin production and resistance, providing direct evidence for these functions being plasmid coded (Ray *et al.*, 1989).

In a study involving a range of bacterial genera, Luchansky *et al.* (1988) found that *Pediococcus* species were successfully electroporated although the frequency ($5x10^1$/ µg DNA) was up to a thousand-fold lower than for *Lactobacillus*.

Table 1.3 Summary of examples of gene transfer in *Pediococcus*.

METHOD	CHARACTER TRANSFERRED	SYSTEM USED	REFERENCE
Conjugation	marker transfer	pIP501	Gonzalez & Kunka, 1983
Conjugation	transfer of bacteriocin production	natural plasmid	Ray *et al.*, 1989
Electroporation	marker transfer	pGK12	Luchansky *et al.*, 1988

LEUCONOSTOC

Leuconostoc species are as economically important as the lactococci and lactobacilli, with their ability to produce C4 compounds (diacetyl and acetoin) and to carry out malo-lactate fermentations, making them important to the dairy and wine industries.

Conjugation within *Leuconostoc* species is well documented. A nisin-encoding plasmid from *Lactococcus lactis* can be conjugally transferred to *Leuconostoc dextranicum* to give a nisin-producing recipient (Tsai & Sandine, 1987). Transfer of broad host range plasmids pIP501 and pAMß1 into *Leuconostoc dextranicum* and *Leuconostoc cremoris* strains from *S. faecalis* and *S. sanguis* donors is also possible (Pucci *et al.*, 1988). Intragenic transfer of both plasmids was also reported but at a low frequency. The "conjugative transposon" Tn919 has also been transferred into a *L. cremoris* strain from *S. faecalis* at a frequency not too dissimilar from that of transfer into lactococci (Hill *et al.*, 1985), although the possibilities of utilising this system for shotgun cloning as with some *Lactococcus* species has still to be evaluated.

Luchansky *et al.* (1988) achieved transformation of *L. dextranicum* and *Leuconostoc lactis* by electroporation at a frequency of 10^3-10^4 transformants /µg DNA. Successful transformation by electroporation of *L. paramesenteroides* has also been achieved (David *et al.*, 1989) with pNZ12 and a deletion derivative of pAMß1, although the frequencies were lower than for lactococci and lactobacilli. *Leuconostoc* spp. do not possess a lactose phosphotransferase system and hence detection of p-ß-gal activity from pNZ12 carrying a fragment of the *Lactococcus lactis* p-ß-gal gene was facilitated. The *lac* Z gene from *E. coli* was also cloned into *L. paramesenteroides*, using *Lactococcus lactis* promoters, on pNZ12 and expression of a ß-gal activity resulted (David *et al.*, 1989).

Hence, although in terms of the number of published reports, the genetics of *Pediococcus* and *Leuconostoc* are rather lagging behind those for *Lactococcus* and *Lactobacillus*, there appears to be equal potential for genetic manipulation in all these genera, with a variety of methodologies available for use.

Table 1.4 Summary of examples of gene transfer in *Leuconostoc.*

METHOD	CHARACTER TRANSFERRED	SYSTEM USED	REFERENCE
Conjugation	transfer of nisin-producing ability	*L. lactis* conjugative plasmid	Tsai & Sandine, 1987
Conjugation	marker transfer	pAMß1 & pIP501	Pucci *et al.*, 1988
Electroporation	marker transfer	pGK12	Luchansky *et al.*, 1988
Electroporation	transfer of *lac* Z gene from *E. coli*	pNZ12	David *et al.*, 1989

ELECTROPORATION

The exact mechanism by which the DNA gains access to the bacterial cell during electroporation is unknown. It is a physical rather than a biochemical process and is thought to involve the formation of transient pores in the cell membrane through which the macromolecules gain entry (Zimmerman *et al.*, 1973). As well as DNA, entry of RNA (Taketo, 1989) and monoclonal antibodies (Chakrabarti *et al.*, 1989) has been achieved. These pores can also result in the fusion of cells (Zimmerman & Vienken, 1982) and be may even be of use as a means of preparing plasmid DNA (Calvin & Hanawalt, 1988) and for plasmid curing (Heery *et al.*, 1989).

Transformation by electroporation is simple, rapid and gives transformation frequencies which are comparable with, and in some cases higher than, those achieved by other transformation methods. Although it is still a relatively new technique, first identified for possible gene transfer by Wong & Neumann (1982), electroporation has found applications in a wide range of cell types. (See Biorad " Gene Pulser " instruction manual, Biorad, Richmond, California for full details). For example, entry of macromolecules by electroporation has been achieved in:

i) Mammalian cells *eg* chinese hamster ovary cells; Burkitt's lymphoma cells; mouse cells

ii) Plant cells *eg* carrot protoplasts; tobacco protoplasts; maize cells

iii) Microbial cells *eg* yeast, both protoplasted and intact; Aspergillus species (Ward *et al.*, 1989).

A major advantage of electroporation with respect to bacterial cells is that the technique can be successful on several strains within a species.

The number of bacterial species successfully transformed by electroporation has grown rapidly since the early published reports on *Lactobacillus casei* by Chassy & Flickinger in 1987, on *Strep. lactis* by Harlander (1987) and on *Strepyomyces* by MacNeil (1987). The genera on which it has been applied now include Gram-positive genera such as *Bacillus* (Belliveau & Trevors, 1989; Mahillon *et al.*, 1989); *Leuconstoc* (Luchansky *et*

al., 1988; David *et al.*, 1989); *Listeria* (Luchansky *et al.*, 1988; Alexander *et al.*,1990); *Lactococcus* (Harlander, 1987; Powell *et al.*, 1988; McIntryre & Harlander, 1989a and 1989b); *Pediococcus* (Luchansky *et al.*, 1988); *Enterococcus* (Fiedler & Wirth, 1988; Solioz & Waser, 1990); *Streptomyces* (MacNeil, 1987); *Staphylococcus* (Luchansky *et al.*, 1988); *Streptococcus* (Somkuti & Steinberg, 1988); *Lactobacillus* (Chassy & Flickenger, 1987; Aukrust & Nes,1988); *Bordetella* (Zealey *et al.*, 1988); and *Clostridium* (Oultram *et al.*, 1988). Gram-negative organisms include *E. coli* (Dower *et al.*, 1988; Calvin & Hanawalt, 1988; Taketo, 1988; Fiedler & Wirth, 1988); *Pseudomonas putida* (Fiedler & Wirth, 1988); *Agrobacterium* (Mattanovich *et al.*, 1989); *Campylobacter* (Miller *et al.*, 1988); and *Pasteurella* (Craig *et al.*, 1989).

Although in application the methodology of electroporation is simple and rapid, there are a number of variables within the process which can be adjusted when optimising the procedure for any given bacterial species. In general, the conditions required for permeabilisation of bacterial cells differ in several aspects from those required for eukaryotic cells. Low voltages and high capacitance are needed for eukaryotic cells while bacterial cells transform under higher voltages and low capacitance because of their much smaller size. The variables included in this study are used in reference to bacterial cells only. (For eukaryotic conditions refer to Gene Pulser Instruction Manual).

ELECTRICAL VARIABLES

All the work included here was done using a Biorad "Gene Pulser" apparatus and hence is subject to the restrictions this unit provides.

The electrical variables include voltage, resistance and capacitance and are fully described in MATERIALS & METHODS, section 2.14.

PROCESS VARIABLES

Within the basic electroporation process which essentially consists of harvesting cells, washing them in electroporation buffer, pulsing and finally incubation and selection,

there are a number of variables and treatments which can be investigated during optimisation.

Pre-treatment

With bacterial cells which seem particularly refractile to electroporation, or simply to enhance achieved transformation frequencies, it is possible to grow cells in the presence of some agents which will result in weakened cell wall structure and hence potentially ease the formation of pores. The extent of this pre-treatment does not generally result in the long regeneration time typical of protoplasts.

1) Glycine

Glycine inhibits cell wall growth by replacing D-alanine residues in the primary structure of the peptidoglycan in such a way that crosslinks cannot form (Hammes *et al.*, 1973).

eg - G- M - G -

L ala

D glu

mDpm---------- D ala

(D ala) mDpm

D glu

L ala

- G - M - G -

Cross linking in peptidoglycan of *L. plantarum* (mDpm = mesodiamino pimelic acid, G = N-acetyl-D-glucosamine, M = N-acetylmuramic acid).

2) Threonine

L-threonine naturally occurs in peptidoglycan. When a mixture of both isomers is added to the bacterial culture, the D isomer replaces some of the L residues and restricts cross linking as a result. L-threonine residues occur in the cross links of peptidoglycan of *Lactococcus* species but not in *Lactobacillus* cross links so the action of the D isomer more pronounced in the former (Schleifer & Kandler, 1972).

eg i) - G - M - G -

L ala

D glu

L lys--L thr--L ala-- D ala

D ala L lys

D glu

L ala

- G - M - G -

Cross linking in *Lactococcus lactis*.

ii)

G - M - G-

D ala

D glu

mDpm---------- D ala

D ala mDpm

D glu

L ala

- G - M - G -

Cross linking in *Lactobacillus plantarum*

3) D-cycloserine (D-4-amino-3-isoxazolidone)

D-cycloserine is an antibiotic which inhibits an early stage in peptidoglycan synthesis; it acts as an analogue of D-alanine and competitively inhibits the two enzymes (alanine racemase and D-alanyl-D-alanine synthetase) involved in the synthesis of D-alanyl-D-alanine, a dipeptide which forms part of the cross link in some types of peptidoglycan.

Growth and harvesting

As with other transformation techniques the growth phase of cells is likely to be important in electroporation. The majority of published papers use mid-exponential phase cells, although one report (McIntyre & Harlander, 1989b) found increased transformation efficiency when using stationary phase cells.

Growth conditions may also affect competence *eg Campylobacter jejuni* (Miller *et al.*, 1988) is only transformed when harvested from an agar plate, not from broth. Similarly, growth medium is also of relevance *eg Lactococcus lactis* gives an increased efficiency when grown on defined media rather than the complex medium traditionally used for laboratory propagation (McIntyre & Harlander, 1989b).

After harvesting the cells (generally by centrifugation) it is important to wash the cells

thoroughly to reduce the ionic strength of the cell suspension; a solution which is too conductive will cause arcing (arcing is an explosive reaction which is fully described in Materials & Methods, section 2.14).

In most of the published literature, electroporation has been carried out at high (10^{10}cells/ml) cell densities. This ensures that although some cell death will result from cell wall damage there will be enough survivors to allow transformation to be successful.

Electroporation buffers

A variety of buffers have been utilised in the electroporation of different bacteria, each one optimised to some extent for the bacterium of choice. Based on information from the Gene Pulser Instruction Manual and from Powell *et al.* (1988), three buffers were used in this study: Hepes buffer; Phosphate buffer; Sucrose buffer (recipes in Materials & Methods).

When using the Pulse Controller it is advisable to use a buffer with a low ionic strength (such as sucrose buffer) to reduce the possibility of arcing at the high field strengths created.

DNA

The source of the DNA used for transformation is important: DNA from another species is generally less efficient than DNA from the host species. This is not an unexpected phenomenon; foreign DNA is more likely to come under attack from the cell's defence mechanisms *eg* restriction-modification.

The amount of DNA also has a marked effect on transformation. In the system described here there is evidence for a DNA saturation level.

Expression conditions

During optimisation procedures, a certain amount of "fine tuning" may be required at first to detect low levels of transformants and then to aid in achieving the maximum

transformation possible. Such fine tuning can involve variations in length of time allowed for expression and dilution prior to expression.

With *E. coli* transformation systems using calcium chloride, incubations on ice are included before and after mixing the cell culture with DNA. The mechanism of entry is thought to involve the DNA binding to a receptor before being taken up and maintained by the cell. Electroporation is unlikely to involve receptors and hence is generally carried out immediately after mixing cells and DNA. An additional fine tuning variable may be the inclusion of an incubation on ice directly after the pulse.

"GENETICS OF SILAGE BACTERIA"

The work presented in this thesis involves genetic manipulation of a number of *Lactobacillus* and *Pediococcus* strains isolated from a grass silage. The ultimate aim of the project was to evaluate these strains for genetic manipulation by introducing characteristics that would benefit the silage fermentation and hence possibly create a new range of inocula as silage additives.

Plasmid profiles and approximate size details of the native plasmids of these strains is presented. From previous work involving the genetic manipulation of members of the LAB, electroporation was selected as the principal method of gene transfer and an optimisation procedure was undertaken for both groups of bacteria. As detailed above, a number of electrical and process variables were studied during the optimisation.

Using this transformation procedure, the maintenance and expression of a foreign characteristic was attempted viz cellulase production.

CHAPTER TWO

MATERIALS AND METHODS

2.1-CULTURES AND CONDITIONS

2.1.1) BACTERIAL STRAINS

All strains of lactobacilli and pediococci in this study were isolated from grass silage by Dr. S. Heron at the University of Edinburgh. A total of six strains of lactobacilli and fourteen of pediococci were isolated. *Lactobacillus* strains were described as follows: 48, 65, 71, 72, 85, Lp; *Pediococcus* strains were numbered: 1, 37, 40, 41, 42, 59, 60, 75, 77, 79, 80, 81, 84, 826.

E. coli MC1022 (plasmid free, *Rec*-, resistance free) was used as recipient strain in all experiments involving transfer of plasmids to *E. coli*. Similarly, *Bacillus subtilis* 1280 (plasmid free) and *Lactococcus lactis* (formerly *Streptococcus lactis*) MG1363 (plasmid free) were also used as recipient strains.

2.1.2) MEDIA

2.1.2.1) Lactobacilli and pediococci were grown on MRS media (supplied dehydrated from Oxoid Ltd.) after de Man, Rogosa and Sharpe, 1960. Ingredients as follows:

Peptone	10g
"Lab Lemco" powder	8g
Yeast extract	4g
Dextrose	4g

Tween 80 (polyoxyethylene sorbitan mono-oleate)	1ml
K_2HPO_4	2g
Sodium acetate .$3H_2O$	5g
$(NH_2)_3$ citrate	2g
$MgSO_4.7H_20$	0.2g
$MnSO_4.4H_2O$	0.05g
Agar	10g
Distilled water to 1000ml	

2.1.2.2) Lactic acid bacteria were selected on Tween Acetate Agar. Ingredients as follows:

"Lab lemco" powder	8g
Peptone	10g
Yeast extract	5g
Glucose	10g
Tween 80	0.5ml
$(NH_2)_3$ citrate	2g
Salts solution	5ml
Agar	20g
Distilled water to 1000ml	

Salts solution: 2M acetic acid, 2M sodium acetate. Salts added after agar was melted.

2.1.2.3) Homo/heterofermentative medium. Ingredients as follows:

Peptone	10g
Yeast extract	5g
Glucose	5g
Fructose	30g

Tween 80	1ml
0.2M KH_2PO_4*	9ml
0.2M Na_2HPO_4*	1ml

Distilled water to 1000ml

* pH to 6.0 before adding salts.

5ml amounts dispensed into test tubes, then an inverted Durham tube was placed in each prior to autoclaving. After inoculation, tubes were plugged with 2ml water agar or liquid paraffin. Gas production in the Durham tube after incubation is indicative of heterofermentative activity.

2.1.2.4) *B. subtilis* was grown on Brain Heart Infusion (BHI) Agar (supplied dehydrated from Oxoid Ltd). Ingredients as follows:

Calf Brain Infusion solids	12.5g
Beef Heart Infusion solids	5g
Proteose peptone	10g
Sodium chloride	5g
Dextrose	2g
Na_2HPO_4	2.5g
Agar	10g

Distilled water to 1000ml

2.1.2.5) *L. lactis* was grown on M17 (supplied dehydrated from Difco Labs.). Ingredients as follows:

Tryptone	5g
Soytone	5g
Meat digest	5g

Yeast extract	2.5g
Ascorbic acid	0.5g
Mg SO_4	0.25g
Disodium-ß-glycerophosphate	19g
Agar	10g
Distilled water to 1000ml	

After autoclaving 5ml of 10% glucose added to 95ml media.

2.1.2.6) *E. coli* was grown on L agar. Ingredients as follows:

Bacto tryptone	10g
Bacto yeast extract	5g
NaCl	10g
pH to 7.0	
Agar	10g
Distilled water to 1000ml	

2.1.2.7) For visualisation of cellulase activity by congo red assay *Lactobacillus* and *Pediococcus* cultures were grown on Fastidious Anaerobe Agar (obtained dehydrated from Lab M). Ingredients as follows:

Peptone mix	23g
NaCl	5g
Soluble starch	1g
Agar No.2	12g
Sodium bicarbonate	0.4g
Glucose	1g
Sodium pyruvate	1g
Cysteine HCl monohydrate	0.5g
Haemin	0.01g

Vitamin K	0.001g
L-Arginine	1g
Soluble pyrophosphate	0.25g
Sodium succinate	0.5g
Carboxymethylcellulose (CMC)	0.5%(w/v)
Distilled water to 1000 ml	

2.1.2.8) For visualisation of cellulase activity from *E. coli* cultures, growth was on cellulolytic medium. Ingredients as follows:

Agar	15g
10x M9 Salts	100ml
1M $MgSO_4$	1ml
0.1M $CaCl_2$	1ml
1M Thiamine.HCl	1ml
20% Glucose	10 ml
ampicillin	40μg/ml (final concentration)
CMC	0.5%(w/v)

Distilled water to 1000 ml.

10x M9 salts:	Na_2HPO_4	6g
	KH_2PO_4	3g
	NaCl	0.5g
	NH_4Cl	1g

added to 1000ml distilled water.

where CMC = carboxymethylcellulose, sodium salt, medium viscosity obtained from Sigma.

2.1.3) CULTURE CONDITIONS

CULTURE	MEDIA	TEMPERATURE	AERATION
E. coli	L broth/agar	37°C	+
B. subtilis	BHI broth/agar	30°C	+
Lactobacillus	MRS broth/agar	30°C	-
Pediococcus	MRS broth/agar	30°C	-
Lactococcus	M17 broth/agar	30°C	-

2.2) SOLUTIONS

2.2.1) BUFFERS AND SOLUTIONS FOR PLASMID PREPARATION

T Buffer: 0.02M Tris-HCl, pH 8.2

TE Buffer: 10mM Tris-HCl, pH 8.O
1mM EDTA

TES Buffer: 0.05M Tris
0.005M EDTA
0.25M NaCl

TEG Buffer: 25mM Tris, pH 8.O
10mM EDTA
50mM Glucose

FLM (final lysis mix): 0.1M Tris
0.05M EDTA
0.8M NaCl
1% SDS

PEG: 24% Polyethylene glycol (MW 20,000) in H_2O.

PROTOPLAST buffer: 0.5M Sucrose

0.04M Sodium acetate

0.001M Magnesium acetate, pH 7.0

2.2.2) AGAROSE GEL RUNNING BUFFER

10x Tris Borate Buffer:	Tris	108g
	Boric Acid	55g
	EDTA(0.5M, pH 8.O)	40ml

Distilled water to 1000ml.

2.2.3) AGAROSE GEL LOADING BUFFER

10x buffer: 0.25% Bromophenol blue

0.25% Xylene cyanol

25% Ficoll (type 400) in H_2O

2.2.4) ELECTROPORATION BUFFERS

2.2.4.1) Hepes buffer: 272mM Sucrose

1mM $MgCl_2$

7mM Hepes

pH 7.4

Distilled water to 100ml.

2.2.4.2) Phosphate buffer: 10ml 10x phosphate solution (see below)

1ml 0.1M $MgCl_2$

17g (w/v) sucrose (equivalent to final concentration of 0.5M).

Distilled water to 100ml.

10x phosphate solution: 19ml solution A (1.092g $NaH_2PO_4.2H_2O$ in 100ml) plus 81ml solution B (1.246g $Na_2HPO_4.2H_20$ in 100ml)

2.2.4.3) Sucrose buffer: 0.5M sucrose in distilled water.

All electroporation buffers were made on day of use and stored at 4 C. NB Water used to make all buffers was double glass distilled and hence had a very low conductivity.

2.2.5) SOLUTIONS FOR SOUTHERN HYBRIDISATION

Denaturation solution:	1.5M NaCl
	0.5M NaOH
Neutralisation solution:	1M Tris-HCl (pH 8.O)
	1.5M NaCl
Transfer buffer (20x SSC):	3M NaCl
	0.3M tri-Sodium citrate, pH 7.O

2.2.6) SOLUTIONS FOR TRANSFORMATION

SMM buffer (x2):	0.5M Sucrose
	0.02M Sodium maleate
	0.02M $MgCl_2$
	pH 6.5 adjusted with NaOH.

2.3) BACTERIAL IDENTIFICATION SYSTEMS

2.3.1) VISUAL IDENTIFICATION

For the genus identification of the isolated grass silage organisms, wet film and Gram

stain film microscopic examinations were used. Gram stain reagents as follows (Preston & Morell, 1962):

Reagent 1)	8g crystal violet
	80ml methylated spirit
	3.2g ammonium oxalate
	320ml distilled water
Reagent 2)	1g iodine
	2g potassium iodide
	100ml distilled water
Reagent 3)	7ml LIF (see below)
	700ml acetone
Reagent 4)	5ml strong carbol fuchsin (see below)
	95ml distilled water

LIF:	10g iodine
	6g potassium iodide
	10ml distilled water
Strong carbol fuchsin:	5g basic fuchsin
	25g phenol
	50ml 95% alcohol
	500ml distilled water

Each reagent was applied for 30 seconds.

2.3.2) GROWTH TEMPERATURE

Preliminary identification of silage isolates was facilitated using growth temperature studies; 40°C and 48°C were used.

2.3.2) API IDENTIFICATION SYSTEM

The API 50 CH system (API, Montalieu Vercieu, France) was used as a further basis for identification. This system allows the study of the carbohydrate metabolism of the organism in question. Three types of reaction can be tested for by these strips (assimilation, oxidation, fermentation) depending on the inoculation medium and conditions used; for the lactobacilli and pediococci studied here the fermentation characteristics were observed. After inoculation and incubation of the strips, reactions were scored as positive or partial and compared to the pattern created by known reference strains.

2.4) PLASMIDS

2.4.1) TABLE OF PLASMIDS AND PROPERTIES

PLASMID	CONSTRUCTION	SOURCE & REFERENCE
pNZ12	replication functions from pSH71 (a cryptic *L. lactis* plasmid), chl resistance gene from pC194, km resistance gene from pUB110.	W. de Vos, Ede Netherlands. De Vos, 1986
pTG262	*Eco*RI-*Hind*III fragment of pCK17 and *Hae*II fragment (polylinker) of pUC18.	M. Gasson Norwich
pIL277	MLS and replication functions from pHV1301 with polylinker	A. Chopin, France. Simon & Chopin, 1988

pIL253	MLS and replication functions from pMT9 with polylinker	As for pIL277
pWM95 & pWM191	*Sau*3A chromosomal fragments from *Cellulomonas flavigena* in *Bam*HI site of pUC18	A. Kelly, Dublin. Akhtar *et al.*, 1988
pMTL500E	replication origin and ery resistance gene from pAMß1 in pMTL20	N. Minton, CAMR Oultram *et al.*, 1988
pCC1	pMTL500E with *cel*C gene from *Clostridium thermocellum*	N. Minton, CAMR

where chl = chloramphenicol, km = kanamycin, ery = erythromycin, MLS = macrolides, lincosamides, streptogramin B resistance determinant

For plasmid diagrams refer to section 2.16.

2.4.2) ANTIBIOTIC SELECTION

Plasmid	Species	Selection	Level
pNZ12	*E. coli*	chloramphenicol	10μg/ml
pNZ12	Lb/Pd	"	20μg/ml
pTG262	*E. coli*	"	15μg/ml
pTG262	*L. lactis*	"	5μg/ml
pTG262	Lb/Pd	"	20μg/ml
pIL277	*L. lactis*	erythromycin	5μg/ml
pIL277	*B. subtilis*	"	5μg/ml
pIL277	Lb/Pd	"	20μg/ml
pIL253 as for pIL277			
pWM95/191	*E. coli*	ampicillin	40μg/ml
pMTL500E	*E. coli*	"	50μg/ml
pMTL500E	Lb/Pd	erythromycin	20μg/ml
pMTL500F, pCC1 as for pMTL500E.			

where Lp/Pd means *Lactobacillus* and *Pediococcus* strains. Spontaneous mutation rates at the above levels were very low and generally nil with respect to selection of transformants.

2.5) PLASMID DNA PREPARATION METHODS

2.5.1) LACTOBACILLI AND PEDIOCOCCI

In the initial stages of this study various methods were utilised in an attempt to extract plasmid DNA from the isolated silage organisms (Anderson & McKay, 1983; Leblanc & Lee, 1979; Klaenhammer, 1984). Finally, the method of Chassy, 1976 was used routinely. Protocol as follows:-

i) Set up 10ml overnight culture in MRS broth.

ii) Harvest by centrifugation and wash with T buffer.

iii) Resuspend in T buffer to 1/40 of original culture volume.

iv) Add PEG (1/20 original culture volume) and mix well.

v) Add lysozyme (Sigma, Grade I) in T buffer to a final concentration of 1 mg/ml.

vi) Incubate at 37°C for 1 hour.

vii) Harvest by centrifugation at 5,000 rpm (Sorval RC-5B centrifuge) for 5 minutes.

ix) Resuspend to 1/10 original culture volume in TE buffer. Protoplasted cells should be very hard to resuspend at this step.

x) Add 1/9 of resuspension volume of 10% SDS and incubate at 37°C for 15 min.

xi) Stir at moderate speed on a magnetic stirrer to shear SDS lysate.

xii) Adjust pH to 12.45 with 1N NaOH. This is a critical step and care when adding the NaOH is advised. For the 10ml culture 1ml NaOH was added. Return to stirrer.

xiii) After 15 min at alkali step add a volume of 2M Tris-HCl that is equal to twice the volume of NaOH added.

xiv) The solution is adjusted to 3% NaCl by addition of a weighed amount of salt. Dissolve by stirring.

xv) Add an equal volume of phenol saturated against 3% NaCl and stir to make an emulsion. Separate the two layers by centrifugation and remove the upper (aqueous) layer to a clean tube.

xvi) Extract the aqueous layer with an equal volume of chloroform-isoamyl alcohol (24:1).

xvii) Combine the upper phase with 2.5 volumes of ethanol to precipitate the DNA and leave at -20°C for a minimum of 30 minutes (generally left overnight).

xviii) Collect the pellet by centrifugation and dry *in vacuo*. Dissolve in TES buffer with warming at 65°C. For the 10ml culture use 5ml TES.

xix) Add RNase A to a final concentration of 10µg/ml and incubate at 55°C for 1 hour.

xx) Adjust to 0.1% SDS and add proteinase K to a final concentration of 10µg/ml. Incubate for a further hour at 55°C.

xxi) Extract with phenol and chloroform as in steps xv) and xvi), except that NaCl is not added.

NB. Use RNase A according to manufacturer's instructions. Some may require heat treatment to remove contaminating DNase.

xxii) Ethanol precipitate the DNA and leave overnight at -20°C.

xxiii) Resuspend the DNA in 1ml TE.

If required, further purify by running on caesium chloride gradients (see below).

Despite repeated attempts involving scaling down of procedures and merging steps from different large scale methods, no mini-prep method consistently gave good yields of plasmid DNA and hence the minimum plasmid preparation volume for *Lactobacillus* and *Pediococcus* cultures remained 100ml as described above.

Purification of *Lactobacillus* DNA on caesium chloride gradients always resulted in a greatly reduced yield and hence further purification was excluded from the standard protocol.

2.5.2) *E. COLI*

E. coli DNA was prepared by the method of Birnboim & Doly (1979).

Protocol as follows(for 40ml):-

i) Grow cells up overnight in selective media.

ii) Harvest by centrifugation.

iii) Resuspend in 1ml TEG and vortex thoroughly.

iv) Add 1ml of 4 mg/ml lysozyme in TEG. Transfer to a sterile Oakridge (30ml) tube and leave on ice for 30 min.

v) Add 4ml 0.2M NaOH, 1% SDS and vortex briefly. Leave on ice 5 min.

vi) Add 3ml 3M Sodium acetate, pH 4.8. Mix by inversion and leave on ice 30-60 min.

vii) Spin at 9,000 rpm for 30 min (in Sorval RC-5B centrifuge).

viii) Remove supernatant, add 16ml cold ethanol and leave 15 min at -20°C.

ix) Spin at 9,000 rpm for 30 min.

x) Dissolve pellet in 2ml 0.1M Na acetate, 1mM EDTA, 0.1% SDS, 40mM Tris, pH8.0.

xi) Add 2ml phenol/chloroform/isoamyl-alcohol, vortex and centrifuge for 10 minutes.

xii) Transfer aqueous phase to a fresh tube and re-extract phenol layer with 2 ml buffer as in step xi).

xiii) Combine both aqueous layers and add 8ml cold ethanol. Leave 15 min at -20°C then spin for 10 min at 9,000 rpm.

xiv) Dissolve pellet in 0.4ml TE and transfer to a 1.5ml microfuge tube. Add 20µl 4M NaCl and 1ml cold ethanol. Leave 10 min at -20°C then spin for 5 min at 4°C.

xv) Dissolve pellet in 0.2ml TE, add 20µl RNase A and incubate for 1 hour at 37°C.

xvi) Add 20µl 4M NaCl, 2ml water and 0.4ml phenol/chloroform/isoamyl-alcohol. Vortex and spin for 1 min.

xvii) Remove aqueous phase and add 0.4ml chloroform, vortex and spin.

xix) Remove aqueous phase and add 8ml cold ethanol. Leave at -20°C for 10 min and spin for 5 min.

xx) Dissolve pellet in TE (0.1 or 0.2ml).

xxi) Put on to caesium chloride gradients (see below).

2.5.3) *B. SUBTILIS*

Bacillus subtilis plasmid DNA was prepared by the method of Gryczan *et al.* (1978).

Protocol as follows:-

i) Grow up overnight culture with required antibiotic selection (250ml).

ii) Harvest and wash cells.

iii) Resuspend in 0.1 volumes of 25% sucrose, 0.1M NaCl, 0.05M Tris, pH 7.5.

iv) Add lysozyme (0.5mg/ml) and incubate for 15 min at 37°C.

v) For every 25 ml suspension add

6 ml	5M NaCl
1.5ml	0.5M EDTA
32.5ml	2% SDS, 0.7M NaCl

Invert gently and keep on ice for 18 hours.

vi) Clear lysate by spinning at 15,000 rpm for 45 min (in Sorval RC-5B centrifuge).

vii) Make supernatant 0.3M in sodium acetate.

viii) Add 2 volumes 95% ethanol and incubate at -20°C for a minimum of 2 hours.

ix) Collect precipitate by centrifugation at 5000 rpm for 20-30 min.

x) Dissolve in 1.2ml TES.

xi) Digest with pancreatic ribonuclease (50μg/ml, final concentration) and T1 ribonuclease (1 unit/μl, final concentration). Maintain at 37°C for 30 min.

xii) Add predigested pronase (500μg/ml) until nearly clear (37°C).

xiii) Put on to caesium chloride gradients (see below).

2.5.4) *L. LACTIS*

L. lactis plasmid DNA was prepared by the standard cleared lysate method of Gasson (1983). Protocol as follows:-

i) Inoculate 500ml of M17 broth with 50ml overnight culture (pre-equilibrate the broth to 30°C).

ii) Grow at 30°C until O.D. 600 nm is 0.7-0.9 (ideally use 0.8).

iii) Harvest cells at 4,000 rpm for 10 min at 4°C (in Sorval RC-5B centrifuge).

iv) Wash in 100ml of cold distilled water.

v) Resuspend in 9ml protoplast buffer and move to Oakridge (30ml) tubes.

vi) Add 1ml 40 mg/ml solution of lysozyme (freshly prepared) in protoplast buffer.

vii) Mix and incubate at 37°C.

viii) After 5 min add 250μl DEPC (Diethylpyrocarbonate, SIGMA).

ix) Mix and continue incubation for 5 min.

x) Remove to ice bath.

xi) Lyse cells by addition of 10ml warmed FLM (final lysis mix) with gentle mixing.

xii) Return to ice, add 5ml of 5M NaCl and gently mix.

xiii) Leave on ice for 30 min.

xiv) Remove chromosome-membrane complex by centrifugation at 15,000 rpm for 30 min at 4°C.

xv) Decant supernatant into fresh Oakridge tubes.

xvi) Deproteinase for 10 min rolling at room temperature with chloroform/isoamyl-alcohol.

xvii) Leave in ice bath for a few minutes, then centrifuge at 10,000 rpm for 20 min at 4°C.

xviii) Remove upper phase with cut off plastic Pasteur pipette into fresh oakridge tubes.

xix) Add 1/10 volume of 3M sodium acetate and 2 volumes absolute ethanol. Leave overnight at -20°C.

xx) Harvest precipitated nucleic acid by centrifugation at 10,000 rpm for 20 min. Pour off ethanol and dry pellet.

xi) Resuspend in a small volume of TES buffer and 1/10 volume ribonuclease and incubate 30-60 min at 37°C.

xii) Put on to caesium chloride gradients (see below).

2.6) CAESIUM CHLORIDE GRADIENTS

Further purification of plasmid DNA from any residual chromosomal DNA was carried out using caesium chloride gradients. The following protocol was used:-

For two gradient tubes (13ml heat-seal polyallomer tubes, 16 x 76 mm from Beckman) 26.32g of caesium chloride (1mg/ml) was dissolved in 23.26ml of TES. The sample in 1.2ml of (10 mg/ml) TES was added to the tube with 0.225ml ethidium bromide to allow visualisation of the DNA. The tube was then filled with the caesium chloride mixture to within 1 cm of the top of the tube. Following balancing, tubes were heat sealed and placed in Beckman L8-70M ultra-centrifuge, type 70.1 Ti rotor. Gradients were spun for 40-50 hours at 40,000 rpm at 10°C (for *B. subtilis* 50-60 hour spin). After centrifugation, the lower (plasmid) DNA band was removed by hypodermic needle and syringe while under examination in UV light. (It is advisable to pierce the neck of the tube above the level of the caesium chloride prior to removing band as this relieves pressure within the tube). The ethidium bromide was removed from the sample by adding 1.2ml isoamyl alcohol saturated with

caesium chloride; 3 to 5 washes may be required.

Residual caesium chloride was removed by dialysis against several changes of TE buffer (generally overnight at 4°C). Finally, the sample was ethanol precipitated, dried and resuspended in a small volume of TE.

2.7) QUANTITATION OF DNA

For an estimation of the amount of DNA present after each purification spectrophotometric quantitation was used (Maniatis *et al.*, 1982, pp468). An O.D. reading of 1 at 260 nm corresponds to approximately 50 μg/ml double stranded DNA. The ratio between the readings at 260 nm and 280 nm gives an estimate on the purity of the nucleic acid: the purest preparations have a ratio (260/280) of between 1.8 and 2. In this study, 2 μl of sample was placed in a quartz cuvette (0.2cm wide) with 1 ml water (*ie* a 1/500 dilution) before readings were taken.

2.8) GEL ELECTROPHORESIS

Separation of plasmid DNA by size was achieved via gel electrophoresis. Agarose gels of 0.7% gels (Sigma, Type II) were made up in TBE buffer and run at 100 volts until the loading dye front had almost reached the end of the gel. For size analysis, two types of standards were used:

i) Linear DNA markers: *Hind*III digest of bacteriophage λ DNA. This gives fragment sizes of 23.15, 9.42, 6.56, 4.38, 2.32, 2.02, 0.56, 0.125 kilobases (kb).

ii) Covalently closed circular (ccc) markers: obtained from Bethesda Research Laboratories. Mixture of eleven supercoiled plasmids. Sizes as follows (in kb): 16.2, 14.2, 12.1, 10.1, 8.1, 7.1, 6.0, 5.0, 3.99, 2.97, 2.1.

5μl of 10 mg/ml ethidium bromide was added to gel mix prior to pouring: this allows visualisation of plasmid bands under UV light.

2.9) NON-RADIOACTIVE DNA LABELLING

The detection of particular plasmids within any bacterial strain may be complicated by the presence of other, similar-sized plasmids. This problem is particularly acute within the *Lactobacillus* strains used in this study as they each have at least two naturally occurring plasmids. In order to overcome any doubt as to which particular plasmid band in a given profile represents the cloning vector, it is possible to label the vector and to use this to probe the plasmid profile for the corresponding plasmid. The most common method for this kind of detection involves the use of radio-labelled plasmid. There are, however, non-radioactive methods available and because of the convenience of a non-radioactive method for a limited number of detections, one of these methods was used.

2.9.1) SOUTHERN HYBRIDISATION.

It is a pre-requisite of any detection procedure that the DNA is first transferred to a solid support. This involves the denaturing of the DNA within the gel matrix by immersion in alkali and its subsequent transfer to the solid support by capillary action. The method is as follows:

i) separate plasmid fractions by gel electrophoresis.

ii) photograph resulting plasmid profile.

iii) Nick the DNA by exposure to ultra-violet light on the transilluminator (245 nm) for a few minutes.

iv) Gently agitate in excess denaturing solution for 30 min.

v) Gently agitate in excess neutralising solution for 15 min. Repeat once.

vi) Place upturned gel tray into buffer reservoir containing 10x SSC.

vii) Pre-soak three pieces of Whatmann 3MM filter paper, cut to the same width but twice the length of the gel, in 2x SSC. Place these on to gel tray with overhang at each end to allow buffer movement up the filter paper.

viii) Invert gel and place onto 3MM paper.

ix) Place membrane (Hybond-N, Amersham) cut to same size as gel on top of the gel.

Ensure that no air bubbles remain between the gel and the membrane.

x) Pre-soak three pieces of Whatmann 3MM paper, cut to same size as the gel, in 2x SSC and place on top of the membrane.

xi) Place about 10 cm of dry tissues on top of 3MM paper.

xii) Place glass plate on top of this, followed by a 0.5 kg weight and leave overnight.

xiii) Carefully dismantle the apparatus and wash the membrane in 2x SSC.

xiv) Place membrane onto a piece of filter paper and allow to air dry.

xv) Put membrane on fresh piece of filter paper and cover with Saran wrap.

xvi) Expose membrane, DNA side down, to UV light on the transilluminator for a few minutes to fix the DNA to the membrane.

2.9.2) NON-RADIOACTIVE DNA LABELLING

Method as detailed in "Non-Radioactive DNA Labelling & Applications" Manual, Boehringer Mannheim.

The process involves incorporation of digoxigenin into the probe DNA, hybridisation of this probe to immobilised (see Southern blot procedure, above) test DNA, detection of the probe with an antibody-enzyme conjugate and finally localisation of this conjugate by an enzyme-linked colour reaction.

2.10) ISOLATION OF PLASMID BANDS

Particular plasmid bands were isolated from agarose gels by the following methods:-

2.10.1) FREEZE-SQUEEZE METHOD (Tautz & Renz, 1983).

Cut required band from 0.7% agarose gel (taking as little excess agarose as possible) and place in a petri dish containing 0.3M Sodium acetate, 1mM EDTA. Seal the petri dish with film and wrap in foil, then shake gently at room temperature for 15 min to remove the ethidium bromide. Transfer DNA band to a 0.5ml eppendorf tube containing some glass wool and with a small hole pierced in the bottom of the tube. Incubate for at least 45 min at -80°C then place the small eppendorf tube into a 1.5ml tube and spin for 10min.

The glass wool effectively acts as a sieve and the DNA solution is collected in the outer tube. Repeat spin to collect as much of the DNA as possible. Add 1/100 volume of 1M $MgCl_2$, 10% Na Acetate and 2.5 volumes ethanol and place at -80°C for at least 30 min. Spin down the DNA and resuspend in a small volume of TE.

2.10.2) MEMBRANE METHOD: DEAE Membranes (NA45) from Schleicher & Schuell were used.

A 0.7% agarose gel run as normal, then beside the required band on the gel a small cut is made perpendicular to the band and a small piece of membrane (pre-soaked in NET buffer: 1.5M NaCl in 1x TE) is inserted into the gel. The gel is then turned round and cut to fit the gel tank in such a way that the plasmid band will run into the membrane when the current is re-applied. The plasmid band will be retained in the membrane. After the whole of the band has run into the membrane, cut away the non-glowing edges of the membrane and put the remainder in an eppendorf tube with 350 µl NET buffer. Vortex briefly then incubate at 65°C for 1 hour. Transfer the NET buffer (which should now contain the DNA) to a fresh tube and wash membrane with some fresh buffer. Pool the two sets of buffer and spin to remove any remaining membrane debris. Remove the supernatant, phenol/chloroform extract, then ethanol precipitate.

2.11) DIGESTION BY RESTRICTION ENZYMES

Table 2.11. Description of restriction enzymes used.

Enzyme	Source	Recognition Sequence
*Bam*HI	*Bacillus amyloliquefaciens* H	GGATCC
*Bgl*II	*Bacillus globigii*	AGATC
*Eco*RI	*E. coli* RY13	GAATTC
*Hind*III	*Haemophilus influenzae* Rd	AGCTT
*Kpn*I	*Klebsiella pneumoniae*	GGTACC
*Pst*I	*Providencia stuartii*	CTGCAG
*Sst*I	*Streptomyces standford*	AGCTC
*Sal*I	*Streptomyces albus* G	GTCGAC

2.12) LIGATIONS

All DNA ligations were carried out at 15°C using T4 DNA Ligase. Final sample volume of 30µl was made up from 1µl enzyme, 2µl buffer, 1µg (1-5µl) of vector, 10µg (5-15µl) proposed insertion fragment. Volume made up with distilled water. All ligations were visualised by agarose gel electrophoresis prior to transformation.

2.13) TRANSFORMATION

2.13.1) *E. COLI*

Genetic transformation of *E. coli* strains was achieved by the standard calcium chloride method of Mandel & Higa (1970). Method as follows:-

i) Inoculate 10ml of L broth with 0.5ml of overnight culture of *E. coli* MC1022.

ii) Grow cells at 37°C shaking for 2-2.5 hours.

iii) Harvest cells by centrifugation.

iv) Discard supernatant and resuspend pellet in 1ml ice-cold sterile 100mM calcium chloride ($CaCl_2$).

v) Leave on ice 20-30 minutes.

vi) Spin down cells in a microfuge and resuspend in 1ml ice-cold 100mM $CaCl_2$.

vii) Leave on ice for at least 2 hours.

viii) Add a small volume of ligation mix (1-10µl) to 200µl of competent cells. Leave on ice for 40 minutes.

Include a control with no DNA.

ix) Heat shock at 42°C for 2 minutes (or 5 minutes at 37°C).

x) Add each mix to 5ml of L broth and grow at 37°C for 1.5-2.5 hours.

xi) Spin down cells and resuspend in 0.2ml L broth (controls) and 0.5ml (samples).

xii) Plate out 0.1ml volumes on to L agar containing the necessary antibiotic selection and incubate the plates at 37°C.

2.13.1.1) Screening for *E. coli* pTG262 and pMTL500 containing inserts.

Plasmid pTG262 contains the *lac*Z gene of the *E. coli* lactose operon; this is the structural gene for ß-galactosidase. In the presence of an inducer (IPTG) and a chromogenic substrate (5-bromo-4-chloro-3-indolyl-ß-D-galactoside, X-gal) bacteria expressing this gene appear as blue colonies. pTG262 contains the pUC18 polylinker within the *lac*Z gene; the construction of a new plasmid by the ligation of another piece of DNA into this region results in the insertional inactivation of the *lac*Z gene and hence recombinants appear as white colonies. For the purposes of this study 150µl each of IPTG (0.2% solution in water) and X-gal (2% solution in dimethylformamide) was added to 100ml agar and colonies were scored after 2 days at 37°C.

2.13.2) *B. SUBTILIS*

The protoplasting technique of Chang & Cohen (1979) was used for *B. subtilis*. Procedure as follows:

i) Grow cells to mid log phase in Penassay broth at 37°C.

ii) Resuspend in 1/10 volume SMMP (see section 2.2.6).

iii) Add lysozyme (2 mg/ml, final concentration) and incubate at 37°C with gentle shaking. Protoplasts form after 30 min but is usual to incubate for 2 hours to ensure

complete protoplasting.

iv) Pellet cells at 2,600 rpm for 15 min (in Sorval RC-5B centrifuge). Wash once by resuspending them gently in SMMP then repellet.

v) Resuspend washed protoplasts in SMMP to 1/10 - 1/15 volume of original culture. Preparation will remain stable at room temp. for 5 hours.

vi) 1-5μg plasmid DNA in 50μl (or less) TE buffer mixed with equal volume of 2 x SMM solution (see below).

vii) Add 0.5ml protoplast solution followed immediately by 1.5ml 40% PEG (w/v, MW 6,000). Mix gently.

viii) After 2 min in presence of PEG add 5ml SMMP.

ix) Recover protoplasts by centrifugation at 2,600 rpm for 10 min.

x) Resuspend protoplasts in 1ml SMMP and incubate for 90 min at 30°C, shaking gently. This allows phenotypic expression before plating onto antibiotic media for direct selection of transformants.

xi) Score plates after 2 days at 37°C.

SMMP medium is prepared by mixing equal volumes of 4x Penassay broth and 2x SMM.

2.13.3) *L. LACTIS*

Transformation of *L. lactis* by electroporation was carried out according to the method of Powell *et al.* (1988). Procedure as follow:-

i) Grow cells up overnight in M17 broth with 0.5% carbohydrate.

ii) Inoculate 1% into fresh broth (pre-warmed to 30°C).

iii) Grow at 30°C to an O.D. of 0.3 - 0.7 at 600 nm.

iv) Take 25ml aliquots of culture and harvest cells at 7,000 rpm for 10 min (in Sorval RC-5B centrifuge).

v) Wash cells in 5ml fresh, ice-cold (phosphate) electroporation buffer (EPB).

vi) Resuspend in 1ml EPB and hold on ice.

vii) Mix 1μg DNA in TE with 0.8ml of cell suspension in pre-chilled Gene Pulser cu-

vette.

viii) Pulse: 1 pulse, 2.5 kV, 25 μFD.

ix) Transfer to eppendorf tube and hold on ice for 25 min.

x) Mix with 10ml M17 with 0.5 % carbohydrate and incubate for 1-2 hours at 30°C.

xi) Concentrate cells; spin down gently and resuspend in 1ml of media.

xii) Plate on selective media with M17 with 0.5M carbohydrate.

2.13.4) LACTOBACILLI AND PEDIOCOCCI

Electroporation method for lactobacilli as follows:

i) Grow 10ml culture to mid-log phase using an inoculant from an overnight culture, generally a 1 in 20 dilution. Growth takes 3 - 3.5 hours and corresponds to an OD 0f 1.2-1.4 at 600nm.

ii) Harvest and wash cells twice in ice-cold (sucrose) electroporation buffer.

iii) Resuspend in 1/10 the original culture volume and store on ice.

iv) Add 1μg of DNA to 100μl cells in a cold, 2 mm diameter cuvette.

v) Pulse: 25 μFD, 2.5 kV, 100 ohms.

vi) Immediately add 1ml MRS broth to the cuvette and transfer to an eppendorf tube.

vii) Incubate for two hours at 30°C.

viii) Harvest cells and resuspend in 0.3ml of MRS. Plate 0.1ml volumes on selective media.

ix) Score for transformants after 3-4 days.

Electroporation method for lactobacilli is based on Chassy & Flickinger (1987) and modified as detailed in chapter four.

Re-use of cuvettes

The gene pulser cuvettes are supplied sterile and are designed for single use. This, however, requires a large stockpile and is obviously expensive. Hence, during the whole of this study, re-use of cuvettes was carried out by rinsing twice with absolute alcohol and then allowing the alcohol to evaporate (residual alcohol can result in arcing).

To counter any possibility of this procedure not resulting in cuvette sterility, each cuvette was used for only one particular strain and during the optimisation, new cuvettes were

used for each variable.

2.14) DESCRIPTION OF GENE PULSER APPARATUS

2.14.1) VOLTAGE

The basic Gene Pulser unit allows a range of voltages from 0 to 2.5 kilovolts (kV) to be applied to the electroporation cuvette. The original cuvettes supplied have an inter-electrode distance of 0.4 cm and hence the field strength (kV/cm) reaches a maximum of 6.25 kV/cm. An additional unit, the Pulse Controller, designed specifically for use with bacterial systems, comes with cuvettes with an inter-electrode distance of 0.2 cm, *ie* a maximum field strength of 12.5 kV/cm can be reached.

2.14.2) CAPACITANCE

Capacitance is the ability to store an electrical charge when a potential difference exists between the conductors *ie* the charge dictated by the voltage is stored and released quickly when the pulser is activated. It is measured in microFarads (μFd). The Gene Pulser allows a choice of four capacitance values (0.25, 1.0, 3.0, or 25 μFd) and a second additional unit, the Capacitance Extender, allows a further four settings (125, 250, 500 or 960 μFd). When the Capacitance Extender is in use the maximum voltage allowed is 0.45 kV.

2.14.3) RESISTANCE

With the basic Gene Pulser unit the main consideration is the resistance of the electroporation medium. This depends upon the medium's ionic strength; as ionic strength increases, resistance of the medium decreases. A pulse delivered into a high ionic strength medium will have a short time constant if all else remains equal. The pulse controller allows the choice of one of six possible resistors to be placed in parallel with the sample chamber. When this resistance is much smaller than the resistance of the sample it is the primary determinant of the resistance across the capacitor.

2.14.4) TIME CONSTANT

By virtue of the involvement of a capacitor unit, the Gene Pulser gives an electrical pulse which has an exponential decay waveform. In this type of waveform the voltage rises very rapidly to a peak amplitude and then declines as an exponential function of the resistance and the capacitance. The time constant (t) is defined as the time it takes for the voltage to decline to 1/e (about 37%) of the initial voltage. t depends on the size of the capacitor and the resistance through which it is discharged. A larger capacitor requires a longer time to discharge through a given resistance. A given capacitor discharges more slowly through a higher resistance. The time constant on the Gene Pulser cannot be altered directly, only by changing the capacitor and/or the resistance.

2.14.5) ARCING

Arcing is caused by the current flowing through the gap between two electrodes, carried by the vapour within the gap. This is an explosive reaction usually caused when the electroporation medium is too conductive and samples are generally discarded as there is no means of establishing how much current has passed through the cuvette. The Pulse Controller reduces the incidence of arcing at high voltage and is also protects the Gene Pulser if an arc does occur. Hence it should always be used when delivering high field strength pulses to samples of small volume and high resistance.

2.15) ENZYME ASSAYS

2.15.1.2) CELLULASE PLATE ASSAY

Congo Red assay of Teather & Wood (1982) used. Media set up containing 0.5% carboxymethylcellulose (CMC). Incubation for 1-2 days followed by flooding with 1 mg/ml congo red for 15 min. Drain, then flood with 1M NaCl for a further 15 min. Cellulolytic activity indicated by zones of clearing in a red background.

2.16) PLASMID DIAGRAMS

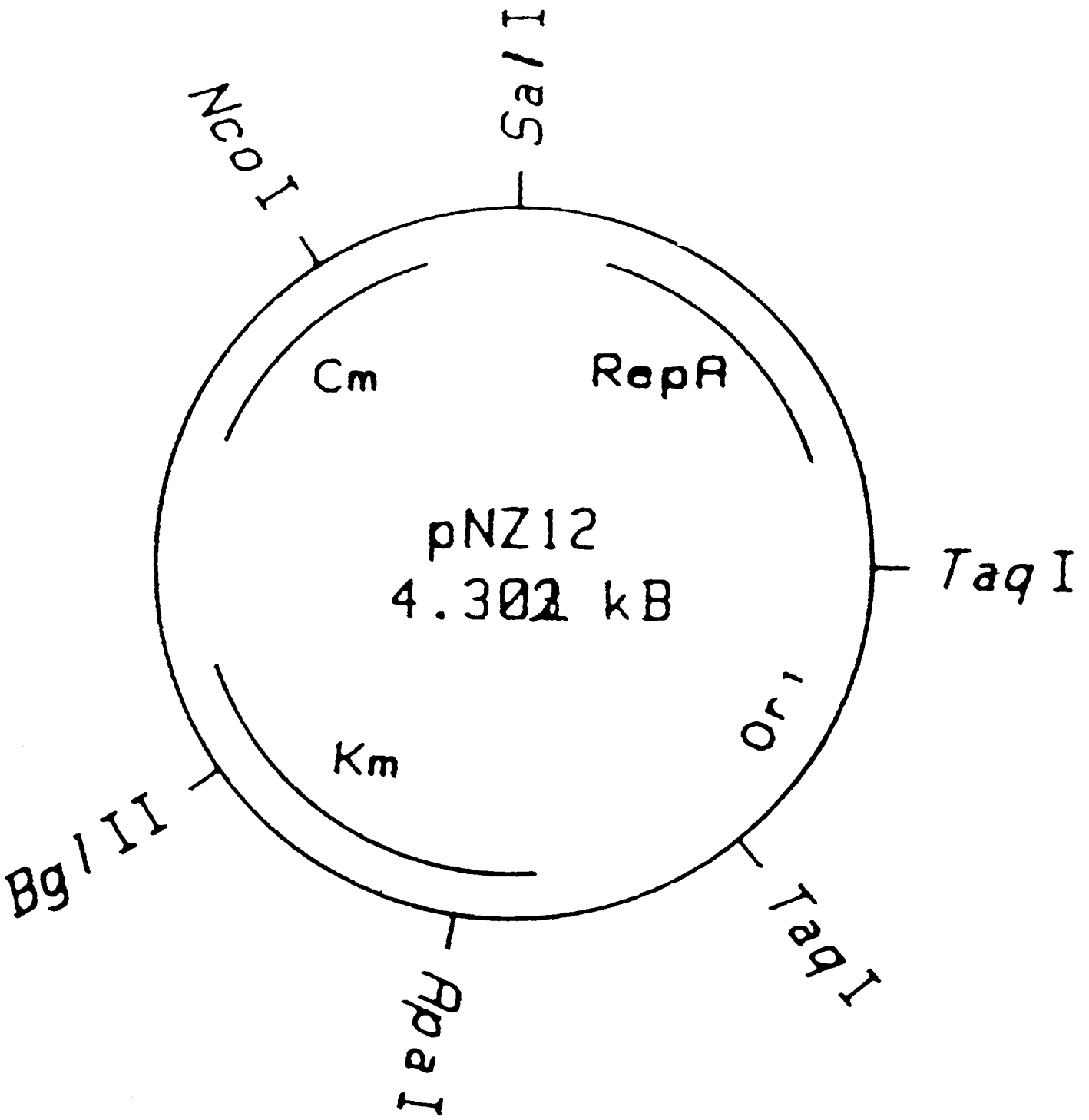


Figure 2.1) pNZ12

Cm = chloramphenicol resistance determinant, km = kanamycin resistance determinant, RepA = replication functions (de Vos, 1986).

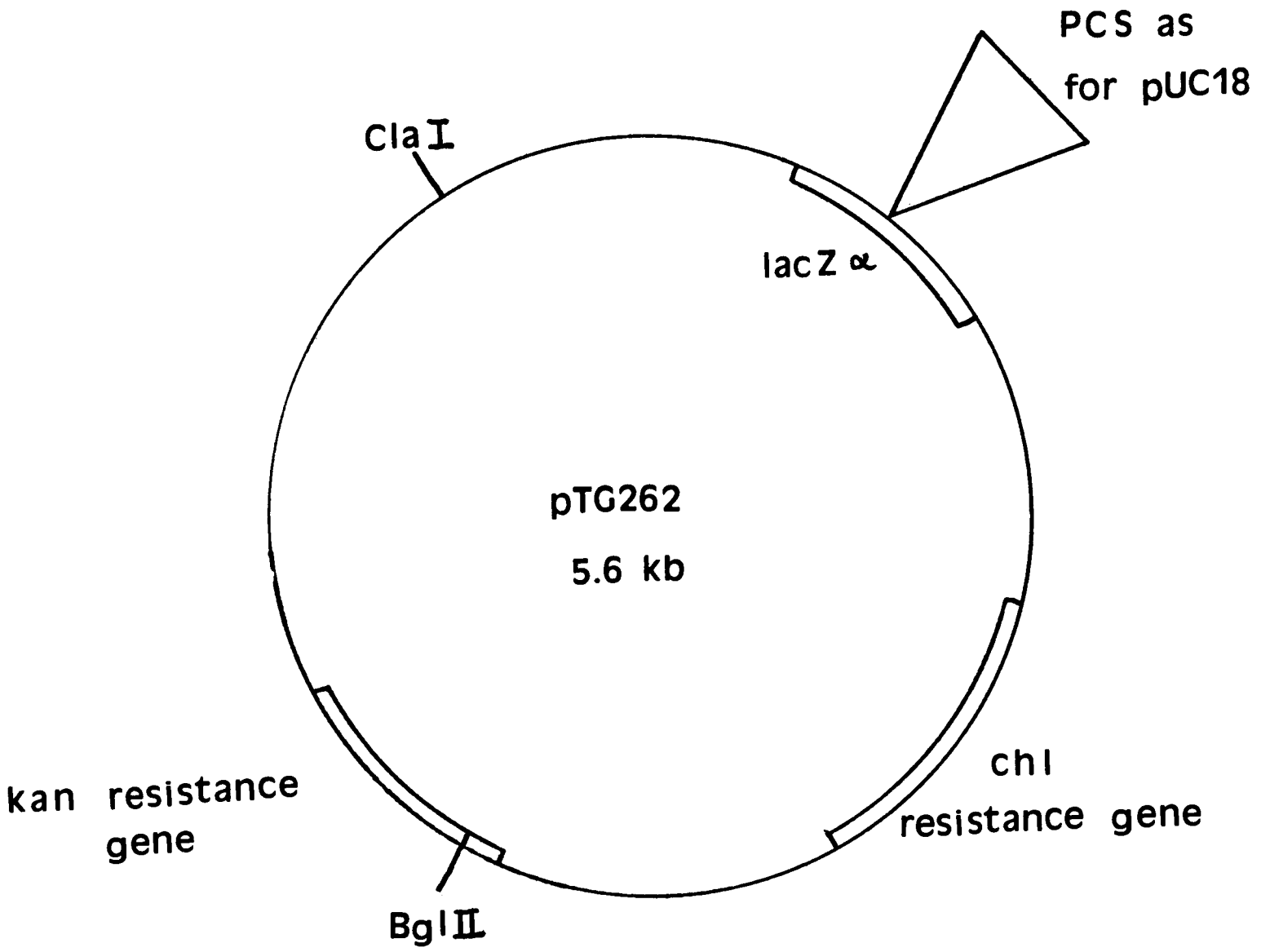


Figure 2.2) pTG262

PCS = polycloning site

The PCS contains the following restriction enzymes sites: *Eco*RI, *Sst*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, *Pst*I, *Sph*I, *Hind*III (Shearman *et al.*, 1989) but its orientation has not been defined

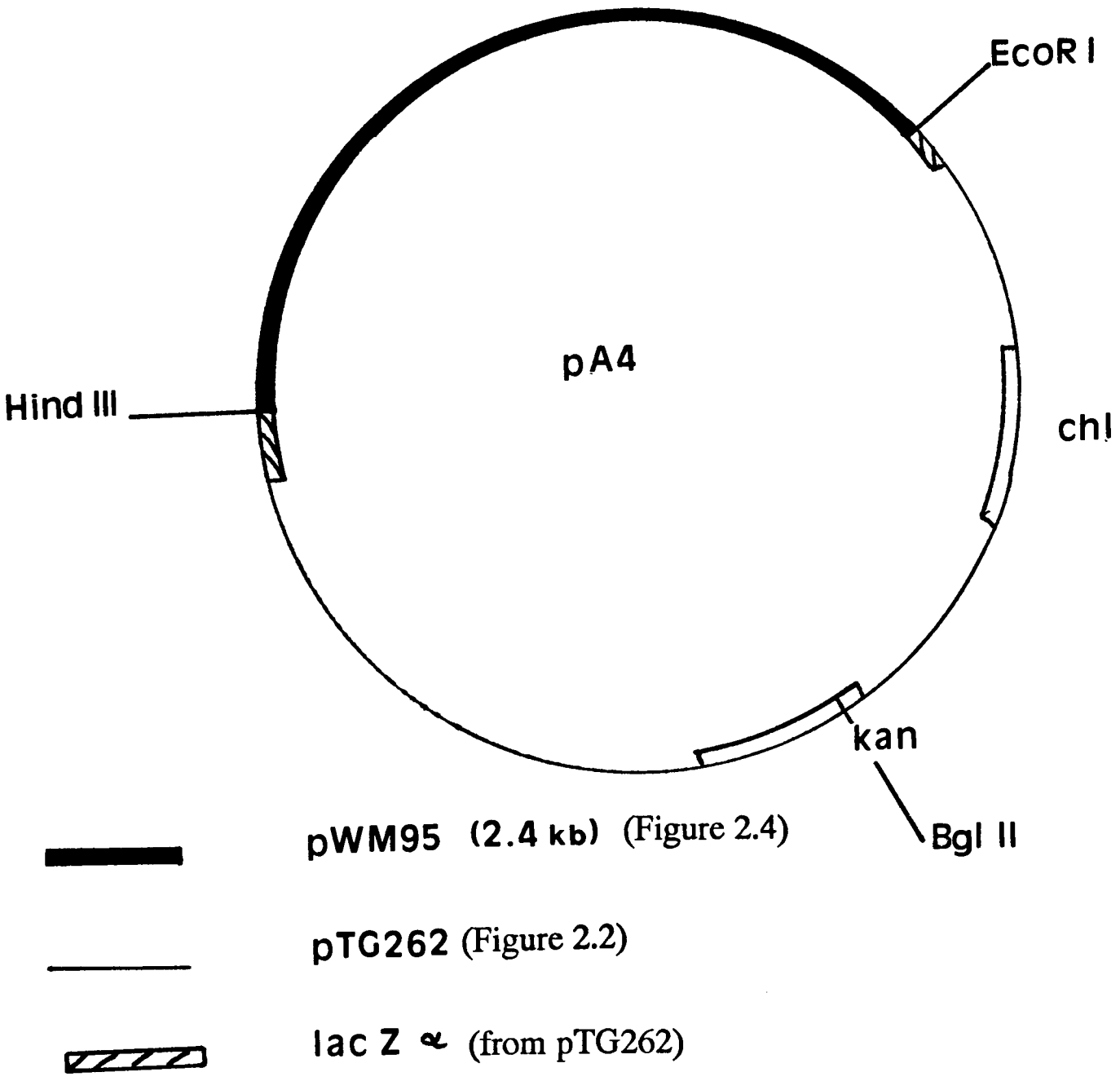


Figure 2.3) pA4 (constructed in the course of this work).

*Eco*RI-*Hind*III (cellulase-encoding) fragment of pWM95 in pTG262. None of original pTG262 PCS remains.
E.coli TG1 colonies are white on media containing IPTG and X-gal due to insertional inactivation of *lac*Z gene.

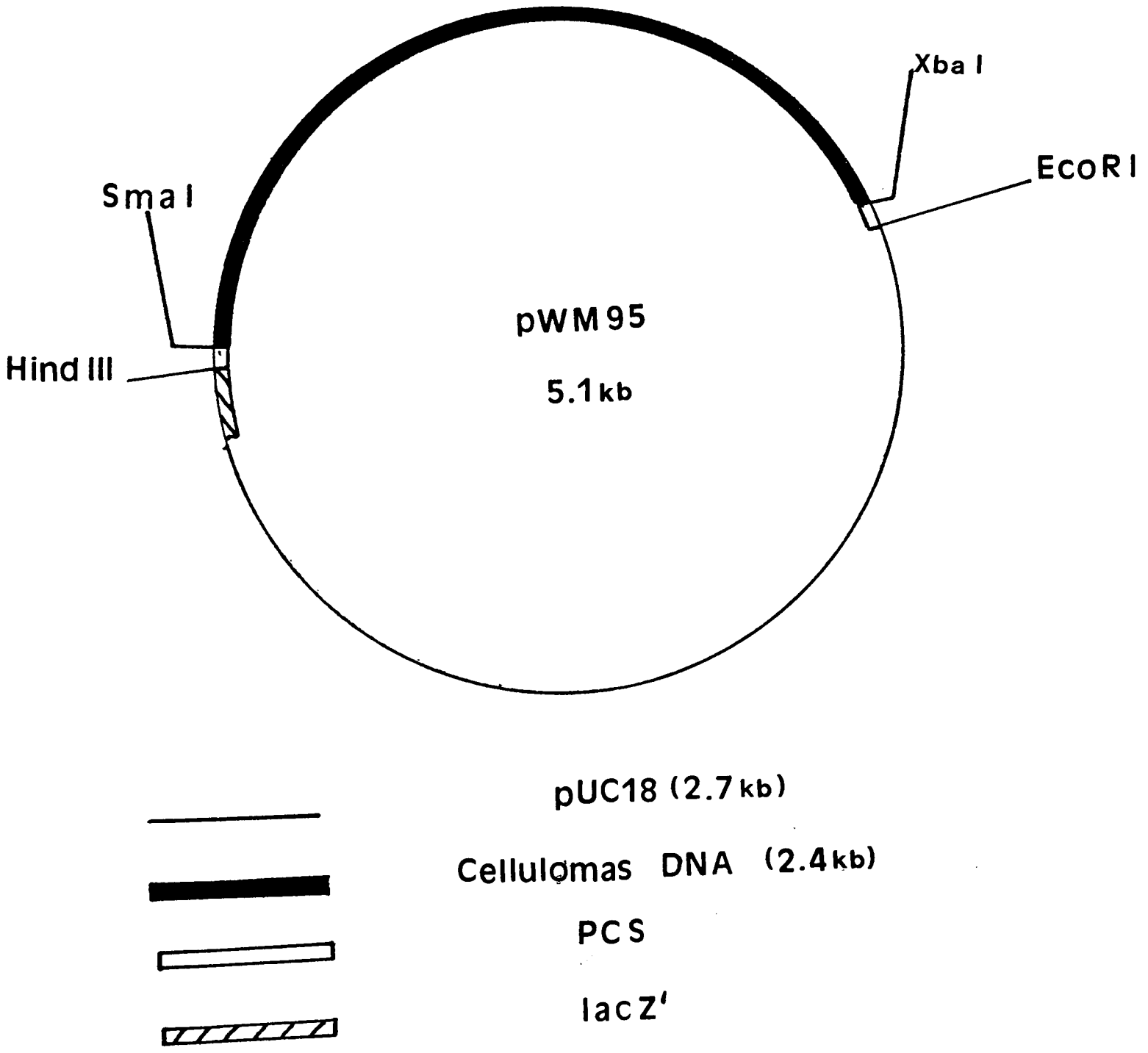


Figure 2.4) pWM95

2.4kb, cellulase-encoding fragment of *Cellulomonas flavigena* DNA in *Bam*HI site of pUC18 (Akhtar *et al.*, 1988). PCS = polycloning site. *Bam*HI site not recreated. Restriction enzymes with sites within the pWM95 fragment include *Sst*I

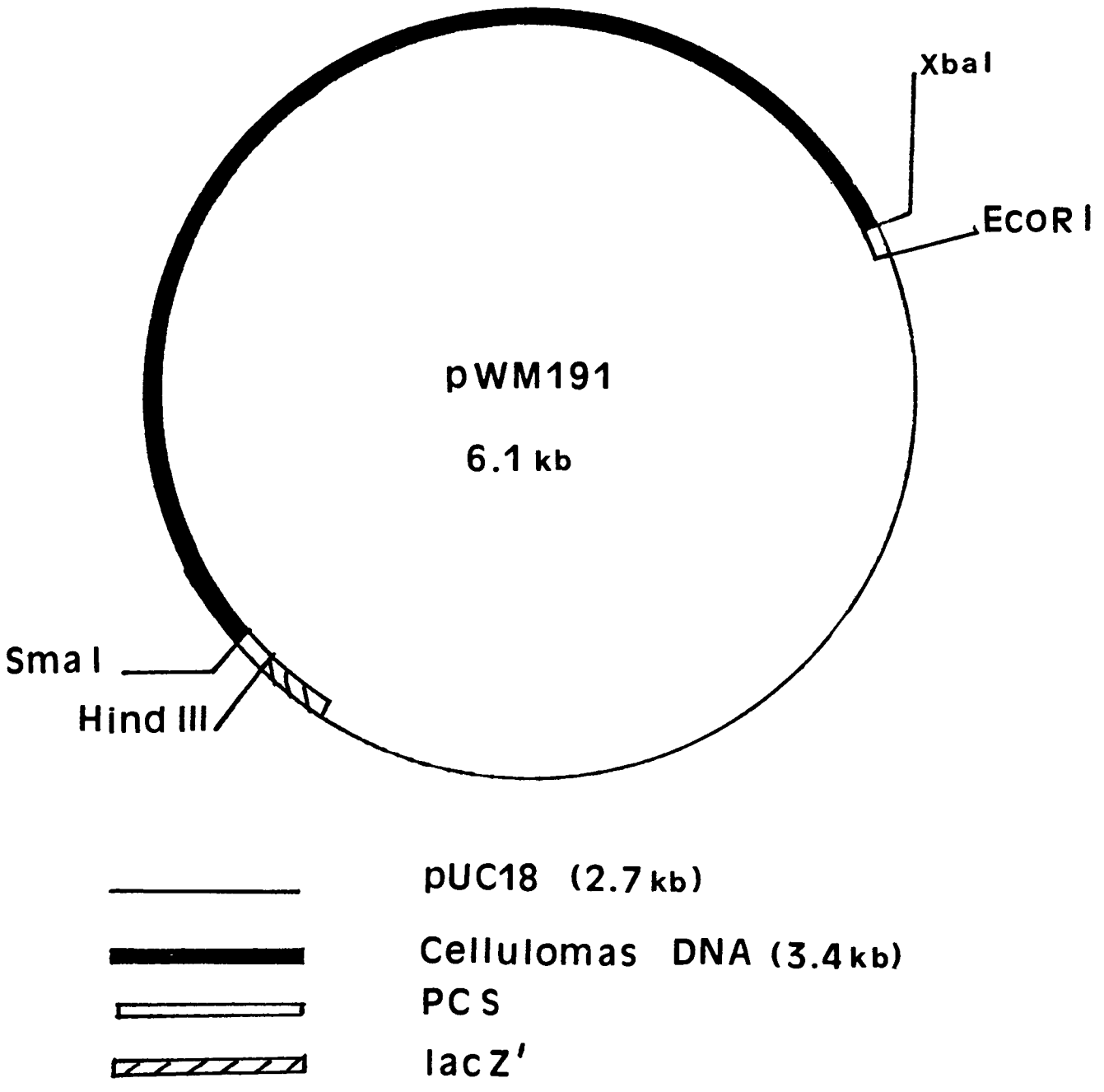


Figure 2.5) pWM191

3.4kb, cellulase-encoding fragment of *Cellulomonas flavigena* DNA in *Bam*HI site of pUC18 (Akhtar *et al.*, 1988). PCS = polycloning site.

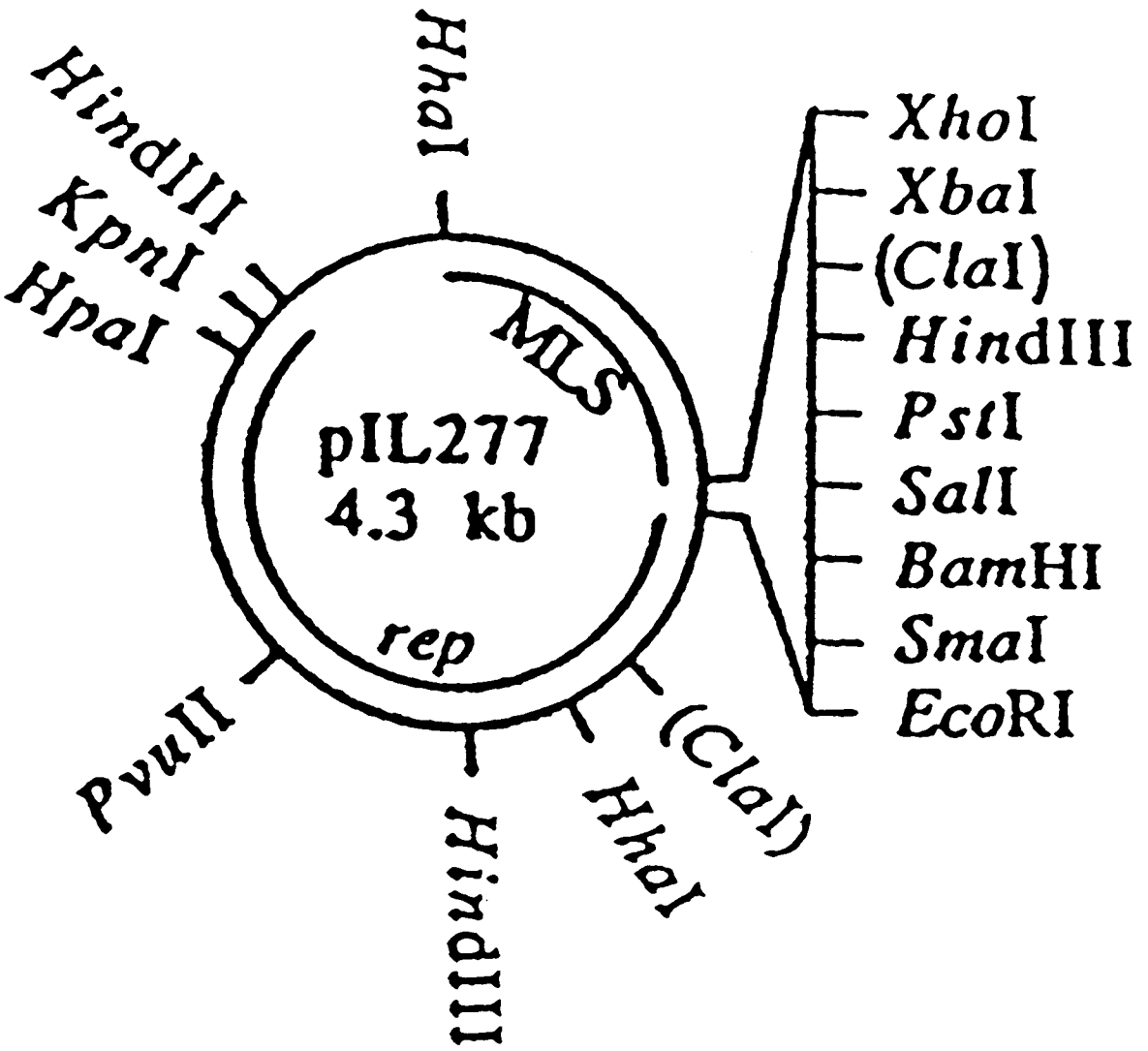


Figure 2.6) pIL277

MLS = Macrolides, lincosamides, streptogramin B antibiotic resistance, rep = replication region, (*Cla*I) = unmapped *Cla*I sites (Simon & Chopin, 1988).

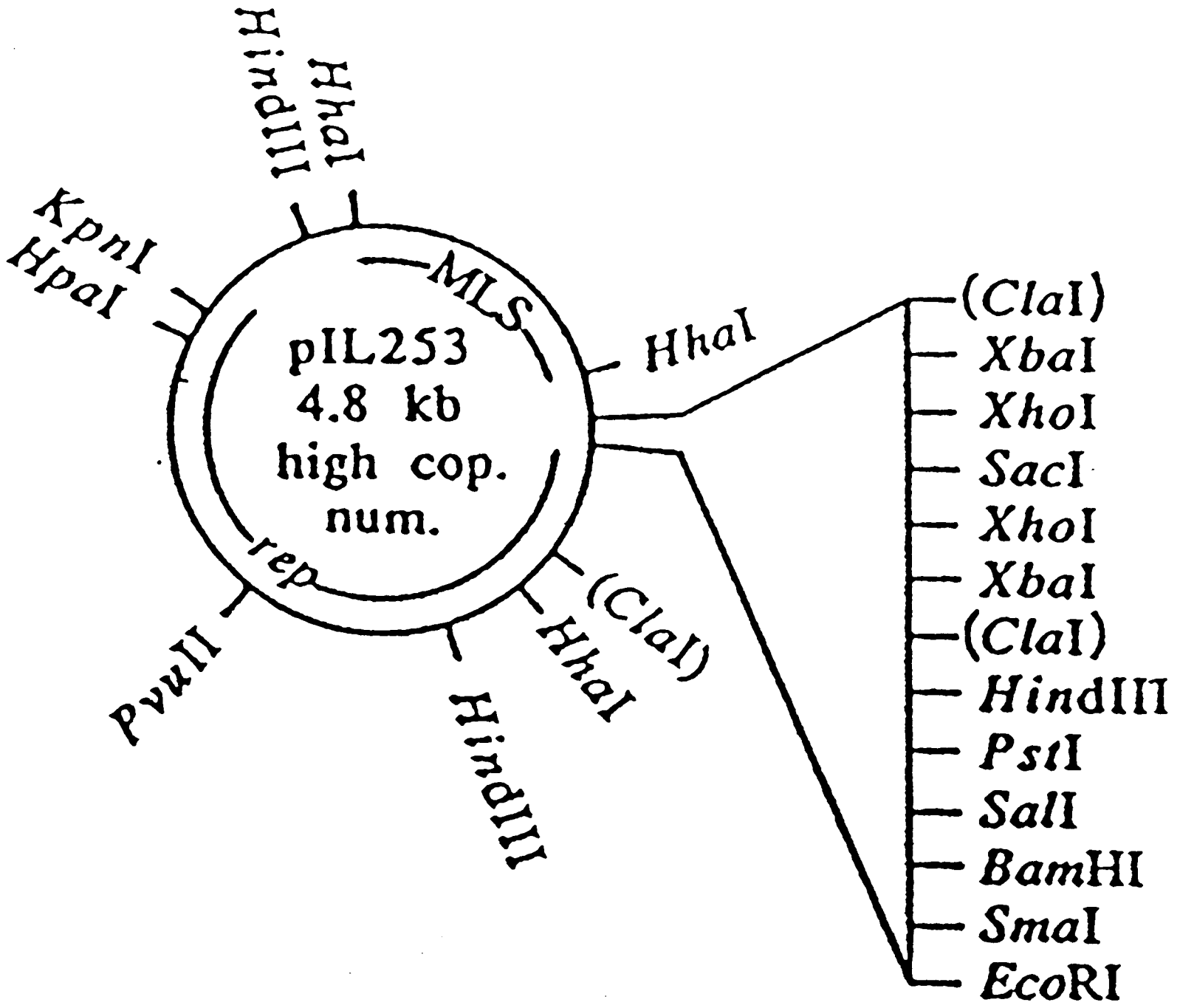


Figure 2.7) pIL253

MLS = macrolides, lincosamides, streptogramin B antibiotic resistance, rep = replication region, (*Cla*I) = unmapped *Cla*I sites (Simon & Chopin, 1988).

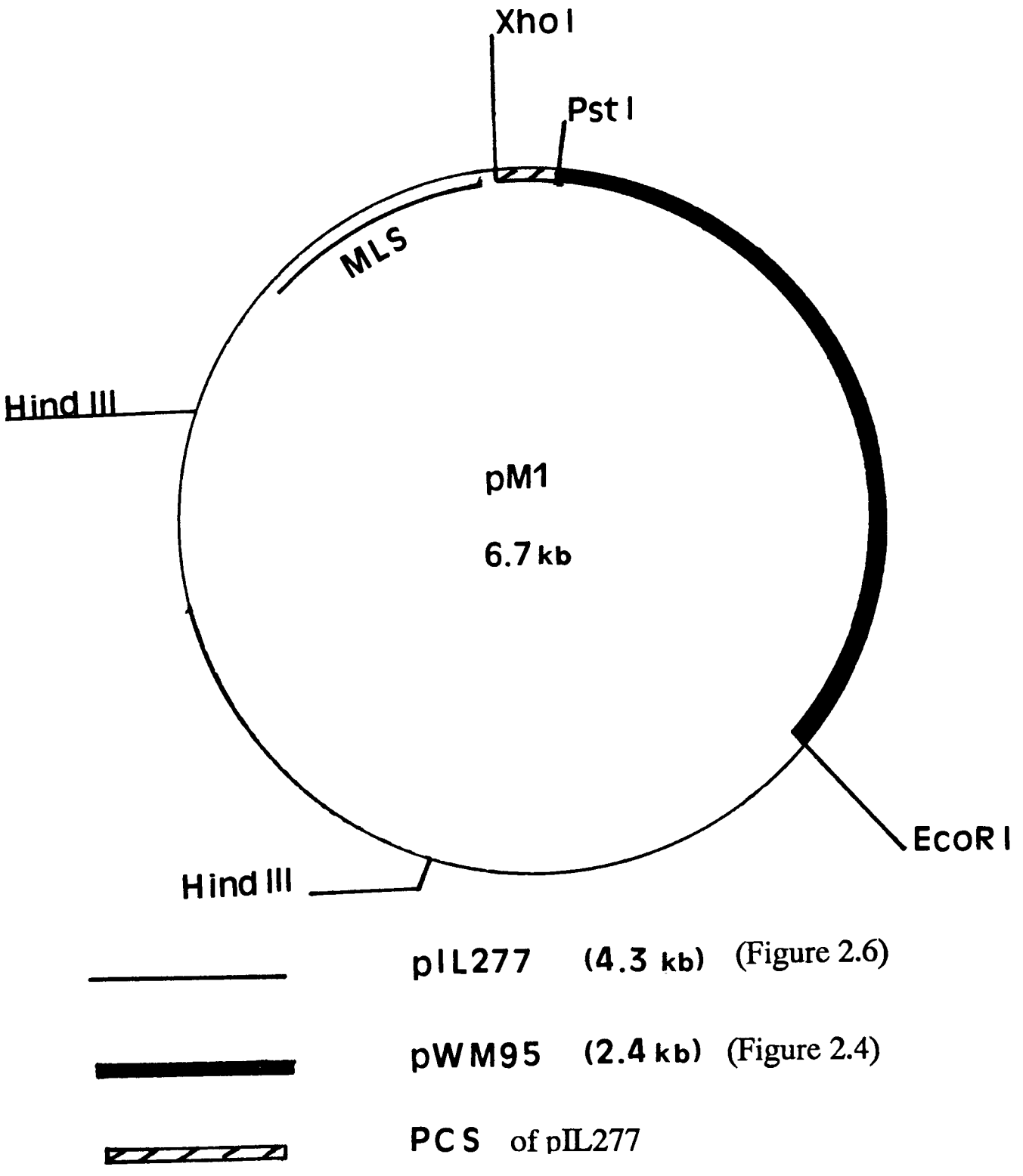


Figure 2.8) pM1 (constructed in the course of this work).

*Eco*RI-*Pst*I fragment (cellulase-encoding) of pWM95 in pIL277. PCS = polycloning site. The following sites were removed from the PCS by the construction of pM1: *Sal*I, *Bam*HI, *Sma*I. MLS = macrolides, lincosamides, streptogramin B antibiotic resistance.

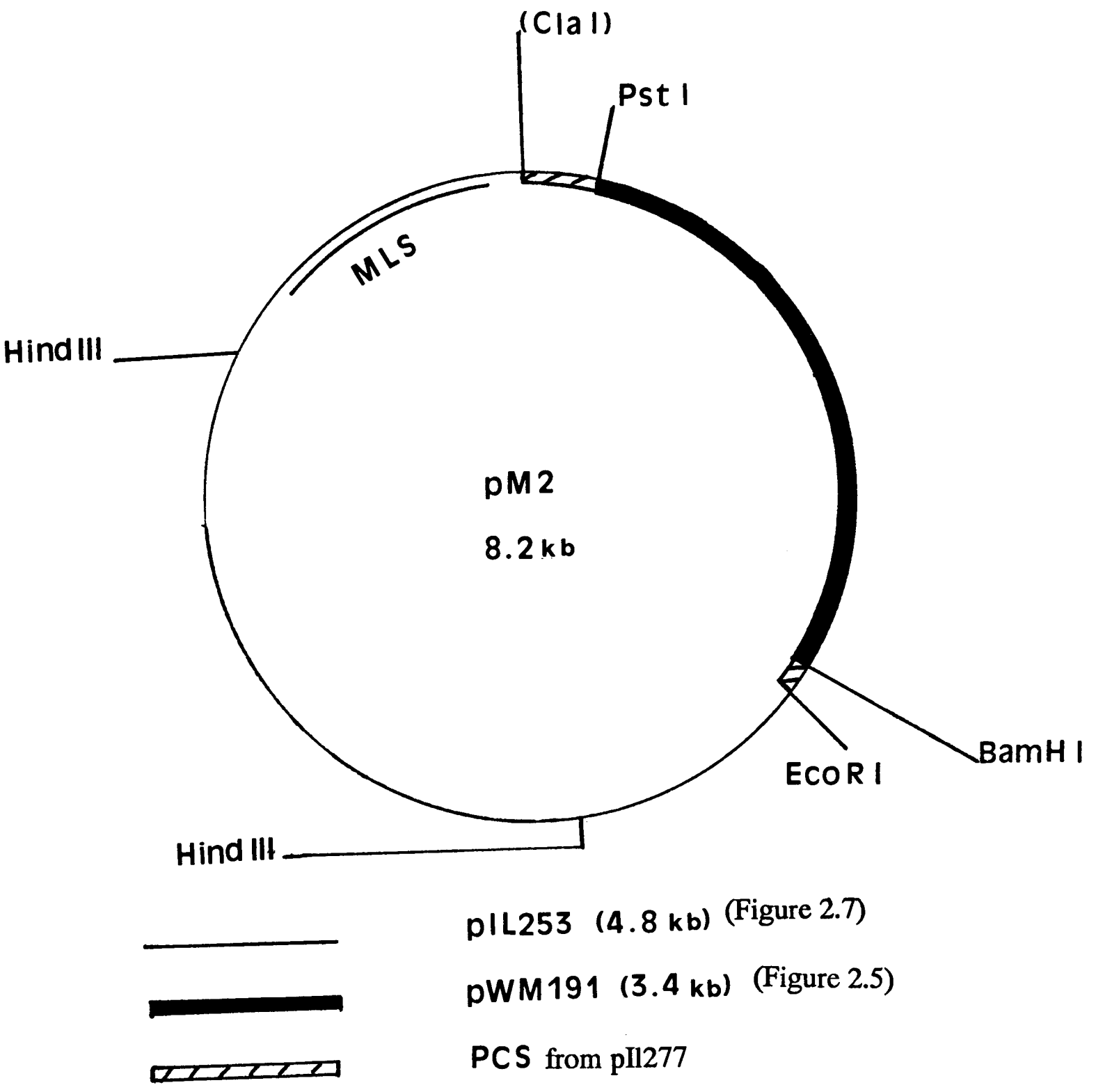


Figure 2.9) pM2 (constructed in the course of this work).

*Bam*HI-*Pst*I fragment (cellulase-encoding) fragment of pWM191 in pIL253. PCS = polycloning site. The *Sma*I site was removed from PCS by the construction of pM2. MLS = macrolides, lincosamides, streptogramin B resistance determinant.

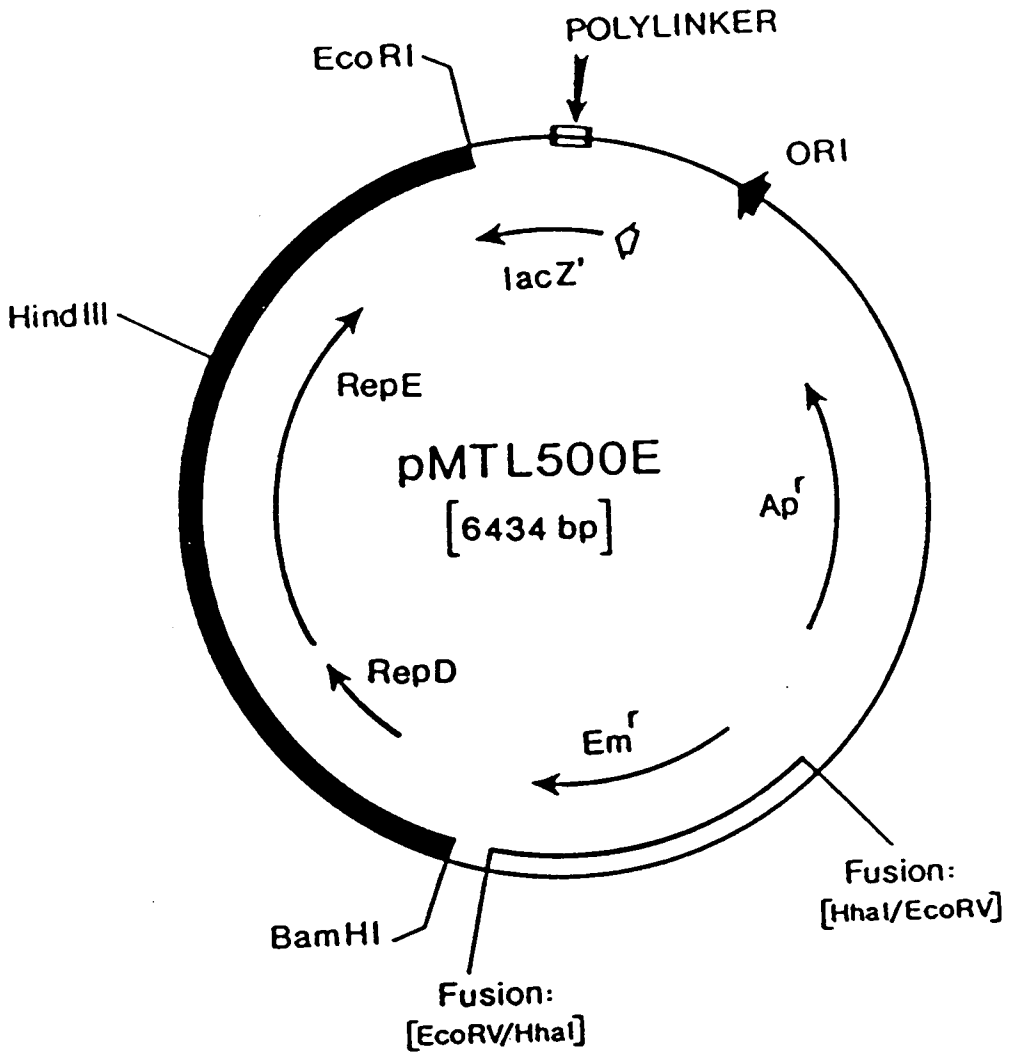


Figure 2.10) pMTL500E (Oultram *et al.*, 1988).

Ap = ampicillin resistance gene, em = erythromycin resistance gene, ori = origin of replication, shaded area = pAMß1 replicon. The following restriction enzymes have sites within the polylinker (reading from nearest to origin): *Eco*RI, *Sst*I, *Kpn*I, *Sma*I, *Xma*I, *Bam*HI, *Xba*I, *Aat*I, *Sal*I, *Sty*I, *Nco*I, *Bgl*II, *Xho*I, *Stu*I, *Pst*I, *Sph*I, *Hind*III.

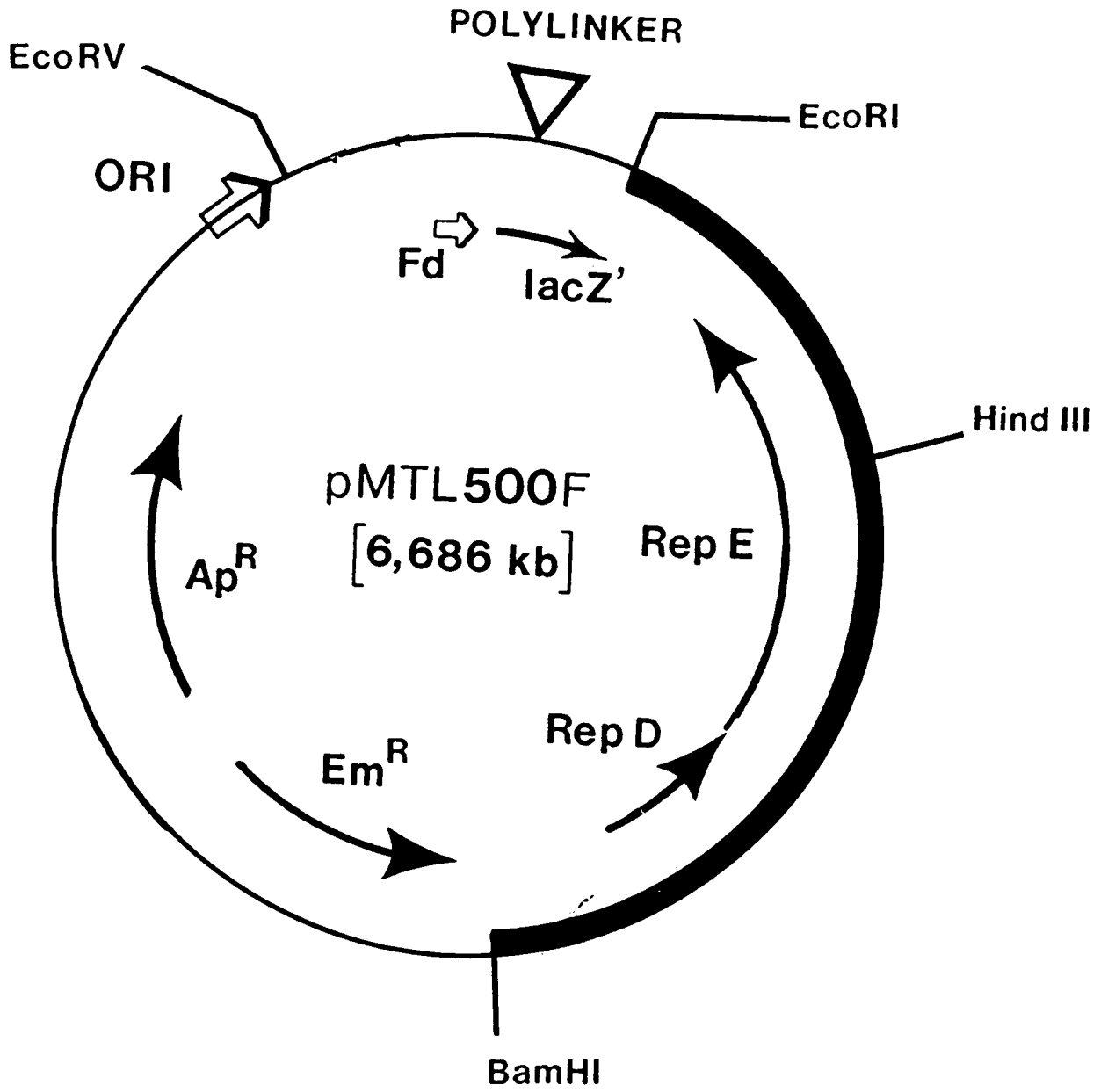


Figure 2.11) pMTL500F (N. Minton, personal communication).

Fd = Gram positive promoter. Polylinker contains the same restriction enzyme sites as pMTL500E.

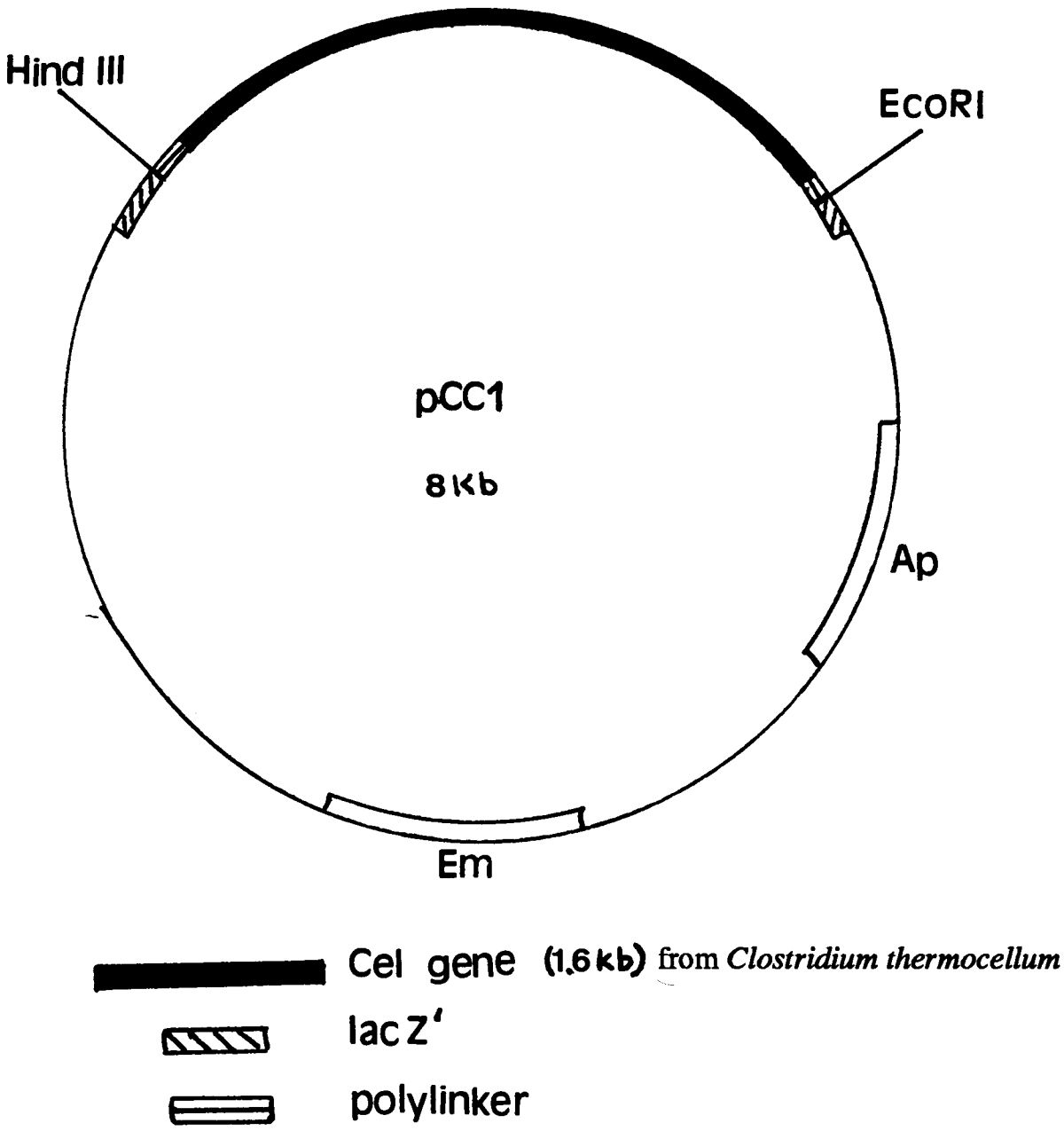


Figure 2.12) pCC1

*Cel*C gene in *Sma*I site of pMTL500E, ap = ampicillin resistance gene, em = erythromycin resistance gene.

E. coli TG1 colonies are white on media containing IPTG and X-gal due to insertional inactivation of *lac*Z' gene. Only the *Sma*I site has been removed from the polylinker.

CHAPTER THREE

INTRODUCTION TO EXPERIMENTAL STRAINS

3.1. STRAIN ISOLATION

The experimental strains used in this study were isolated from a successful grass silage fermentation by Dr. S Heron at the University of Edinburgh. Lactic acid bacteria were selected using MRS agar and Tween acetate agar.

3.1.2. HOMO/HETEROFERMENTERS

One of Whittenbury's (1961) criteria for the selection of LAB strains which have potential as silage additives was homofermentative ability *ie* the ability to produce only lactic acid from carbohydrates. This achieves maximum lactic acid production from the WSC within the crop and hence helps to lower the pH of the silage. Each of the isolated organisms was tested for gas production, which is indicative of a heterofermentative organism, using homo/heterofermentative medium. All of those which tested positive for heterofermentative ability were excluded from this study.

3.2. INITIAL IDENTIFICATION

3.2.1. GRAM STAINING

The remaining strains were all subjected to Gram staining in an attempt to achieve preliminary genus identification. Since the primary objective of this study was the manipulation of lactobacilli and pediococci, all those strains which were identified as Gram-positive cocci occurring in chains, which is indicative of lactococci, were excluded.

Six *Lactobacillus* isolates were thus characterised as non-sporing, Gram-positive rods occurring singly or in pairs and assigned numbers 48, 65, 71, 72, 85 and Lp. These strains had approximate dimensions of 2-3μm in length by 0.5μm width. Twelve *Pediococcus* strains, identified as Gram positive cocci occurring in pairs or tetrads and of

approximately 1μm in diameter were also characterised and numbered as strains 1, 37, 40, 41, 42, 60, 75, 77, 79, 80, 84 and 826. The numbering of the isolates was purely arbitrary.

3.2.2. CATALASE TEST

As further confirmation of the genus identification of the experimental strains, a catalase test was performed on all of the isolates. Both *Lactobacillus* and *Pediococcus* species are catalase negative (Kandler & Weiss, pp1029 and Garvie, pp1075 respectively in Bergey's Manual of Systematic Bacteriology, Volume Two, First Edition, 1986). All of the isolates described here were also catalase negative.

The overall aim of this study was to achieve genetic manipulation of environmental (silage) isolates of LAB *ie* strains from nature rather than strains from culture collections. The source of the organisms gives an obvious advantage in that environmental isolates will already be adapted to survival within a silage system whereas laboratory strains may be much less efficient. The work reported within this study is unlikely to be exactly reproducible due to the unique culture collection used. Hence, it was not considered vital to identify the strains beyond the species level. For the purposes of this thesis each isolate was considered to be a unique strain although some evidence will be presented which suggests that some of the isolates are identical (see below).

3.3. FURTHER IDENTIFICATION

3.3.1. API SYSTEM

In order to identify the isolates to the species level, the API (Montalieu Vercieu, France) 50 CH identification system was used. The fermentation characteristics of each isolate using the given range of 49 carbohydrates was recorded and compared to that obtained with known reference strains.

On the basis of the API system, strains 85, 65, 71, 72, 48 and Lp were all identified as *Lactobacillus plantarum*. The reference strains *Pediococcus acidilactici* and

Pediococcus pentosaceus gave very similar results with the API system and hence the pediococci could not be identified on the basis of this result alone. Both of these *Pediococcus* species occur commonly in grass silage (McDonald, 1981) and the definitive separation of these two species is based on growth temperature (Genus *Pediococcus*, Garvie, pp1075 in Bergey's Manual of Systematic Bacteriology, Volume Two, First Edition, 1986): *P. acidilactici* grows at 48°C whereas *P. pentosaceus* does not. The experimental strains did not grow at 48°C and hence were all classified as *P. pentosaceus*.

All of the strains described in this study are capable of both aerobic and anaerobic growth *ie* are facultative anaerobes. This is in agreement with the genera descriptions given in Genus *Lactobacillus*, Kandler & Weiss, pp1209 and Genus *Pediococcus*, Garvie, pp1075 in Bergey's Manual of Systematic Bacteriology, Volume Two, 1986.

3.3.2. DNase ACTIVITY

The genetic manipulation of bacterial cells is dependent on the entry of DNA into the cell and the maintenance of some of the characteristics which it encodes within the cell. This relies on the DNA not being destroyed by a defensive restriction enzyme system or, at least some form of recombination occurring before the destruction of the foreign DNA. Within a transformation system, the production of exogenous DNase activity by a cell can be particularly catastrophic since the foreign DNA would have very little chance of entering the cell. The production of exogenous DNase can be detected by a plate assay using agar containing salmon sperm DNA. Areas on the plate which do not contain DNA after growth and flooding with ethidium bromide show up as zones of clearing under UV light and are indicative of exogenous DNase activity.

None of the experimental strains were positive for exogenous DNase activity under the above test conditions.

3.3.3. AMYLASE & CELLULASE ACTIVITY

Amylase and cellulase production were ear-marked as potential functions to be introduced by genetic manipulation as both of these activities should result in better

substrate utilisation and hence a more efficient silage fermentation. Prior to the introduction of these functions it was necessary to determine whether or not the experimental strains used already possessed such activities. All the experimental strains were tested for amylase and cellulase production using the plate assays described in Chapter Two. Negative reactions were recorded for all the experimental strains for both amylase and cellulase production.

3.4. NATIVE PLASMID PROFILES

3.4.1. *LACTOBACILLUS* STRAINS

Many laboratory and natural strains of LAB have been found to have native plasmids (*eg* Chassy *et al.*, 1976; Klaenhammer & Sutherland, 1980; Vescovo *et al.*, 1981; West & Warner, 1985; Hill & Hill, 1986) and hence it was not unexpected to find that all the *Lactobacillus* strains used in this study also had native plasmids. The possible function of these plasmids was not investigated during this study although it is possible that they play some beneficial role since disadvantageous functions are probably quickly lost in nature. The presence of native plasmids can be a potential problem to genetic manipulation. Some plasmids cannot be maintained within the same bacterial cell and are said to be incompatible. Incompatible plasmids generally have the same replication system and compete with each other during replication (Novick, 1987). Hence, if any potential vector is in the same incompatibility group as one or more of the native plasmids it is unlikely to be maintained and is obviously of limited use. There are, however, several reports where the presence of native plasmids has not been a disadvantage to experimentation (West & Warner, 1985; Shrago & Dobrogosz, 1988).

Several restriction modification systems (*ie* defence systems designed to limit the entry of exogenous DNA into a bacterial cell) are plasmid coded and indeed there is evidence of this within other members of the LAB (*eg* Chopin *et al.*, 1984; Gautier & Chopin, 1987; Langella & Chopin, 1989). Therefore, it is possible that the native plasmids within these strains may also encode a restriction modification system which could be detrimental to

vector DNA.

Both of these problems can be overcome by curing the strains *ie* attempting to remove the native plasmids. This is generally achieved by a variety of methods *eg* heat treatment, ultra-violet light, mutagenic chemicals (*eg* acriflavin & ethidium bromide) all of which are designed to lower the stability of the plasmids and hence select for their absence (Vescovo *et al.*, 1982; Schillinger & Lucke, 1989; Rinckel & Savage, 1990). This will, of course, lead to the loss of the functions encoded by these plasmids and potentially lead to a decrease in the efficiency of the strain. This, in turn, could result in the organism playing a less significant role within a silage fermentation and hence reduce the potential for use as a silage additive. Therefore, no attempt was made to cure any of the experimental strains used.

Another disadvantage of the presence of native plasmids can be the masking of vector plasmids after agarose gel electrophoresis if the two are of similar size. This is a much less important point and can be overcome with care and, if necessary, the use of plasmid-free laboratory strains to confirm the presence of a particular vector or construct.

The native plasmids were all extremely stable; strains were routinely maintained on agar plates and repeated sub-culturing did not lead to a loss of any of the plasmids. This is not unexpected since they are likely to have been maintained over many generations in nature. Examples of long-term stability of native plasmids have been documented for other members of the LAB (Davies *et al.*, 1981; Nes, 1984; von Husby & Nes, 1986; Tannock *et al.*, 1990).

It is possible to give an approximate size measurement of the native plasmids by agarose gel electrophoresis by using covalently closed circular DNA markers of known size. (Different forms of DNA such as linear or covalently closed circular will separate during electrophoresis and hence it is important to run markers which have the appropriate conformation). A standard curve of the distance moved by the markers on a particular gel versus the size of the markers can be created and used to gain an approximate size

measurement for any unknown plasmids. The following size measurements (in kb) were achieved using the above method:

1) *Lactobacillus* strain 85: 8.9, 3.7, 2.0

2) *Lactobacillus* strain Lp: 8.9, 3.7, 2.0

3) *Lactobacillus* strain 48: 8.9, 2.0

4) *Lactobacillus* strain 65: 8.9, 2.0

5) *Lactobacillus* strain 72: 11.5, 7.1, 5.0, 3.7, 1.9

6) *Lactobacillus* strain 71: 11.5

The band running between 12 and 14 kb in all lanes is chromosomal DNA.

Figure 3.1 shows examples of agarose gel electrophoresis of wild-type plasmid DNA from the *Lactobacillus* strains used in this study. Appropriate size measurements of marker plasmids are indicated.

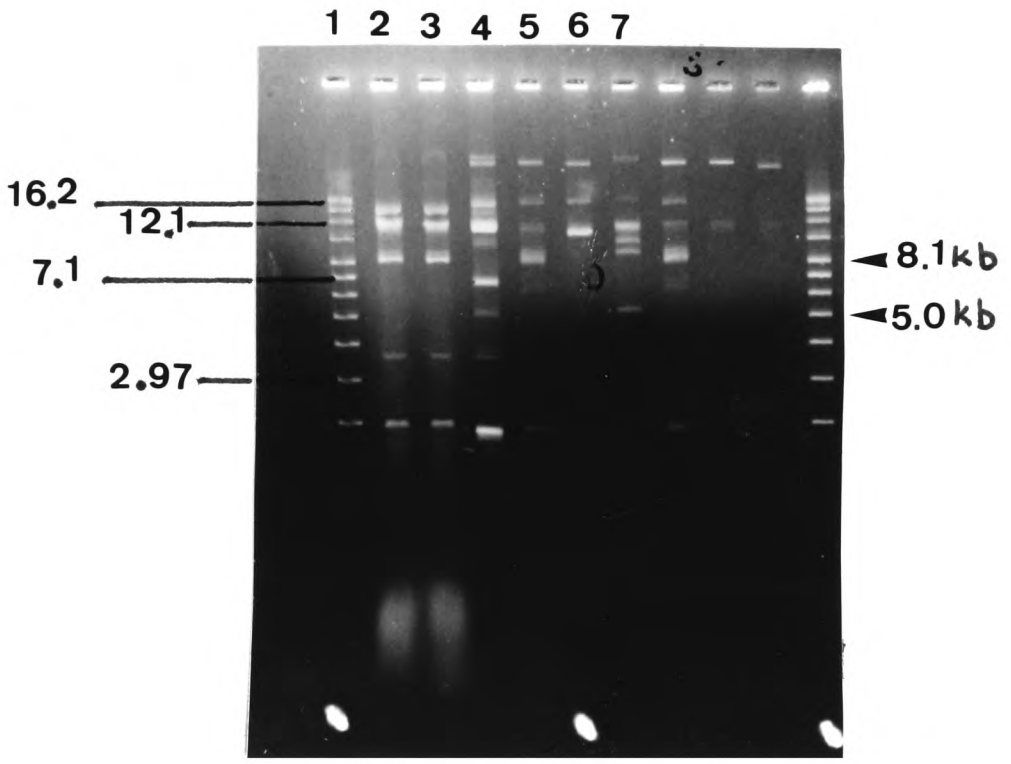
The size analysis indicates that the six *Lactobacillus* strains fall into at least two groups. The first comprises strains 65, 85, 48 and Lp all of which contain two plasmids of the same size (8.9 and 2.0 kb). In addition, a third plasmid band (3.7 kb) is visible with strains 85 and Lp. This band is of much lower intensity than the other plasmid bands and, with some preparations, was not visible at all. This would perhaps indicate a plasmid of lower copy number compared with the others or perhaps a multimeric form of the smallest plasmid. This 3.7 kb band is not visible in either strain 65 or 48; it is possible that it is indeed absent or that the preparation method was simply not sufficiently sensitive to detect it.

The similarity in plasmid profiles and also the very similar reactions with respect to transformation (detailed in Chapter Four) led to the conclusion that strains 85, Lp, 48 and 65 are quite probably repeat isolates of the same strain.

Strains 71 and 72 both have unique plasmid profiles which are distinct from the other lactobacilli although the size of two of the plasmid bands in strain 72 (3.7 and 1.9 kb) is similar to those occurring in the group including strain 85. It is quite possible that these

Figure 3.1. Wild-type (WT) plasmid profiles of *Lactobacillus* strains.

Lane 1, covalently closed circle DNA markers; Lane 2, *Lb* Lp; lane 3, *Lb* 85; lane 4, *Lb* 72; lane 5, *Lb* 65; lane 6, *Lb* 71; lane 7, *Lb* 48.



Sizes (in kb) refer to markers.

similar-sized bands are indeed the same plasmids. By definition, plasmids are capable of replication independently of the chromosome and in some cases also encode genes responsible for the transfer to other cells. Numerous methods of natural DNA transfer *eg* transduction, transposition, conjugation can result in plasmid transfer *in vivo* and bacteria which tend to occur within a common environment *eg* silage, plant surfaces, farm machinery may well undergo natural transfer of genetic material. Hence the occurrence of the same plasmids in apparently different strains is not entirely surprising and indeed has been documented (Nes, 1984; Axelsson *et al.*, 1988; Bringel *et al.*, 1989). These reports propose the possibility of common ancestry for apparently different strains and this could also help to explain the similarity in plasmid profiles of the strains used in this study.

The single band in strain 71 is of a similar size to one of the plasmids visible in strain 72 (11.5 kb) but this similarity is not sufficient to suggest that 71 and 72 are identical isolates.

Five plasmid band sizes are included in the description of strain 72. Two of these bands (5.0 and 3.7 kb) are again of lower intensity than the others and were found to be absent from some preparations. Plasmids with low copy number would be expected to produce such low intensity bands, as would other less frequently occurring molecules such as dimers or other multimers.

It has been proposed here that the similarity of the plasmid profiles of some of these *Lactobacillus* strains can be used as evidence for strain identity. No attempt was made within this study to establish the degree of homology between plasmids from different strains.

Plasmid profiling is one of a number of means which can be used to confirm the identity of a strain or to trace common ancestry between apparently different isolates *eg* Davies *et al.*, 1981; Nes, 1984; Axelsson *et al.*, 1988; Bringel *et al.*, 1990.

3.4.2) *PEDIOCOCCUS* STRAINS

The presence of native plasmids within *Pediococcus* spp. has also been well documented (Gonzalez & Kunka, 1983; Graham & McKay, 1985; Ray *et al.*, 1989). Almost all of the *Pediococcus* strains used in this study had a more limited number of native plasmids than the *Lactobacillus* strains.

Figure 3.2 shows agarose gel electrophoresis of the *Pediococcus* wild-type plasmid DNA with appropriate marker sizes indicated.

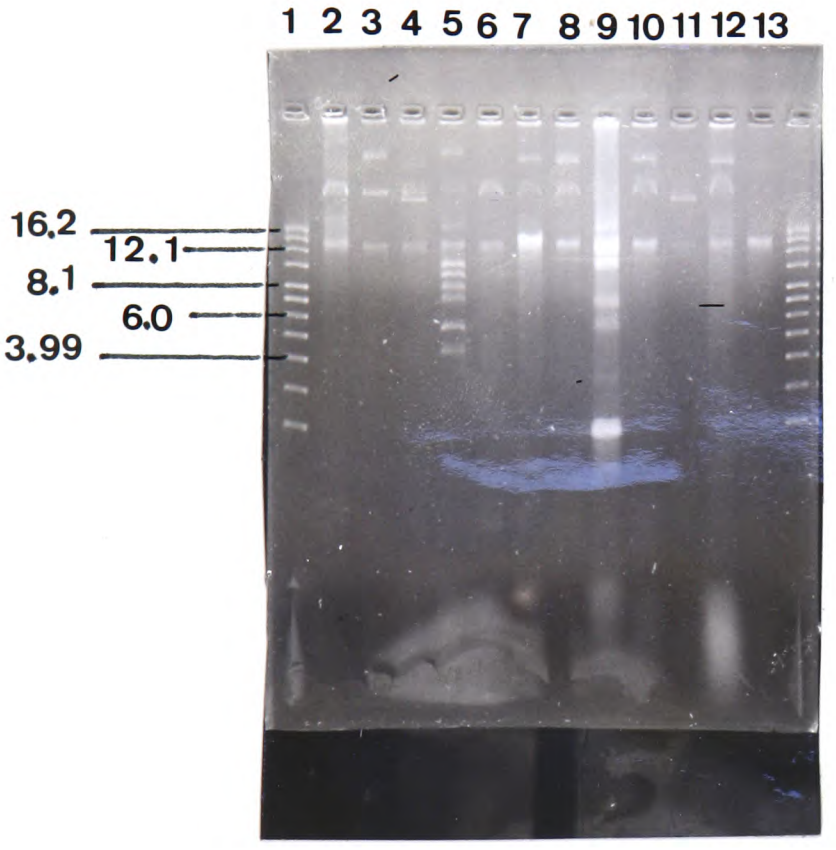
All of the *Pediococcus* strains exhibited a plasmid band at approximately 14kb in size which is probably chromosomal DNA. Two larger plasmid bands are visible within most of the *Pediococcus* strains. As with the lactobacilli, it is quite possible that these are indeed the same plasmids occurring in more than one strain. There is not, however, sufficient evidence to confirm this possibility. Common ancestry has also been proposed for other *Pediococcus* isolates (Hoover *et al.*, 1988; Kayahara *et al.*, 1989).

Pediococcus strain 60 was identified as the only strain with plasmids bands other than the two large plasmids. A further six possible bands were observed, corresponding to sizes of 4.4, 5.6, 7.1, 8.1, 9.1 and 10 kb.

By virtue of their comparative lack of plasmid DNA, the *Pediococcus* strains would seem to be much more suitable recipient strains for plasmid vectors as no masking effect or confusion over the correct identification of particular bands would occur. This potential, however, was unfulfilled because all the *Pediococcus* strains were notably less transformable than the lactobacilli. Full details are given in the following chapter.

Figure 3.2. WT plasmid profiles of *Pediococcus* strains.

Lane 1, covalently closed circle DNA markers; lane 2, *Pd* 1; lane 3, *Pd* 40; lane 4, *Pd* 41; lane 5, *Pd* 60; lane 6, *Pd* 826; lane 7, Lb 48; lane 8, *Pd* 37; lane 9, *Pd* 75; lane 10, *Pd* 79; lane 11, *Pd* 80; lane 12, *Pd* 42; lane 13, *Pd* 77.



Sizes (in kb) refer to markers.

ADDENDUM TO CHAPTER THREE

The method of Hintermann *et al.* (1981) was utilised to distinguish the molecular forms of the native plasmid bands within the experimental strains. This method is based on ultraviolet irradiation between two sets of electrophoresis in perpendicular directions. During the irradiation single-stranded nicks occur within the DNA converting some closed covalently closed circular molecules to the open circular form and the two forms will separate during the second electrophoresis stage making it possible to distinguish between the two forms. Linear and open circular forms will tend to move faster than their equivalent covalently closed circular form.

Figure 3.3 shows the result of two directional electrophoresis on *Lb* 85. Following such analysis the following sizes (in kb) were considered to represent plasmids within the experimental strains:

1) *Lb* 85: 8.9, 3.7, 2.0

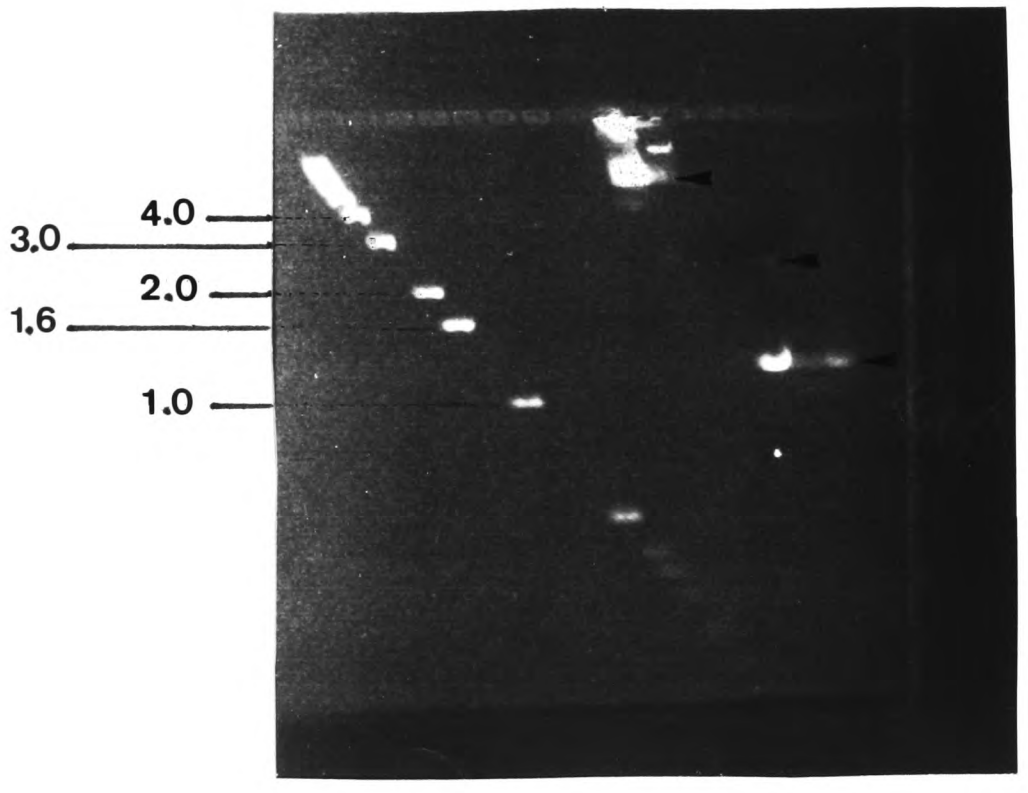
2) *Lb* 72: 11.5, 7.1, 1.9

3) *Lb* 71: 11.5

4) *Pd* 60: 10, 9.1, 5.6

All the other plasmid bands detailed earlier must therefore be considered as different molecular forms of the plasmids listed above. The band common to all of the *Pediococcus* strains (12.9 kb) behaved as a linear fragment and hence was probably a remnant of chromosomal DNA.

Figure 3.3. Two directional electrophoresis on WT *Lb* 85 showing newly formed open circular molecules (arrowed).



Sizes (in kb) refer to markers.

CHAPTER FOUR

OPTIMISATION OF DEVISED ELECTROPORATION PROCEDURE

A) LACTOBACILLI

4.1) EVIDENCE FOR SUCCESSFUL TRANSFORMATION BY ELECTROPORATION

For the purposes of this study, the transformation efficiency (TE) is given as the number of transformants per μg DNA and the transformation frequency (TF) is given as the number of transformants as a proportion of the number of survivors. In most transformation experiments, it is the absolute number of transformants obtained which is most important (transformation being a means to obtain new phenotypic characteristics) and hence this optimisation was based around the determination of parameters which increase the transformation efficiency. The transformation frequency is, however, also important and therefore was also recorded

Prior to any optimisation, attempts were made to transform the *Lactobacillus* strains with pNZ12 from *E. coli* by electroporation using information supplied by the Gene Pulser instruction manual and Chassy & Flickinger (1987). These original conditions were as follows (using the protocol as detailed in Materials & Methods):

Mid exponential-phase cells (3-4 hours growth, OD 600nm 1.2-1.4, corresponding to 10^9-10^{10} cells/ml).

Hepes buffer (recipe as section 2.2.4.1)

Single pulse at 2.5kV, 25μFD

1μg DNA: pNZ12 from *E. coli*

Incubate on ice for 10 minutes

Overnight expression at 30°C in MRS broth (cells diluted 1 in 10 for expression)

* Selection on 20μg/ml chloramphenicol

* The original selection was performed on 10μg/ml chloramphenicol but this led to a slight background lawn appearing on the plates. When replated out on to other 10μg/ml chloramphenicol plates, this lawn failed to grow. This suggests that the lawn was result of the total cell population growing for a few generations before becoming inhibited by the chloramphenicol. Increasing the selection level to 20μg/ml chloramphenicol eliminated this background lawn and hence removed any possibility of it "masking" true transformants.

Transformation was successful, giving the following results for *Lactobacillus* (*Lb*) strain 85:

TE: 1.2

TF: $1.5x10^{-9}$

DNA used was pNZ12 with selection on 20μg/ml. NB In most cases the spontaneous mutation rate at the given selection was nil. When any spontaneous mutants were recorded, the level was subtracted from the experimental TF value to give the true TF which is the value stated in all cases.

Control experiments involving no DNA and cells incubated with DNA but not pulsed, gave negative results suggesting that the colonies selected were indeed true transformants.

Transformation of *Lb* strain 85 was also possible using total plasmid DNA from transformants of 85 (*ie* using a mixture of DNA containing strain 85's own plasmids as well as pNZ12; plasmid DNA preparation method as in section 2.5.1).

Using 1μg DNA from 85 containing pNZ12:

TE: 2.2

TF: 2.9×10^{-9}

As no transformation resulted from wild-type (WT) DNA from strain 85, this result was taken as further proof of the authenticity of the transformants.

It is generally considered that DNA from the host organism will give more efficient transformation than DNA from a different organism. This is perhaps not too surprising since exogenous DNA from the same host is less likely to be damaged by restriction modification than DNA derived from a clearly different host.

There is no statistically significant difference between the results for *E. coli* DNA and the results for *Lactobacillus* DNA within the system detailed here.

Transformation levels obtained under the same conditions using the other *Lactobacillus* strains are shown in Table 4.1.

Table 4.1. Transformation of *Lactobacillus* strains with pNZ12 from *Lb* 85.

Strain	TE	TF
Lp	10	9.5×10^{-9}
71	0.8	1.5×10^{-9}
72	7	8×10^{-9}

Following agarose gel electrophoresis of plasmid DNA from these transformants, it was possible to see a plasmid band corresponding to pNZ12, as shown in Figure 4.1.

The total plasmid DNA containing pNZ12 from each of the transformed *Lactobacillus* strains was used to transform *E. coli* MC1022. Mini-preps. of plasmid DNA from the *E. coli* transformants were then prepared and digested with restriction enzymes. As shown in Figure 4.2 the restriction fragments generated by this treatment corresponded with the

fragments from the control (*E. coli*) DNA. This was final confirmation that transformation of *Lactobacillus* strains with pNZ12 by electroporation had occurred even though the TE and TF values obtained were very low.

Figure 4.1. Evidence of successful transformation of *Lactobacillus* strains with pNZ12. Lane 1, λ*Hind*III markers; lane 2, *Lb* 65 pNZ12; lane 3, *Lb* 65 WT; lane 4, *Lb* 85 pNZ12; lane 5, *Lb* 85 WT; lane 6, *Lb* 71 pNZ12; lane 7, *Lb* 71 WT; lane 8, pNZ12 from *E. coli.* Sizes refer to λ*Hind*III markers. A band corresponding to pNZ12 (4.3kb) is evident within the proposed transformant preparations (lanes 2, 4 & 6) but absent from the WT preparations.

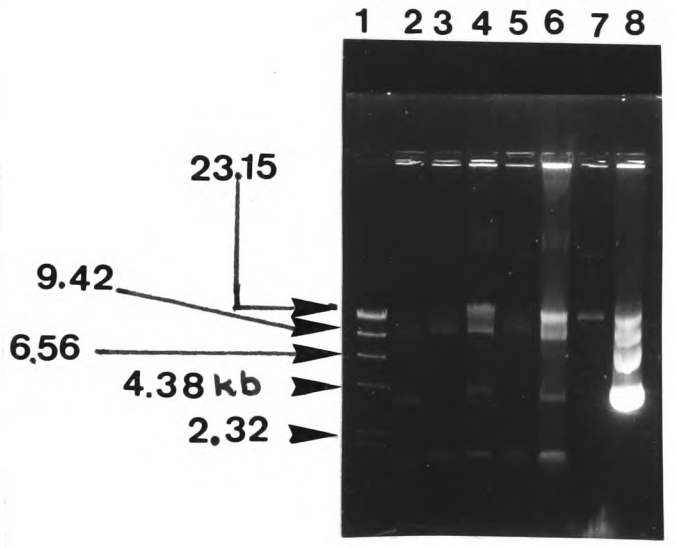
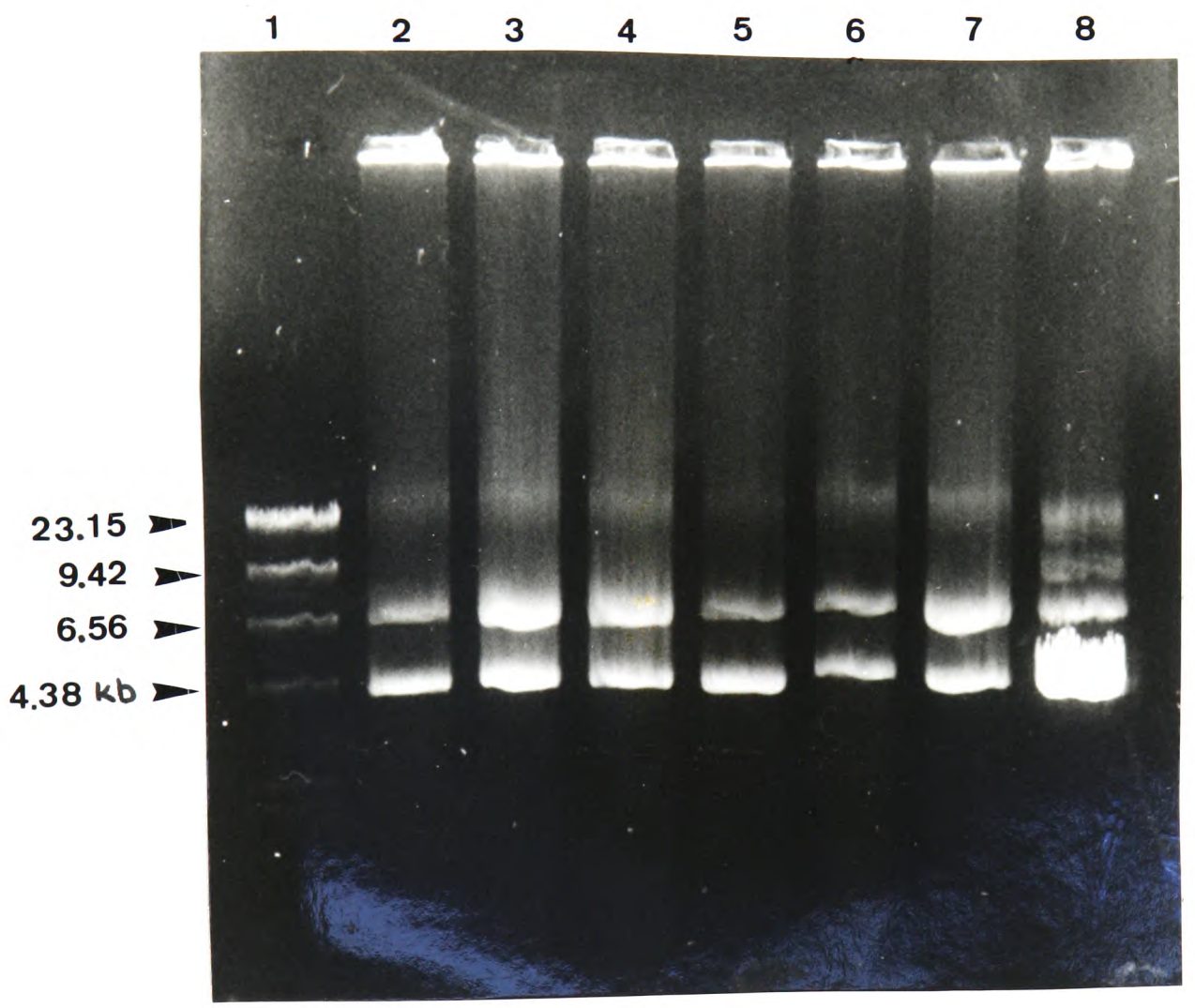


Figure 4.2. Restriction enzyme (*Taq*I) analysis of pNZ12 originally isolated from *Lactobacillus* strains.

Lane 1, λ*Hind*III markers; lanes 2 & 3, *Lb* 65 pNZ12; lanes 4 & 5, *Lb* 71 pNZ12; lane 6, *Lb* 85 pNZ12; lane 7, *Lb* Lp pNZ12; lane 8, pNZ12 from *E. coli*.

Sizes refer to λ*Hind*III markers. The top band in all lanes is probably uncut plasmid DNA. Although pNZ12 has two *Taq*I sites, the smaller band is likely to be too small to show up on a gel of this type.



NOTES ON ELECTROPORATION

The Biorad Gene Pulser (the electroporation apparatus used throughout the work presented in this thesis) is not the only electroporation apparatus on the market although most of the others also have an exponential decay pulse. Early papers on electroporation of bacteria, such as Dower *et al.* (1988), established that transformation is dependent on two major parameters, the field strength and the pulse length. Hence procedural variations which alter these may be expected to have the most obvious effect on transformation levels. In the majority cases, the frequency of transformation is a linear function of the DNA concentration and the efficiency of transformation is a function of cell concentration. All of the results presented in this chapter comply with these findings and this will be fully discussed at the end of the chapter.

4.2) OPTIMISATION RESULTS

Although it was possible to transform the lactobacillus strains under the initial conditions (as shown in section 4.1), the TE thus obtained was very low and therefore an optimisation procedure was instigated.

Unless otherwise stated, all the procedures which follow were based on the conditions described in the previous section with only one experimental variable under investigation at a time. The DNA used in most cases (differences will be noted) was 1μg *Lb* 85 pNZ12 *ie* total plasmid DNA containing pNZ12. All the results reported here are based on more than one experiment. The experiments are listed sequentially *ie* any conditions changed in a given section can be taken as standard in all subsequent experiments (unless otherwise stated).

Most experiments were performed using more than one *Lactobacillus* strain. In some cases, to aid comparison between results, relative values compared with a baseline (control) value are given beside the experimental figures.

Colony counts were taken from an average of two agar plates. Horizontal lines within the tables mark divisions between experiments.

4.2.1) Electroporation Buffers

4.2.1.a) Hepes buffer v. phosphate buffer

The buffer used for electroporation can have a number of important effects on transformation:

1) The resistance of the medium has an effect on the time constant (more resistant buffers give longer time constants).

2) The buffer must act as an osmotic stabiliser in the event of membrane damage.

3) Buffers with high ionic strengths can lead to an increase in the incidence of arcing.

All initial experiments (section 4.1) were carried out using hepes buffer (Chassy & Flickinger, 1987) which was originally used for electroporation of *Lactobacillus casei*. Using later information (Powell *et al.*, 1988) on work carried out on *Lactococcus lactis* subsp. *lactis*, a comparison was made between hepes and phosphate buffers. NB All electroporation buffers were made on day of use and stored at 4°C. Water used was double glass distilled and hence had low conductivity.

The results are shown in Table 4.2.a.

Table 4.2.a. Comparison between hepes and phosphate electroporation buffers.

STRAIN	BUFFER	TE actual	TE relative	TF
85	H	0.10	1.0	3.9×10^{-9}
85	P	0.80	8.0	7.5×10^{-9}
85	H	0.02	1.0	6.9×10^{-9}
85	P	0.20	10.0	5.8×10^{-8}
Lp	H	0.01	1.0	2.1×10^{-10}
Lp	P	0.08	13.3	6.9×10^{-8}
Lp	H	0.01	1.0	6.9×10^{-10}
Lp	P	0.10	12.5	1.5×10^{-10}

H= hepes buffer

P= phosphate buffer (recipes in section 2.2.4)

Single pulse at 2.5kV, 25μFD for 800μl cells in 0.4ml cuvette. Baseline value taken for hepes buffer.

When phosphate buffer was used there was approximately a ten-fold increase in the number of transformants compared with the results using hepes buffer. Phosphate buffer was concluded to be a better electroporation buffer than hepes under these conditions and was used in subsequent experiments.

4.2.1.b) Phosphate buffer v. Sucrose"buffer"*

During further optimisation experiments using phosphate buffer and the pulse controller (see 4.2.2), arcing caused severe problems and the loss of many experiments. Since no other parameter seemed to be consistently linked to arcing, it was concluded that the

problem was the conductivity of the buffer. In an attempt to limit arcing, a series of experiments was set up to investigate the role of each of the components of the phosphate buffer. The results are shown in Table 4.2.b.

Table 4.2.b. Comparison of the effect of the constituents of phosphate buffer on transformation.

STRAIN	BUFFER	TE actual	TE relative	TF
85	1	0.9	1.0	$6.1x10^{-9}$
85	2	1.5	1.7	$6.5x10^{-8}$
85	3	11.4	12.7	$9.0x10^{-7}$
85	4	15.0	16.9	$5.4x10^{-7}$
85	1	0.07	1.0	$3.7x10^{-9}$
85	2	0.08	1.1	$3.7x10^{-9}$
85	3	10.1	144	$4.2x10^{-8}$
85	4	16.6	237.0	$8.8x10^{-7}$
85	3	6.5	--	$5.5x10^{-9}$
85	4	23.0	--	$3.6x10^{-8}$
85	3	3.1	--	$4.8x10^{-8}$
85	4	21.9	--	$3.2x10^{-8}$
Lp	2	0.4	--	$4.5x10^{-8}$
Lp	3	2.6	--	$3.4x10^{-7}$
Lp	3	3.1	--	$8.5x10^{-7}$
Lp	4	6.3	--	$6.8x10^{-7}$

From recipe in section 2.2.4.2, buffer constituents as follows: Buffer 1 = sucrose, 10x phosphate solution, $MgCl_2$ and water; Buffer 2 = sucrose, 10x phosphate solution and water; Buffer 3 = sucrose, $MgCl_2$ and water; Buffer 4 = sucrose and water. Single pulse at 2.5kV, 25μFD for 800μl cells in 0.4cm cuvette. Baseline taken with buffer 1 when this was included.

All the variations of phosphate buffer that excluded one or both of the ionic constituents

(phosphate, $MgCl_2$) gave better transformation levels than complete phosphate buffer. Removal of the phosphate ions (buffer three) led to more than a ten-fold increase in the number of transformants. Removal of all the ions (buffer four) resulted in a further, though less marked, increase. Hence, sucrose solution, consisting simply of 0.5M sucrose, was substituted as the routine electroporation" buffer".

* As the definition of a true buffer requires a solution to be ionic, 0.5M sucrose does not actually qualify for the term. In order to avoid confusion, however, the description sucrose buffer will be retained where it refers to the solution used to resuspend the cells prior to electroporation.

4.2.2) Use of the Pulse Controller

The pulse controller is an additional unit designed for use with the gene pulser and provides further control over the pulse length by introducing resistors in parallel with the sample chamber. The higher the resistance value, the longer the time constant. The pulse controller allows a choice of six resistors from 100 to 1000 ohms.

The introduction of this piece of equipment coincided with the arrival of a set of new cuvettes for the gene pulser. These cuvettes had a smaller inter-electrode distance than the original ones, 0.2cm compared with 0.4cm. This allowed a greater variation in field strengths (V/cm) to be achieved; using the maximum voltage in combination with the new cuvettes results in a doubling of the field strength from 6.25 kV/cm to 12.5 kV/cm. The new cuvettes were also designed for use with a much smaller volume of cell suspension; 40µl to 400µl compared with 800µl in the original cuvettes. This smaller volume increased the possibility of arcing and these cuvettes were not used unless the pulse controller was connected. Arcing can seriously damage the gene pulser and the pulse controller can act as useful protection in this event.

The results from a comparison of transformation levels obtained with and without the use of the pulse controller are shown in Table 4.3.

Table 4.3. Comparison of transformation with and without the Pulse Controller.

STRAIN	CONDITIONS	TE actual	TF	% SURVIVAL
85	1	2	$3.0x10^{-10}$	100
85	2	28	$1.1x10^{-7}$	17
Lp	1	3	$6.9x10^{-10}$	100
Lp	2	17	$3.6x10^{-8}$	7
85	1	2	$6.3x10^{-10}$	100
85	2	33	$1.4x10^{-7}$	37
Lp	1	2	$4.9x10^{-10}$	100
Lp	2	33	$3.4x10^{-8}$	4

Conditions: 1= 800µl cell suspension (0.4cm cuvette) with pulse controller

2= 800µl (0.4cm cuvette) without pulse controller

Percentage survival measured immediately after the pulse.

Voltage=2.5kV, capacitance=25µFD, pulse controller at 100ohms, cells resuspended in sucrose buffer.

When the pulse controller was used, the efficiency of transformation was at least ten-fold lower. The TF was also considerably reduced (over 100-fold). From this experiment alone no benefit from the inclusion of the pulse controller was obvious.

The use of the pulse controller resulted in much reduced time constants, from 20ms to 2ms. It is possible that this shorter pulse caused less cell damage to the same volume of cells and, as a consequence of this, a lower TE (this is assuming that some degree of cell damage precedes transformation). This hypothesis was confirmed by the percentage survival after the pulse as shown in Table 4.3 (above).

4.2.3) Use of the 0.2cm cuvettes

The use of the 0.2cm inter-electrode distance cuvettes doubles the field strength and when the pulse controller (set at 100 ohms) was used with the 0.2cm inter-electrode distance cuvettes containing 100µl cell suspension, both the TE and TF were increased at least ten-fold to levels similar to those achieved without the pulse controller using 0.4cm cuvettes (Table 4.3).

Table 4.4. Effect of inter-electrode distance (and hence field strength) on transformation.

STRAIN	CONDITIONS	TE actual	TF	% SURVIVAL
85	1	203.0	$7.8x10^{-7}$	31
85	2	2.0	$3.0x10^{-10}$	100
Lp	1	94.0	$9.4x10^{-7}$	30
Lp	2	3.0	$6.8x10^{-10}$	100
85	1	72.0	$1.6x10^{-7}$	59
85	2	1.5	$6.3x10^{-10}$	100
Lp	1	122.0	$2.7x10^{-7}$	8
Lp	2	2.0	$4.9x10^{-10}$	100

Conditions: 1= 100µl cell suspension in 0.2cm cuvette with pulse controller

2= 800µl in 0.4cm cuvette with pulse controller

Voltage=2.5kV, capacitance=25µFD, pulse controller at 100ohms, with field strengths of 12.5kV/cm and 6.25kV/cm corresponding to conditions 1 and 2 respectively. Cells resuspended in sucrose buffer. Percentage survival measured immediately after pulse.

The TE with these strains of *L. plantarum* is probably related to the amount of cell damage. When the pulse controller was used with 0.4 and 0.2 cm cuvettes, the length of the pulse was approximately 2ms in both cases but with the smaller volume, it is possible that a greater proportion of the cells were damaged and hence a greater number were

potentially transformable.

The level of survival after the pulse for each of these conditions, shown in Table 4.4, confirms that this was probably the case.

For practical reasons, the smaller cuvettes and hence the pulse controller, were used to complete studies on the optimisation of conditions for electroporation. Using a volume of 100µl cell suspension reduced the volume that had to be prepared and it was also possible to make a one-in-ten dilution for expression within an Eppendorf tube thus removing the need for larger, bulkier vessels.

Most of the relevant published data which involves the use of the 0.2cm cuvettes and pulse controller are presented in terms of achieving a range of pulse lengths (see section 4.2.5) rather than a comparison between the small and large cuvettes.

4.2.4) Length of expression time

The original, unoptimised electroporation conditions (section 4.1) involved an expression time of 24 hours. This extended period was used in an attempt to achieve isolation of the maximum number of transformants from what was originally a rather inefficient process. Such a long expression time, however, allows the total number of cells to increase and so the number of transformants will increase accordingly. The true TE is likely to be less than that obtained from 24 hours.

Table 4.5 shows the results of a comparison of expression times of two and 24 hours.

Table 4.5. Effect of the length of expression incubation on transformation by electroporation.

STRAIN	EXPR. TIME	TE actual	TE relative	TF
85	24	8300	45	$4.3x10^{-6}$
85	2	183	1	$1.0x10^{-6}$
Lp	24	5300	48	$5.8x10^{-6}$
Lp	2	111	1	$4.8x10^{-7}$
85	24	3780	47	$9.4x10^{-7}$
85	2	80	1	$1.6x10^{-7}$
Lp	24	4680	77	$1.2x10^{-6}$
Lp	2	61	1	$2.8x10^{-7}$

Expression time given in hours. Single pulse at 2.5kV, 25μFD, 100ohms for 100μl cells resuspended in sucrose buffer in 0.2cm cuvette. Baseline value taken as 2 hour expression time.

The TE after 24 hours is at least ten-fold higher than at two hours. This corresponds to an increase in total cell number during the same period as indicated in Table 4.6.

Table 4.6. Viable counts (VC) of cell suspensions at different expression times.

STRAIN	EXPR.TIME	VC after pulse	VC after expression
85	24	$1.2x10^9$	$1.9x10^{10}$
85	2	$1.2x10^9$	$1.8x10^9$
Lp	24	$1.5x10^9$	$9.2x10^9$
Lp	2	$2.7x10^9$	$2.3x10^9$
85	24	$1.7x10^9$	$4.0x10^{10}$
85	2	$2.7x10^9$	$4.9x10^9$
Lp	24	$1.6x10^9$	$4.0x10^{10}$
Lp	2	$2.1x10^9$	$2.2x10^9$

Viable count expressed in cfu/ml. Expression time given in hours. Single pulse of 2.5kV, 25μFD, 100 ohms for 100μl cells in sucrose buffer in 0.2cm cuvette.

None of the values at two hour expression showed a significant increase in the total cell population (using chi^2 test) whereas all of the values at 24 hours were significantly increased. Hence the number of transformants can also be expected to be significantly increased and for the continuation of the optimisation, a two hour expression time was introduced as standard.

4.2.5) Varying parallel resistors

The pulse controller allowed one of six resistors to be placed in parallel with the sample chamber viz 100, 200, 400, 600, 800, 1000 ohms (within the gene pulser itself a 20 ohm resistor is in series with the sample). The greater the resistance, the longer the pulse length and a longer pulse may be expected to cause more damage to cells.

A comparison of different resistances at constant voltage and capacitance was set up.

Table 4.7. Effect of resistance (ohms) on transformation by electroporation.

STRAIN	RESISTANCE	TE	TF	TC	% SURVIVAL
85	100	151	$6.4x10^{-8}$	2.3	ND
85	200	3	$2.2x10^{-9}$	4.5	ND
85	400	0	-	7.9	ND
85	100	395	$6.8x10^{-7}$	2.3	ND
85	200	95	$9.1x10^{-7}$	4.5	ND
85	400	6	$8.7x10^{-9}$	7.9	ND
65	100	98	$3.5x10^{-7}$	2.3	21
65	200	28	$9.3x10^{-7}$	4.5	8
65	400	8	$2.0x10^{-7}$	7.9	3
65	100	33	$2.3x10^{-7}$	2.3	36
65	200	19	$2.1x10^{-7}$	4.5	31
65	400	4	$4.1x10^{-8}$	7.9	24
72	100	13	$6.5x10^{-7}$	2.3	11
72	200	4	$1.8x10^{-7}$	4.5	3
72	400	0	-	7.9	0
72	100	6	$6.0x10^{-8}$	2.3	67
72	200	4	$2.2x10^{-8}$	4.5	31
72	400	0	-	7.9	14

ND = not done. TC = time constant in ms. % survival levels measured immediately after pulse. Values given are for 100 µl cell suspensions in sucrose buffer, with single pulse of 2.5kV, 25µFD.

With a parallel resistor value of 600 ohms, or above, arcing occurred routinely and such samples were discarded.

The results of these experiments (Table 4.7) indicated that a resistance of 100 ohms gave an optimal TE under these conditions, so this value was used as standard. The overall

general trend of the TF was also to decrease as resistance increased.

It is probable that varying the field strength will result in a different optimum time constant. If the hypothesis of damage being related to transformation potential is correct then it might be expected that at a lower field strength, a longer time constant would be necessary to achieve optimum transformation. The results shown in Table 4.8 concur with this hypothesis.

Table 4.8. Correlation between field strength and time constant during electroporation.

STRAIN	FIELD STRENGTH	TE	TF	TC
Lp	12.5	260	$1.1x10^{-6}$	2.3
Lp	10	313	$2.6x10^{-6}$	4.1
85	12.5	620	$3.3x10^{-6}$	2.3
85	10	670	$1.9x10^{-6}$	4.1
Lp	12.5	50	$1.8x10^{-7}$	2.3
Lp	10	70	$1.6x10^{-7}$	4.1

Field strength in kV/cm, where 12.5kV/cm = 2.5kV and 10kV/cm = 2.0kV. Time constant in ms. Single pulse at 25μFD and 100ohms for 100μl cells in 0.2cm cuvette.

For the completion of the optimisation studies 2.5kV, 25μFD and 100 ohms were taken as the standard conditions.

4.2.6) Varying voltage at constant resistance

Keeping the parallel resistance constant at 100 ohms, a comparison of the levels of transformation at various field strengths was conducted. Results are shown in Table 4.9.

Table 4.9. Transformation levels obtained at varying field strengths (FS).

STRAIN	VOLTAGE	TE	TF	FS	% SURVIVAL
85	1.5	127	$1.2x10^{-7}$	7.5	-
85	2.0	253	$6.0x10^{-7}$	10.0	-
85	2.5	164	$2.3x10^{-6}$	12.5	-
85	1.5	43	$4.1x10^{-8}$	7.5	69
85	2.0	68	$3.4x10^{-7}$	10.0	25
85	2.5	95	$3.4x10^{-7}$	12.5	10
Lp	1.5	70	$8.3x10^{-8}$	7.5	-
Lp	2.0	125	$6.3x10^{-7}$	10.0	-
Lp	2.5	138	$1.4x10^{-6}$	12.5	-
65	1.5	5	$4.1x10^{-9}$	7.5	86
65	2.0	30	$6.5x10^{-8}$	10.0	54
65	2.5	25	$4.8x10^{-8}$	12.5	38
65	1.5	4	$5.6x10^{-9}$	7.5	42
65	2.0	9	$3.0x10^{-8}$	10.0	22
65	2.5	12	$9.2x10^{-8}$	12.5	9
72	1.5	12	$1.8x10^{-8}$	7.5	25
72	2.0	11	$8.5x10^{-8}$	10.0	20
72	2.5	33	$6.6x10^{-7}$	12.5	12
72	1.5	2	$3.8x10^{-9}$	7.5	12
72	2.0	0	-	10.0	20
72	2.5	4	$7.0x10^{-8}$	12.5	10

Voltage expressed in kV, field strength in kV/cm. Single pulse at 25μFD, 100 ohms for 100μl cells in sucrose buffer in 0.2cm cuvette. Percentage survival measured immediately after the pulse.

In almost all cases, the highest TE and TF occurred at the highest field strength used *ie* 12.5kV/cm. This value was thus selected as the optimum value for transformation.

Table 4.10 also shows the percentage survival levels after the pulse at each field strength. In all cases the greatest cell death occurs at the highest field strength, a further indication that, with this system, the amount of cell damage caused by the pulse seems to be related to the number of transformants gained.

4.2.7) DNA concentration

The effect of increasing DNA concentration with this experimental system is shown in Table 4.10 and Figure 4.3.

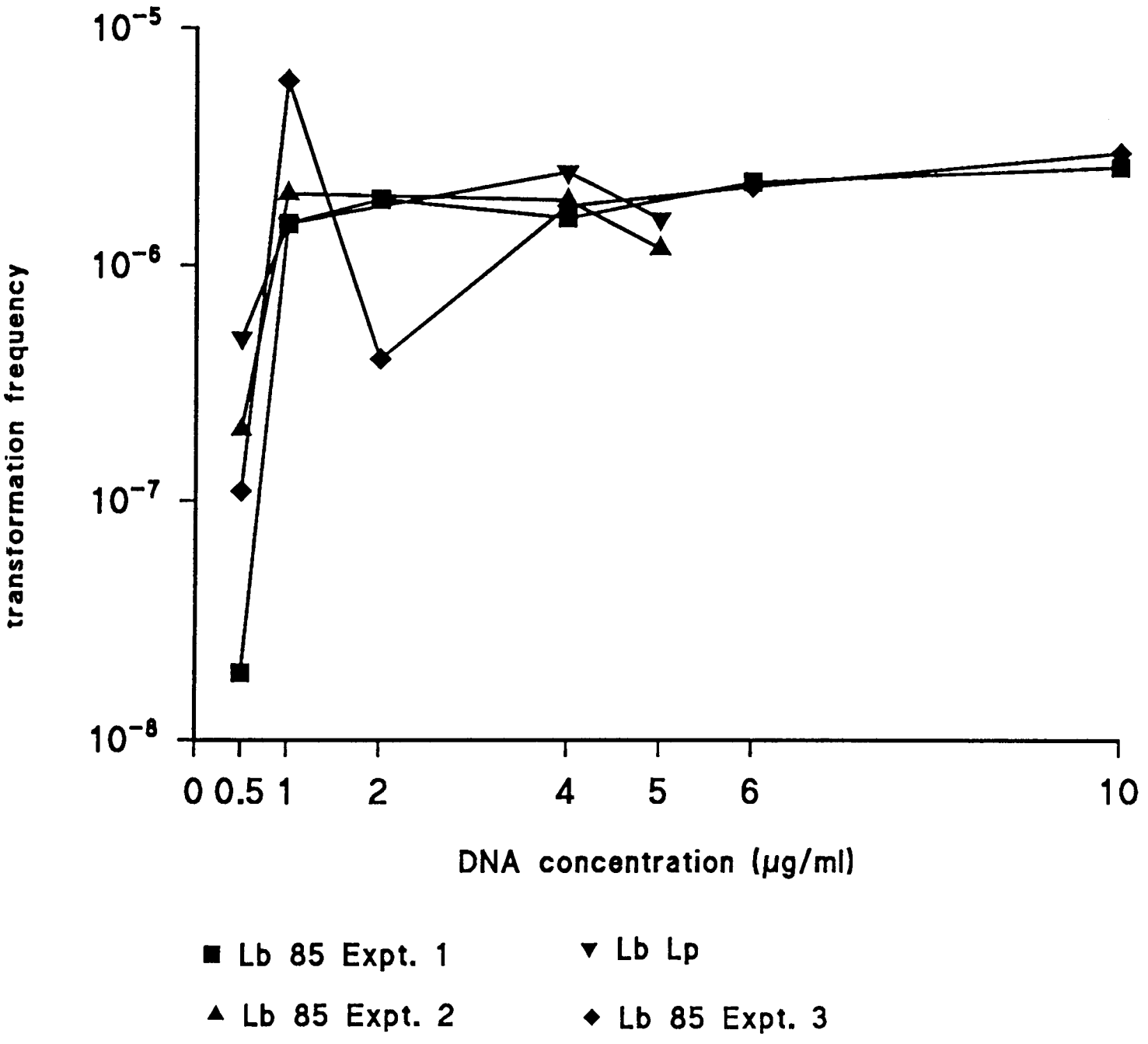
Table 4.10. Effect of DNA concentration on transformation by electroporation.

STRAIN	µg DNA	TE actual	TE relative	TF
85	0.5	38	0.30	$1.9x10^{-8}$
85	1.0	118	1	$1.5x10^{-6}$
85	2.0	47	0.40	$1.9x10^{-6}$
85	4.0	32	0.30	$1.6x10^{-6}$
85	6.0	30	0.25	$2.3x10^{-6}$
85	10.0	16	0.10	$2.7x10^{-6}$
85	0.5	179	0.20	$2.0x10^{-7}$
85	1.0	826	1.0	$2.0x10^{-6}$
85	4.0	186	0.2	$1.9x10^{-6}$
85	5.0	121	0.15	$1.2x10^{-6}$
85	0.5	24	0.5	$1.1x10^{-7}$
85	1.0	48	1.0	$6.0x10^{-6}$
85	2.0	26	0.5	$4.0x10^{-7}$
85	4.0	18	0.4	$1.8x10^{-6}$
85	6.0	32	0.7	$2.2x10^{-6}$
85	10	21	0.44	$3.1x10^{-6}$
85	13	23	-	$4.0x10^{-7}$
85	32.5	6	-	$2.9x10^{-7}$
Lp	13	17	-	$3.0x10^{-7}$
Lp	32.5	6	-	$1.8x10^{-7}$
Lp	0.5	502	0.6	$4.9x10^{-7}$
Lp	1.0	804	1.0	$1.5x10^{-6}$
Lp	4.0	277	0.3	$2.5x10^{-6}$
Lp	5.0	152	0.2	$1.6x10^{-6}$

Single pulse at 2.5kV, 25µFD, 100 ohms for 100µl cells in sucrose buffer in 0.2cm cuvette. Baseline taken as 1µg DNA. Cell concentration 10^9-10^{10} cells/ml.

As the saturation level was recorded as 1µg DNA, this level was retained as the working concentration.

Figure 4.3 Effect of increasing DNA concentration on transformation frequency



4.2.8) Pre- and post-pulse incubation on ice.

An investigation into the effects of a post-pulse incubation in this system was set up and the results are shown in Table 4.11. It should be pointed out that until this point in the optimisation, a ten minute post-pulse incubation had been standard.

Table 4.11. The effect of post-pulse incubation on ice on transformation.

STRAIN	CONDITIONS	TE actual	TE relative	TF
72	+	26	1.0	$3.9x10^{-8}$
72	-	165	6.3	$4.6x10^{-7}$
65	+	297	1.0	$4.6x10^{-7}$
65	-	313	1.1	$4.3x10^{-7}$
Lp	+	27	1.0	$3.0x10^{-7}$
Lp	-	190	7.0	$1.2x10^{-6}$
72	+	14	1.0	$7.4x10^{-8}$
72	-	103	7.4	$1.0x10^{-7}$
65	+	35	1.0	$4.4x10^{-7}$
65	-	73	2.1	$3.0x10^{-7}$
85	+	114	1.0	$6.3x10^{-7}$
85	-	216	1.9	$1.0x10^{-6}$
85	+	98	1.0	$8.9x10^{-7}$
85	-	143	1.5	$2.9x10^{-6}$

Conditions: + = with 10 minute post-pulse incubation on ice

- = without post-pulse incubation

Single pulse at 2.5kV, 25μFD, 100ohms for 100μl cells in sucrose buffer in 0.2cm cuvette. Baseline taken with post-pulse incubation.

A consistent increase in the TE occurred by omitting the post-pulse incubation although the size of the increase appeared to be strain dependent. The TF was also increased by this procedure. Although the increases did not involve large differences, in cloning procedures the isolation of even a very small number of transformants is vital and the inclusion of a post-pulse incubation could lower the frequency below detectable levels. As a result of this comparison, all samples from this point were routinely diluted into the expression medium immediately after the pulse.

4.2.9) Freezing of cell culture prior to pulse.

As is evident from the results listed above, there was considerable day-to-day variability in survivor and transformant counts between experiments. Despite growing the cells to approximately the same optical density each time, the exact number of cells will obviously differ and this will effect the level of transformation. Slight, unavoidable differences in routine sample preparation will also lead to variation in results. To overcome some of these problems, it might have been beneficial to prepare a large batch of cells and freeze them for later use.

The effect of freezing on the *Lactobacillus* strains in this study are shown in Table 4.12. Samples were grown to mid-log phase, washed in electroporation buffer, then resuspended in 10% glycerol: 90% sucrose buffer prior to pulse. Samples for freezing were stored at -80°C.

Table 4.12. The effect of freezing of the cell culture prior to transformation by electroporation.

STRAIN	CONDITIONS	TE	TF	VIABLE COUNT*
Lp	0	14	$1.5x10^{-7}$	$1.3x10^{10}$
Lp	72	4	$8.0x10^{-9}$	$1.6x10^{10}$
Lp	96	3	$5.8x10^{-9}$	$1.8x10^{10}$
85	0	21	$3.0x10^{-7}$	$1.6x10^{10}$
85	72	1	$3.7x10^{-9}$	$1.7x10^{10}$
85	96	0.5	$6.7x10^{-10}$	$1.8x10^{10}$

Conditions: Hours frozen at -80°C in 10% glycerol: 90% sucrose buffer.

* Viable count measured on thawing and prior to pulse. Single pulse at 2.5kV, 25μFD, 100ohms for 100μl cells in 10% glycerol: 90% sucrose buffer in 0.2cm cuvette.

It is evident that freezing of cells under the conditions used here resulted in a significant reduction in transformation and hence was not a suitable method to employ to overcome day-to-day variation.

The observed reduction in transformation efficiency was not related to a loss of viability caused by freezing (Table 4.13). No statistically significant reduction in total cell number occurred.

4.2.10) Use of capacitance extender.

All the optimisation experiments were performed at 25 μFD, the highest choice of capacitance within the gene pulser. The capacitance extender (an additional unit) allowed the choice of four higher capacitance values (125, 250, 500, 960 μFD). The voltage, however, was limited to a maximum of 0.45 kV when the extender was in use. The capacitance extender resulted in longer time constants at given resistance and voltage values.

No transformants were recorded after the use of extender at any of the higher capacitance

levels.

4.2.11) Growth phase of cells used for electroporation.

Experiments performed during the present study failed to give transformants using stationary phase cells under conditions where the use of mid-log phase cells resulted in successful transformation (results not shown).

4.2.12) Osmotic stabilisation during electroporation.

The results of the addition of 0.5M sucrose to MRS at all points in the electroporation procedure (growth, expression, selection) compared to the use of normal, unsupplemented MRS are presented in Table 4.13.

Table 4.13. The effect of osmotic stabilisation with sucrose during transformation.

STRAIN	CONDITIONS	TE actual	TE relative	TF
85	-	431	1.0	$1.7x10^{-6}$
85	+	183	0.4	$8.3x10^{-7}$
Lp	-	503	1.0	$2.8x10^{-6}$
Lp	+	385	0.8	$1.2x10^{-7}$
65	-	49	1.0	$3.0x10^{-7}$
65	+	59	1.2	$9.8x10^{-7}$
85	-	209	1.0	$2.0x10^{-6}$
85	+	64	0.3	$2.3x10^{-7}$
Lp	-	108	1.0	$6.3x10^{-7}$
Lp	+	82	0.8	$3.4x10^{-7}$
72	-	2	1.0	$1.2x10^{-8}$
72	+	6	3.0	$2.0x10^{-8}$

Conditions: + = with 0.5M sucrose in growth medium (MRS broth)

- = without 0.5M sucrose in growth medium (MRS broth)

Baseline taken without sucrose in growth medium with single pulse of 2.5kV, 25μFD, 100ohms for 100μl cells in sucrose buffer in 0.2cm cuvette.

The requirement for sucrose appears to be strain dependent with two of the four strains tested showing an increase in transformation efficiency and frequency due to osmotic stabilisation. (Only the result for strain 72 is statistically significant).

4.2.13) Use of cell wall weakening agents.

The mechanism of electroporation is believed to involve formation of transient pores in the cell membrane. The membrane of bacterial cells is protected by a cell wall, one of the

main components of which is peptidoglycan. It has been proposed that the use of agents which weaken this cell wall will increase transformation frequency by helping to overcome the physical barrier presented by this wall (Mercenier & Chassy, 1988). Cultures grown in the presence of cell wall weakening agents do not grow to the same cell density as normal cultures and hence require to be resuspended in a smaller volume of buffer prior to electroporation in order to compensate for this and lead to comparable cell numbers.

4.2.13.a) DL-Threonine

The results of experiments using MRS broth supplemented with 40mM DL-threonine are presented in Table 4.14.

Table 4.14. Effect of the addition of DL-threonine on transformation.

STRAIN	CONDITIONS	TE actual	TE relative	TF
85	-	62	1.0	$3.3x10^{-6}$
85	+	89	1.4	$6.8x10^{-7}$
Lp	-	5	1.0	$1.8x10^{-7}$
Lp	+	48	9.6	$1.5x10^{-7}$
72	-	1.5	1.0	$1.0x10^{-8}$
72	+	1.3	0.87	$3.4x10^{-10}$
65	-	455	1.0	$6.5x10^{-6}$
65	+	1050	2.3	$7.5x10^{-6}$
85	-	890	1.0	$2.2x10^{-5}$
85	+	725	0.8	$1.2x10^{-5}$
Lp	-	190	1.0	$1.5x10^{-6}$
Lp	+	280	1.5	$5.6x10^{-6}$
85	-	115	1.0	$1.7x10^{-6}$
85	+	106	0.9	-
Lp	-	110	1.0	$8.5x10^{-7}$
Lp	+	60	0.5	$5.5x10^{-7}$

Conditions: + = growth in MRS containing with 40mM DL-threonine

- = growth in MRS without DL-threonine

Baseline taken with no DL-threonine, single pulse of 2.5kV, 25μFD, 100ohms for 100μl cells in sucrose buffer in 0.2cm cuvette.

These results show a general trend for the number of transformants to be increased following the treatment with DL-threonine. In comparison with the increases gained with some of the buffer changes (10-fold), the increases exhibited here are small. However, as

with the other procedures which also slightly increase the TE, any increase can be vital in a transformation experiment where it may be necessary to compensate for conditions which will lower the number of transformants (*eg* ligated DNA).

It was concluded from the above data that DL-threonine treatment of these strains could be potentially useful for achieving increased transformation levels.

4.2.13.b) D-Cycloserine

D-cycloserine is an antibiotic which competitively inhibits two enzymes (alanine racemase and D-alanyl-D-alanine synthetase) that catalyse the formation of a dipeptide (D-alanyl-D-alanine) which is part of the peptide cross-link in some types of peptidoglycan. This inhibition results in weakened peptidoglycan.

The concentration of D-cycloserine which was chosen for the experimental procedures was the value below that which inhibits growth (growth determined by increase in OD at 600nm after overnight incubation). Thus, using 50μg increments, growth occurred at concentrations of 50μg/ml and 100μg/ml but not at 150μg/ml or 200μg/ml. Hence, the experimental concentration was defined as 100μg/ml.

The results of the use of D-cycloserine are shown in Table 4.15.

Table 4.15. Effect of growth in D-cycloserine on transformation by electroporation.

STRAIN	CONDITIONS	TE actual	TE relative	TF
72	-	1	1.0	$5.0x10^{-8}$
72	+	22	22.0	$4.4x10^{-7}$
65	-	288	1.0	$2.4x10^{-6}$
65	+	196	0.7	$2.9x10^{-7}$
72	-	12	1.0	$1.6x10^{-7}$
72	+	2	0.2	$6.7x10^{-8}$
65	-	110	1.0	$5.5x10^{-7}$
65	+	216	1.9	$1.1x10^{-6}$
72	-	9	1.0	$6.5x10^{-8}$
72	+	14	1.7	$1.0x10^{-7}$
65	-	92	1.0	$5.7x10^{-7}$
65	+	468	5.1	$2.3x10^{-6}$
85	-	191	1.0	$5.5x10^{-7}$
85	+	620	3.2	$4.1x10^{-6}$

Conditions: - = without cycloserine in growth medium

+ = with 100μg/ml cycloserine in growth medium

Baseline taken with no cycloserine in growth medium with single pulse of 2.5kV, 25μFD, 100ohms for 100μl cells in sucrose buffer in 0.2cm cuvette.

While these results show some experimental inconsistencies which are probably related to the amount of day-to-day variability inherent in these experiments, the general trend seems to be that the use of D-cycloserine leads to an increase in the number of transformants.

This result is perhaps surprising since in the cross link structure proposed by Schleifer &

Kandler (1972), no D-alanyl-D-alanine dipeptide occurs within *L. plantarum* peptidoglycan. It may be that the site of action in these experimental strains differs to some extent from that described or that the strains used here have a different cross-link structure to that proposed.

As with the use of DL-threonine as a cell wall weakening agent, the effect of D-cycloserine on *L. lactis* would be expected to be more pronounced than that on *L. plantarum* due to the presence of the D-alanyl-D-alanine dipeptide in the cross link of *L. lactis*. No published data are available on the use of D-cycloserine with *L. lactis*.

From the results presented above, it was concluded that the use of D-cycloserine could be beneficial in achieving an increased number of transformants.

4.2.13.c) Glycine.

Glycine acts as an analogue of D-alanine and hence prevents cross-link formation within the peptidoglycan of lactobacilli (Hammes *et al.*, 1973).

Table 4.16 shows the effect on transformation of the experimental strains over a range of glycine concentrations.

Table 4.16. Effect of growth in glycine on transformation by electroporation.

STRAIN	% GLYCINE	TE actual	TE relative	TF
85	0.0	30	1.0	$3.0x10^{-7}$
85	1.0	350	11.6	$1.1x10^{-6}$
85	2.5	1000	33.3	$2.1x10^{-6}$
85	5.0	1700	56.7	$6.8x10^{-6}$
85	7.5	360	12.0	$1.3x10^{-6}$
85	10.0	180	6.0	$6.2x10^{-7}$
85	0.0	1200	1.0	$1.0x10^{-7}$
85	1.0	3400	2.8	$1.6x10^{-6}$
85	2.5	1500	1.3	$6.8x10^{-7}$
85	5.0	17000	14.0	$1.1x10^{-5}$
85	7.5	18000	15.0	$1.3x10^{-5}$
85	10.0	5400	4.5	$3.0x10^{-6}$
72	0.0	0.5	1.0	$5.0x10^{-8}$
72	2.5	1.5	3.0	$1.3x10^{-8}$
72	5.0	2	4.0	$1.0x10^{-7}$
72	7.5	14	27.0	$1.4x10^{-6}$
65	0.0	288	1.0	$2.4x10^{-6}$
65	2.5	133	0.5	$5.5x10^{-8}$
65	5.0	488	1.7	$1.9x10^{-6}$
65	7.5	315	1.1	$1.7x10^{-6}$
65	0.0	110	1.0	$5.5x10^{-7}$
65	2.5	219	2.0	$1.3x10^{-6}$
65	5.0	628	5.7	$2.7x10^{-6}$
65	7.5	640	5.8	$1.6x10^{-6}$

Percentage glycine given in terms of concentration in growth medium. Baseline taken with no glycine. Single pulse at 2.5kV, 25μFD, 100ohms for 100μl cells in sucrose buffer in 0.2cm cuvette.

The efficiency of transformation increases over all of the glycine concentrations measured although an optimum of between 5% and 7.5% is evident. The increase in

efficiency at these optimal levels is quite distinct; at least a ten-fold difference is observed.

The conclusion from this experiment is, therefore, that growth in 5%-7.5% glycine prior to electroporation is a beneficial method of enhancing the number of transformants obtained during a transformation experiment.

4.2.13.d) Lysozyme and mutanolysin.

Perhaps the obvious first choice of an agent to weaken the cell wall is lysozyme, which is commonly used in many cell lysis procedures. At concentrations lower than those used for lysis, it may be possible to weaken the cell wall enough to allow increased transformation. In the case of the strains studied here, which are apparently quite resistant to lysozyme treatment alone (see Introduction), mutanolysin was also used.

Experimentation showed that the optimum concentrations for formation of spheroplasts *ie* cells seen by microscopy to be spherical after treatment at 37°C for 15 minutes, were 1 mg/ml lysozyme and 10 units/ml mutanolysin. Spheroplasts probably retain some cell wall structure whereas true protoplasts have none.

Within this study, all experiments where lysozyme and mutanolysin treatment preceded electroporation, no transformants were found. This is most likely to be due to the pulse causing lysis of the cells and it could be that treatment to a point before true protoplasts are formed may be optimal for transformation. Due to the success of the other pre-treatment procedures, no further analysis of the effect of lysozyme/mutanolysin was made.

Lysozyme treatment of cells has been used successfully to increase the transformation frequency with other members of the LAB *eg* Powell *et al.* (1988).

4.2.14) Other parameters.

4.2.14.a) Multiple pulses.

The single electrical pulse used in electroporation obviously mediates the entry of DNA into cells (controls involving DNA but no pulse were all negative). It can be postulated, therefore, that if the pulses have an additive effect, more than one pulse might lead to increased transformation.

All experiments which involved more than one pulse (all of the same voltage, capacitance and resistance) failed to give any transformants despite decreasing the percentage survival. These results agree with published reports involving the use of multiple pulses *eg* Powell *et al.* (1988) using *L. lactis*; Zealey *et al.* (1988) using *Bordetella* spp.

4.2.14.b) Other plasmids

All of the optimisation experiments were performed using pNZ12. Different cloning vectors were also used within this experimental system *eg* pTG262, pIL277, pIL253, pMTL500F. Table 4.17 lists representative data for the transformation of the *Lactobacillus* strains with vectors other than pNZ12.

Table 4.17. Transformation of *Lactobacillus* strains with other plasmids.

STRAIN	PLASMID	TE	TF
85	pIL277	275	$3.2x10^{-7}$
Lp	pIL277	241	$2.4x10^{-7}$
65	pIL277	299	$2.5x10^{-7}$
85	pTG262	2500	$3.1x10^{-6}$
Lp	pTG262	2500	$4.4x10^{-6}$
65	pTG262	2500	$2.7x10^{-6}$
72	pTG262	169	-
Lp	pIL253	387	$3.6x10^{-7}$
85	pIL253	362	$7.5x10^{-7}$
65	pIL253	428	$3.2x10^{-6}$
72	pIL253	15	-
85	pA4	760	$6.9x10^{-6}$
Lp	pA4	332	$6.6x10^{-6}$
65	pA4	459	$4.2x10^{-6}$
72	pC3	362	$5.0x10^{-7}$
72	pC3	164	-

The origins of these plasmids are detailed in Table 4.18 (see also section 2.4 in Materials & Methods). Electroporation conditions in all cases were single pulse of 2.5kV, 25μFD, 100ohms for 100μl cells in sucrose buffer in 0.2cm cuvette.

Table 4.18. Description of plasmids successfully transformed into *Lactobacillus* strains by electroporation.

PLASMID	BASIS OF GRAM +VE REPLICON	REFERENCE
pNZ12	cryptic *L. lactis* plasmid pSH71	de Vos, 1986
pTG262	cryptic *L. lactis* plasmid pSH71	Horn *et al.*, 1991
pIL253 & pIL277	broad host range plasmid pAMß1	Simon & Chopin, 1988
pMTL500F & pCC1	broad host range plasmid pAMß1	Oultram *et al.*, 1988 N. Minton, personal communication

Some degree of variation for the level of transformation is evident from these data but the overall trend is for a TE of approximately $3x10^2$ and a TF in the region of $5x10^{-6}$ *ie* the use of other vectors does not obviously lower the efficiency of the system.

B) PEDIOCOCCI

All the strains of *Pediococcus* involved in this study were consistently less transformable than any of the *Lactobacillus* strains used. Indeed, all attempts to transform the *Pediococcus* strains with DNA from heterologous hosts (*E. coli*, *B. subtilis*) were unsuccessful.

Working on the hypothesis that members of the LAB are likely to react similarly to each other (although the many differences between *Lactobacillus* spp. and *Lactococcus* spp. show that not all responses correspond to this), all of the changes implemented in the optimisation of electroporation for the lactobacilli were also used in attempts to transform the *Pediococcus* strains.

The few successful transformations were performed with *Pediococcus* cultures grown in 5% glycine and using DNA derived from *Lb* 85. All other conditions were the same as those used for the *Lactobacillus* strains.

Figures 4.4 and 4.5 show evidence for positive transformation of some of the *Pediococcus* strains obtained from agarose gel electrophoresis.

The efficiency of these transformations were considerably less than those achieved using the lactobacilli. Whereas a routine electroporation with a *Lactobacillus* strain generally yielded 100-1000 colonies per plate from undiluted transformation mix, the equivalent number of colonies from the *Pediococcus* strains were one to five. This corresponds to a TE in single figures and a TF of approximately $1x10^{-9}$.

It is noteworthy that the percentage survival of the *Pediococcus* strains after the pulse is always higher than those of the *Lactobacillus* strains under the same conditions. Following a pulse of 2.5kV, 25μFD, 100 ohms, the percentage survival of the *Lactobacillus* strains is generally in the region of 30-50%, whereas, with the *Pediococcus* strains, over 50%, sometimes up to 80-90%, of the total population survive.

These differences in survival may be related to cell size. The lactobacilli are rod-shaped,

2-3μm in length, while the pediococci are spherical and approximately 1μm in diameter. As has been noted previously, it is considered that smaller cells require higher field strengths for successful transformation (Shigekawa & Dower, 1988). Hence, it could be that the maximum field strengths available here (12.5kV/cm) are not sufficient to achieve optimal transformation of the *Pediococcus* strains. The conformation of the two types of cells may also be important: pediococci tend to occur in pairs or in tetrads rather than single cells whereas the lactobacilli occur singly or in pairs and are hence probably more exposed to damage by the pulse (and therefore may have a greater transformation potential).

The single published report on electroporation of *Pediococcus* spp. (Luchansky *et al.*, 1988) also found that transformation was consistently lower than for *Lactobacillus* spp. *P. acidilactici* LA-82 gave a TE of $5x10^1$ per μg DNA whereas the lactobacilli gave a corresponding TE in the region of $3x10^2$ to $9x10^5$. The results for the *Pediococcus* strains used in this study are, therefore, in agreement with these published data.

Figures 4.4. Evidence of successful transformation of *Pediococcus* strains.

Lane 1, λ*Hind*III markers; lane 2, pIL277 from *L. lactis*; lane 3, *Pd* 40 pIL277; lane 4, *Pd* 40 WT; lane 5, *Pd* 60 pIL277; lane 6, *Pd* 60 WT. Sizes refer to λ*Hind*III markers.

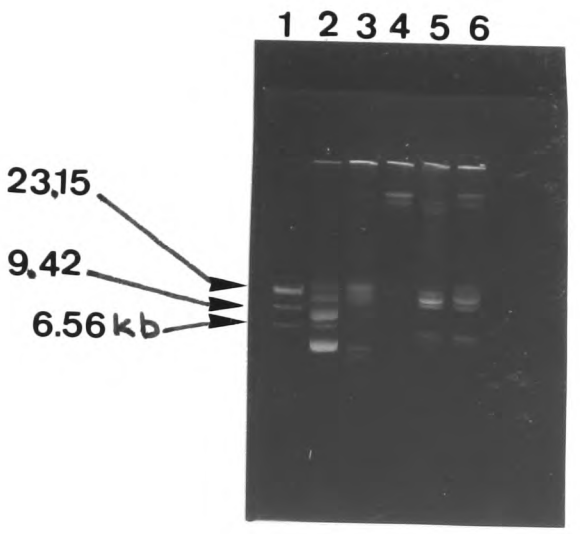
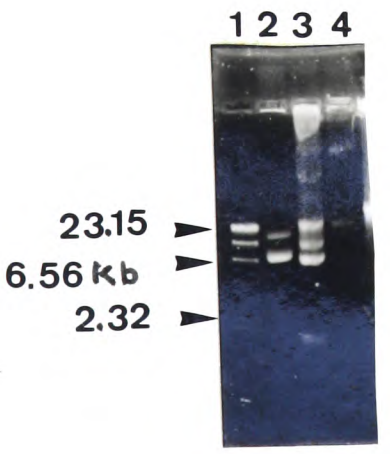


Figure 4.5. Evidence for successful transformation of *Pediococcus* strains.

Lane 1, λ*Hind*III markers; lane 2, pM1 from *B. subtilis*; lane 3, *Pd* 37 pM1; lane 4, *Pd* 37 WT. Sizes refer to λ*Hind*III markers.



DISCUSSION of ELECTROPORATION

The main requisite for genetic manipulation is the ability to transfer DNA between different organisms. Conjugative transfer is well documented within the LAB (*eg* Kempler & McKay, 1979; Chassy & Rokaw, 1981; Shrago *et al.*, 1986) but there is no natural transformation. The technique of electroporation has great potential for facilitating the transfer between many different types of bacteria since it has a relatively simple methodology and is theoretically not bound by the strain- and species-dependencies inherent in other techniques.

Although one of the initial reports of electroporation (Chassy & Flickinger, 1987) used a member of the LAB, it was first necessary to demonstrate that the experimental strains isolated from silage could be susceptible to this form of DNA transfer. The initial electroporation experiments were successful but the yield of transformants and hence the process of transfer itself were found to be rather inefficient. Therefore, an optimisation study was set up to attempt to achieve an optimal level of transformation and also to increase the total yield of transformants. Screening of many transformants is much more likely to allow detection of recombinants than screening of small numbers.

ELECTROPORATION BUFFER (section 4.2.1)

From the optimisation results, sucrose buffer was found to be the most successful of the buffers tested. Sucrose buffer has a low ionic strength and hence has a higher resistance compared with phosphate buffer, resulting in longer time constants *ie* 2.3ms compared with 1.9ms (using the pulse controller). It is possible that the increase in transformation efficiency with sucrose buffer is related to these longer time constants; perhaps the (theoretically) longer pulse causes greater damage to individual cells or damages a greater proportion of the cell population and hence renders them susceptible to transformation.

The finding that the best buffer contained no divalent cations (calcium or magnesium)

was unexpected since it is generally accepted that these enhance the uptake of DNA. Transformation of *E. coli* is dependent upon the presence of calcium ions to facilitate the uptake of DNA (Mandel & Higa, 1970; Dagert & Ehrlich, 1979). Similarly, DNA uptake in *Streptococcus pneumoniae* is absolutely reliant on calcium ions (Smith *et al.*, 1981) while, in *B. subtilis*, binding of DNA can occur in the absence of divalent ions but magnesium is essential for DNA uptake (Smith *et al.*, 1981). Magnesium ions are also used in the medium for transformation of *L. lactis* subsp. *lactis* protoplasts (Gasson, 1980) and electroporation of *L. lactis* subsp. *lactis* (Powell *et al.*, 1988) as well as the electroporation of other lactobacilli (Chassy & Flickinger, 1987). The mechanism of action of these divalent cations is unclear; they may shield the phosphate groups of the DNA in such a way as to facilitate the otherwise unlikely association of two phosphate-rich structures, the DNA and the bacterial cell (Hanahan, 1983).

Variations of the phosphate buffer used in the experiments described in this thesis (generally differing in the amount of sucrose) are the most common buffers used in the relevant published data. Luchansky *et al.* (1988) working with a range of LAB and Badii *et al.* (1989) and Bates *et al.* (1989) both working with *L. plantarum*, all used potassium phosphate buffers while Aukrust & Nes (1988) used a sodium phosphate buffer for *L. plantarum*. Holo and Nes (1989) used 0.5M sucrose as the buffer for *Lactococcus lactis* subsp. *cremoris*.

Two of these studies (Luchansky *et al.* (1988) and Bates *et al.* (1989)) used higher strength buffers *eg* 1.5x, 2x. In this study, all attempts to use higher strength buffers resulted in either arcing or in fewer transformants than normal strength buffers (data not shown).

It is noteworthy that the only published report in which the 0.2cm cuvettes were used (thus achieving a higher field strength and hence increasing the possibility of arcing), that of Holo & Nes (1989), also used a buffer of low ionic strength (0.5M sucrose).

USE of PULSE CONTROLLER (section 4.2.2)

As can be seen in Table 4.3, the use of the pulse controller resulted in a marked loss of viability when measured immediately after the pulse. It has been proposed (Chassy *et al.*, 1988) that some loss of viability will occur at the most effective voltage for transformation, although this is not always the case *eg Bordetella* spp. (Zealey *et al.*, 1988) and *Campylobacter jejuni* (Miller *et al.*, 1988) do not exhibit any significant loss even at the highest field strengths tested. Loss of viability can serve as a useful indicator that damage is being caused by the pulse even though transformation is unsuccessful although there does not appear to be any general rule of what level of cell death is optimal for transformation (Chassy *et al.*, 1988).

The use of the pulse controller resulted in much reduced time constants, from 20ms to 2ms. It is possible that this shorter pulse caused less cell damage to the same volume of cells and, as a consequence, a lower TE (assuming that some degree of cell damage precedes transformation).

LENGTH OF EXPRESSION PERIOD (section 4.2.4)

An expression period of between one and five hours is common in most traditional transformation methods and there is no exception in other published electroporation protocols. Expression is required for entry of exogenous DNA into the cell, its stable maintenance within the cell and expression of the gene in terms of its phenotype. Expression time will depend on the phenotype being assayed.

VARYING PARALLEL RESISTORS (section 4.2.5)

The results presented in Table 4.7 indicate that a resistance level of 100ohms was found to be optimal for transformation. The level of 100 ohms generally gave a pulse length of 2.3 to 2.4 ms and was lower than the values quoted in the few published reports which used the pulse controller (all other reports were published prior to its introduction). For electroporation of *Lactococcus lactis* subsp. *lactis* (McIntyre & Harlander, 1989b) and *L.*

lactis subsp. *cremoris* (Holo & Nes, 1989), 200 ohms was used, while 400 ohms was used for *Lactobacillus hilgardi* and *L. plantarum* (Josson *et al.*, 1989). In none of these accounts were there any details of the selection of these resistance levels and none were carried out under the conditions as stated here. As well as being dependent on the procedural conditions, electrical variables such as resistance and voltage may need varying with different species and strains.

Interestingly, the longer pulse lengths led to greater cell death as indicated in Table 4.7.

It has been proposed that cell damage is related to transformation potential *ie* the more cells damaged, the greater the number available for transformation. Therefore, it may be expected that an increase in cell death would be accompanied by an increase in transformation. This does not appear to be the case here and these results indicate that there may be a maximum level of cell damage which is beneficial for transformation under these conditions.

From published data, it is evident that the time constant at which maximum transformation occurs is species (and strain) dependent. Some examples of published values are given in Table 4.19.

Table 4.19. Examples of time constant values from published reports of transformation by electroporation.

ORGANISM	TIME CONSTANT*	VOLTAGE	REFERENCE
E. coli	7	2.5	a
L. monocytogenes	4.4	8	b
Staph. epidermidis	2.5	10	c
B. amyloliquefaciens	8-10	7.5	d
E. faecalis	7.1	6.25	e
L.lactis	5	17	f

where a = Dower *et al.*, 1988; b = Alexander *et al.*, 1990; c = Augustin & Gotz, 1990; d = Vehmaanperä, 1989; e = Fiedler & Wirth, 1988; f = McIntyre & Harlander, 1989b.

* Time constant at maximum levels of transformation. Voltage in kV.

VARYING VOLTAGE AT CONSTANT RESISTANCE (section 4.2.6)

It has been proposed (Shigekawa & Dower, 1988) that the field strengths (V/cm) required for electroporation of bacteria will be higher than those for eukaryotic cells because bacteria are much smaller. In practice, most bacterial protocols require a field strength of at least 3kV/cm whereas mammalian cells undergo transfection at 1kV/cm (Potter *et al.*, 1984) and below.

Electroporation procedures involving members of the LAB are consistent with this use of high field strengths; a range between 5kV/cm (Chassy & Flickinger, 1987) and 8.5 kV/cm (Josson *et al.*, 1989) have been utilised. Some studies (*eg* Powell *et al.*, 1988; Holo & Nes, 1989) have found that the highest transformation frequencies are achieved using the highest field strengths available. This was also the case for the results presented here (Table 4.9).

The data generated from the experiments using the pulse controller, the 0.2cm inter-electrode distance cuvettes and increased voltages, all indicated a connection between the

level of survival immediately after the pulse and the transformation efficiency; the TE increased as the survival level dropped. In contrast, increases in resistance (and hence time constant) also led to increases in levels of cell death but corresponded to a decrease in TE. It would appear that the time constant is the critical factor; increases in pulse length may push the level of cell death beyond that which is optimal for transformation.

DNA CONCENTRATION (section 4.2.7)

The amount of DNA used to facilitate transformation is also a variable within electroporation. In practise, however, the amount of DNA available for any transformation process is generally limited because of the time and effort required to extract it and also, with some bacterial species, the relatively low yields. Therefore, it is common to add a standard amount of DNA, usually 1μg, to a transformation reaction.

It is common to find that the total number of transformants obtained by electroporation increases linearly with increasing DNA concentration *eg* Luchansky *et al.* (1988), McIntyre & Harlander (1989a and b), David *et al.* (1989). Any advantage to be gained by the addition of higher DNA concentrations has to weighed against DNA availability.

The extent of this dose response is, however, generally limited by DNA saturation. At DNA concentrations above 10μg each of the above reports records a levelling off of the curve and only minimally increased transformation efficiencies.

The expected saturation level typical of traditional transformation experiments (Hayes, 1968) was observed (Table 4.10) but at a lower DNA concentration (1μg) than most published data (10μg). Beyond the DNA saturation concentration of any transformation experiment the TE is expected to fall because the ratio of transformants to DNA concentration falls, while the TF levels off since the overall population numbers do not alter. The observed TE and TF values are consistent with these statements.

The variation in TF for values lower than 1μg of DNA do not strictly abide to the expected pattern; 10-fold and 100-fold increases in TF were recorded for a doubling of

DNA concentration. This may be partly a result of inaccurate dilution steps.

The lower saturation level recorded for this study as compared to the published reports may be related in part to the purity of the DNA. None of the *Lactobacillus* plasmid DNA used in the optimisation studies was purified on caesium chloride gradients (see note section 2.5.1). The successful use of crude DNA for electroporation confirms that such purification steps are not always necessary.

Varying the level of DNA used to perform the transformation experiments led to the generation of a DNA saturation curve consistent with those achieved during classical transformation experiments (Hayes, 1968). DNA saturation is a function of the binding of the DNA to the cell or of the uptake of the DNA by the cell. This suggests that the mechanisms of DNA uptake are the same for electroporation as for other transformation methods. There is some published evidence to suggest that uptake of DNA during electroporation does not involve prior binding to the cell surface (Dower *et al.*, 1988). More recent published reports suggest the opposite (Xie *et al.*, 1990; Tsong, 1990) and indeed these experiments have shown that transformation efficiency is increased dramatically by prior DNA binding (all of these reports used strains of *E. coli*).

While recent studies into the mechanisms of electroporation have been able to demonstrate the occurrence of transient pores in a (human erythrocyte) cell surface by rapid freezing electron microscopy, they have also suggested that uptake of DNA through these pores is unlikely because of the relatively small surface area that the pores occupy (Tsong, 1990).

PRE AND POST-PULSE INCUBATION ON ICE (section 4.2.8)

To maintain bacterial cells in a uniform state, many transformation procedures are carried out as far as possible below room temperature, generally by placing the cell suspension on ice.

In the traditional calcium chloride method for the transformation of *E. coli* (Mandel & Higa, 1970), the cell suspension is incubated with DNA for 40 minutes prior to selection.

During this time it is thought that the DNA binds to receptors on the cell and then gains entry into the cell.

In terms of transformation protocols, electroporation is quite a violent procedure which is thought to involve the formation of transient pores in the cell membrane (Zimmerman *et al.*, 1973) through which the DNA gains entry. In such a mechanism, a step involving the binding of the DNA to a cell receptor is unlikely. Dower *et al.* (1988) found that a pre-pulse incubation on ice ranging from 0.5 to 30 minutes had very little effect on transformation in *E. coli.*

During the optimisation procedure described here, no pre-pulse incubation was included in the protocol. All pulses were performed immediately after mixing the cell suspension with the DNA.

A post-pulse incubation may be necessary to allow uptake of the DNA. Many published reports on electroporation of LAB include a post-pulse incubation on ice of ten minutes *eg* Chassy & Flickinger (1987); Powell *et al.* (1988); Luchansky *et al.* (1988); McIntyre & Harlander (1989b); Holo & Nes (1989); Badii *et al.* (1989).

Working with *E. coli* however, Dower *et al.* (1988) found that a post-pulse incubation actually lowered the TE. A similar result was obtained by Fiedler & Wirth (1988) using *Enterococcus faecalis* and by Augustin & Gotz (1990) using *Staph. epidermidis.*

FREEZING OF CELLS PRIOR TO PULSE (section 4.2.9)

The indication from the experimental results that freezing of cell culture prior to electroporation greatly reduced the efficiency was mildly surprising since glycerol is a recognised cyroprotectant and, as indicated in section 4.2.9, this reduction in transformation was not simply due to a loss of viability caused by freezing. With *L. lactis* LM0230, McIntyre & Harlander (1989b) found that while freezing and storage in 10% glycerol at -20°C and -60°C did cause a transformation drop, freeze-drying at -60°C did not. No investigation of the benefits or otherwise of freeze-drying were undertaken in

the present study. Storage at -85°C for several months has not been found to be detrimental to *Lactobacillus curvatus* (Gaier *et al.*, 1990) while no reduction in the number of transformants after freezing has also been recorded for *L. plantarum* CCM 1904 (Bringel & Hubert, 1990). From the differences in the published methods it would seem that the effect of freezing on transformation is strain dependent. The major disadvantage caused by the inability to freeze cultures to be used for electroporation is the lack of a common source from which a standard level of transformants may be gained.

Holo & Nes (1989) found that freezing samples of *L. lactis* in buffer containing glycerol did not reduce the level of transformation. Similar results were achieved by freeze-drying *L. lactis* samples (McIntyre & Harlander, 1989b).

USE OF CAPACITANCE EXTENDER (section 4.2.10)

No transformants were recorded from the use of the capacitance extender in this study. This result is perhaps not too surprising since it is generally agreed that the size of an electrical field required to generate membrane pores is inversely related to cell size *ie* smaller (bacterial) cells require higher field strengths than larger ones (Potter, 1988).

A capacitance value of 25 μFD is the level most commonly used in the published data over a range of bacterial genera *eg* Chassy & Flickinger, 1987; Fiedler & Wirth, 1988; Dower *et al.*, 1988; Powell *et al.*, 1988; McIntyre & Harlander, 1989b.

GROWTH PHASE OF CELLS USED FOR ELECTROPORATION (section 4.2.11)

All of the early published experiments on electroporation on members of the LAB (*eg* Chassy & Flickinger, 1987; Powell *et al.*, 1988; Luchansky *et al.*, 1988) and also on members of other bacterial genera (*eg* Fiedler & Wirth, 1988; Dower *et al.*, 1988) used mid-exponential phase cells. At this point in their growth cycle, bacterial cell walls are still actively growing and hence are more susceptible to processes designed to increase permeability *eg* electroporation.

McIntyre & Harlander (1989a and b) tested *L. lactis* subsp. *lactis* at a range of optical densities corresponding to early-log, mid-log and stationary phase cells. With cells resuspended at a high concentration ($5x10^{10}$ cfu/ml) in each case, the best transformation efficiencies were obtained with stationary phase cells. Since the initial total cell counts were very similar, this difference could not simply be ascribed to a greater cell density at stationary phase. It has been suggested (McIntyre & Harlander, 1989a) that the difference may be caused by strain-dependent variations in the optimal growth phase for electroporation.

OSMOTIC STABILISATION DURING ELECTROPORATION (section 4.2.12)

If electroporation is dependent on membrane pore formation as is proposed (Zimmerman *et al.*, 1973) then it is possible that the cells become osmotically fragile during the

procedure. If this occurs to a major degree, the cells will lyse in a medium which is hypertonic or hypotonic compared with the intracellular environment and hence viability will be lost. Protoplast formation also involves the production of osmotically fragile cells and sucrose is generally added as an osmotic stabiliser. There is, however, no evidence that sucrose acts in a similar manner during electroporation (Chassy *et al.*, 1988) although it is has been commonly used to supplement the expression medium prior to selection (Powell *et al.*, 1988; Holo & Nes, 1989; van der Lelie *et al.*, 1988 all of whom used *Lactococcus* spp.). There is no evidence that growth of electroporated cells on regeneration media (required for protoplast regeneration) or osmotically-stabilised media increases either the recovery of viable cells or the transformation frequency (Chassy *et al.*, 1988).

None of the published reports on electroporation of lactobacilli (Chassy & Flickinger, 1987; Luchansky *et al.*, 1988; Aukrust & Nes, 1988; Badii *et al.*, 1989; Bates *et al.*, 1989) used sucrose as an osmotic stabiliser in the expression media, although it must be pointed out that all of the electroporation buffers used in these studies contained sucrose. It is therefore possible that the level of sucrose contained in these buffers already acts as an osmotic stabiliser. Since the electroporation buffer used in the experiments shown here is simply a solution of 0.5M sucrose, the same may be said of these results. For simplicity, sucrose was not added to the growth media at any point during the completion of the optimisation.

USE OF CELL WALL WEAKENING AGENTS (section 4.2.13)

L-THREONINE (section 4.2.13a)

L-threonine occurs naturally in the cross-linking peptides within the peptidoglycan of some members of the LAB. When growth occurs in a mixture of both the D and the L isomers of this amino acid, the D isomer can be incorporated into the peptidoglycan resulting in a weaker linked structure.

Growth in media supplemented with 40mM DL-threonine has been successfully used to enhance transformation frequency by McIntyre & Harlander (1989b) with *L. lactis* subsp. *lactis*, by van der Lelie *et al.*, (1989) with *L. lactis* subsp. *cremoris* and by David *et al.*, (1989) using *Leuconostoc paramesenteriodes.*

The cross-link structures within the peptidoglycan of both *L. lactis* subsp. *lactis* and *L. plantarum* have been elucidated (Schleifer & Kandler, 1972; introduction of this thesis). L-threonine residues only occur within the *L. lactis* subsp. *lactis* cross-link whereas L-threonine residues occur in the peptidoglycan of *L. plantarum* although not necessarily in the cross-links (Ikawa & Snell, 1960). It is therefore expected that the use of DL-threonine will be more effective at increasing transformation in strains of *Lactococcus* rather than *Lactobacillus*. McIntyre & Harlander (1989b) showed a consistent four-fold increase in the number of transformants when DL-threonine treatment was used with *L. lactis* subsp. *lactis*.

GLYCINE (section 4.2.13c)

The addition of glycine (1%) to the growth medium has been successful in increasing the transformation efficiency when applied to *L. plantarum* (Aukrust & Nes, 1988). Glycine (2.5%) also results in increased transformation when used with *L. lactis* subsp. *cremoris* (Holo & Nes, 1989). Other Gram positive organisms have also exhibited an increase in transformation efficiency when grown in glycine prior to electroporation *eg B. amyloliquefaciens* (Vehmaanperä, 1989) and *Corynebacterium glutamicum* (Haynes & Britz, 1990).

Following the buffer changes at the beginning of the optimisation, the most successful procedures for increasing the TE were the use of the cell wall weakening agents, especially glycine. Other experimental parameters such as field strength and time constant exhibited a relationship between the level of cell damage and transformation, both of which increased until an optimal of cell damage was reached. The use of glycine

in particular also reflected this relationship; maximal levels of cell damage were reached at concentrations of glycine over 7.5%.

PUBLISHED METHODS

Most of the published reports on the methodology of transformation of lactobacilli using electroporation show very little variation from the first report on *L. casei* (Chassy & Flickinger, 1987).

One exception is the method of Josson *et al.* (1989) who recorded that the use of 30% polyethylene glycol (PEG) 1000 as the electroporation buffer increased the transformation efficiency on *L. hilgardi* and *L. plantarum*.

No transformants were found when this method was used on the experimental strains in this study.

A review of the published reports on electroporation of the LAB demonstrates that while many species and strains react similarly, there is still a substantial degree of variation. This was also evident from the data presented here where all of the *Pediococcus* strains were transformed much less efficiently than the *Lactobacillus* strains. Despite this disadvantage, transformation was still successful and both species were proved to be potential candidates for further cloning work.

CONCLUSIONS

Upon conclusion of the optimisation study, the following modifications were made to the standard electroporation conditions (compare with section 4.1):

Mid-exponential phase cells grown in 5% glycine

Cells resuspended in sucrose buffer (recipe in section 2.2.4)

Single pulse at 2.5kV, 25μFD, 100ohms

1μg DNA per 100μl cell suspension in 0.2cm cuvette

Sample immediately added to expression medium (MRS broth) to give a 1 in 10 dilution

Two hour expression at 30°C

The optimised protocol generally gave a TE of 3 to $5x10^2$ and a TF near $3x10^{-6}$ for the *Lb* strains 85, 65, 48 and Lp. This represents a hundred-fold increase in the TE level from the unoptimised conditions (section 4.1).

This observed increase in TE was cumulative *ie* resulted from a combination of modifications and hence it was not possible to define to what degree each single change would affect the TE.

While the optimised transformation efficiency for the *Lactobacillus* strains was sufficient for routine transformations involving intact plasmid DNA from heterologous and homologous sources, it was probably not sufficient to allow direct transformation of ligated plasmid DNA (the efficiency of transformations with ligated DNA in most species of bacteria is generally always 100-1000 fold lower than that of intact plasmid DNA). It was, therefore, generally necessary to use intermediate hosts (*E. coli*, *B. subtilis*, *L. lactis*) which had higher TE values for the entry of ligated DNA, prior to transformation of the experimental strains used here.

NB Most of the direct transformations attempted using ligated DNA with these experimental strains resulted in arcing, probably a result of the conductivity of the ligation buffer. It is, therefore, suggested that ligated DNA is ethanol precipitated before

electroporation. None of the direct transformations which did not arc were successful.

From the described optimisation, it is evident that small procedural changes *eg* inclusion of a post-pulse incubation on ice, can have an effect on the rate of transformation achieved by electroporation and hence it is important not to ignore such parameters.

While this optimisation was performed using a single plasmid (pNZ12), successful transformation is not exclusive to this vector as shown by the transfer of other plasmids (eg pTG262, pIL277).

A comparison between the transformation efficiency obtained within this study and other, published data for *Lactobacillus* spp. indicates a general transformation efficiency in the region of 10^2-10^5 transformants per μg DNA. Examples of some of the published results are given in Table 4.20.

Table 4.20. Comparison of transformation efficiencies of *Lactobacillus* spp.

Strain	Maximum TE	Reference
L.casei ATCC 393	$8.5x10^4$	Chassy & Flickinger, 1987
L. casei ATCC 4646	$2.6x10^4$	"
L. plantarum NCDO 343	$5x10^2$	Badii *et al.*, 1988
L. plantarum NCDO1193	$6.4x10^4$	"
L. plantarum ATCC8014	$2x10^2$	Aukrust & Nes, 1988
L. plantarum	$5x10^3$	Bates *et al.*, 1989
L. acidophilus ADH	$3x10^5$	Luchansky *et al.*, 1989
L. acidophilus 89	$4x10^4$	"
L. plantarum MOP-3	$8x10^3$	"
L. plantarum C-11	$9x10^3$	"
L. casei 393	$1.7x10^2$	"
L. fermentum 1750	$9.7x10^5$	"

The strains used in the present study are obviously at the lower end of this scale but the

similarity to other, published data indicates that this still represents a quite respectable transformation level. The strain variation observed within this study is also exhibited within the published data. Successful transformation of environmental isolates such as the strains used in this study confirm the vast potential of electroporation. A significant result from the published data is that of Bates *et al.* (1989) who recorded a maximum transformation efficiency of $5x10^3$ transformants per μg DNA for a *L. plantarum* strain isolated from a grass silage. This value is not too dissimilar from the maximum TE values recorded for the environmentally isolated strains used within the present study.

The investigation of agents which may enhance transformation of the *Lactobacillus* strains also proved useful in achieving transfer to some of the *Pediococcus* strains and hence some of the information noted during this study could be helpful when applied to other, related strains. Varying degrees of species and strain variation must, however, also be expected.

In conclusion, a reliable system for the transfer of (plasmid) DNA between environmental *Lactobacillus* strains has been achieved and hence a method is now available for the genetic manipulation of these organisms.

CHAPTER FIVE

USE OF CLONING VECTORS

5.1) pNZ12

For use with the system under investigation here, pNZ12 had all the advantages of many other plasmid vectors *ie* small size, high copy number, shuttle vector potential between Gram-positive and Gram-negative species. The main limitation with pNZ12 is the lack of a multi-cloning site (MCS) and the presence of only four unique restriction enzyme sites. However, since the initial use was simply as an indicator of positive transformation, none of these disadvantages were considered major.

Due to a high background level of resistance to kanamycin in the experimental *Lactobacillus* strains, only the selection for chloramphenicol was utilised in this study with pNZ12 (de Vos, personal communication).

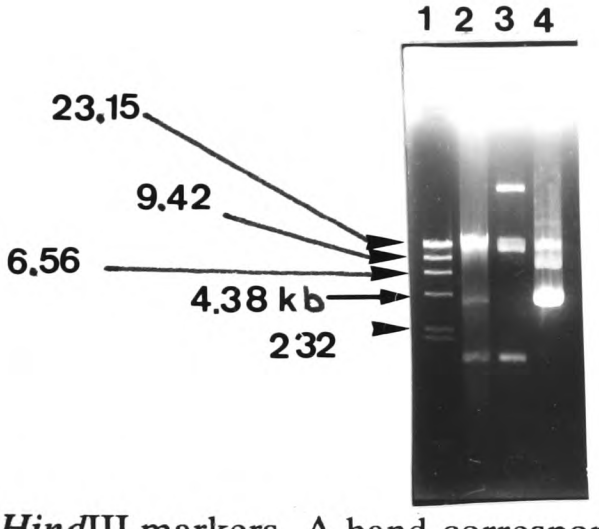
CONFIRMATION OF TRANSFORMATION USING pNZ12.

Transformation of the experimental LAB strains with pNZ12 was carried out using the electroporation conditions described previously. Figure 4.1 (in Chapter Four) shows a band corresponding to pNZ12 after agarose gel electrophoresis of plasmid DNA from some of the experimental strains. No similar band was visible in the plasmid profiles of wild-type (WT) DNA from the experimental strains.

Further evidence for successful transformation with pNZ12 was obtained by using plasmid DNA preparations from some of these proposed transformants to transform other isolates. Following agarose gel electrophoresis, a band corresponding to pNZ12 was seen in all these new transformants (Figure 5.1).

Figure 5.1. Plasmid profile of *Lb* Lp after transformation with pNZ12 from *Lb* 85.

Lanr 1, λ*Hind*III markers; lane 2, *Lb* Lp pNZ12; lane 3, *Lb* Lp WT; lane 4, *E. coli* pNZ12.



Sizes refer to λ *Hind*III markers. A band corresponding in size to pNZ12 can be clearly seen in Lane 2.

A common method used to confirm the presence of a particular plasmid is restriction enzyme analysis. Due to the presence of other plasmids within the experimental strains, such analysis was not possible here. It was, however, possible to transform a plasmid-free *E. coli* strain (MC1022) with pNZ12 from the *Lactobacillus* strains and perform restriction enzyme digests on the plasmid DNA thus obtained. Figure 4.2 shows restriction enzyme analysis of original pNZ12 (lane 8) and pNZ12 transformed into *E. coli* via *Lactobacillus*. As expected, the band patterns obtained were identical thus finally confirming that pNZ12 had indeed been successfully transformed into the experimental strains. No further experimental proof was considered necessary.

As detailed above, pNZ12 had certain disadvantages for use as a cloning vector and hence other plasmids were also investigated for their use within the experimental LAB system.

5.2) pTG262

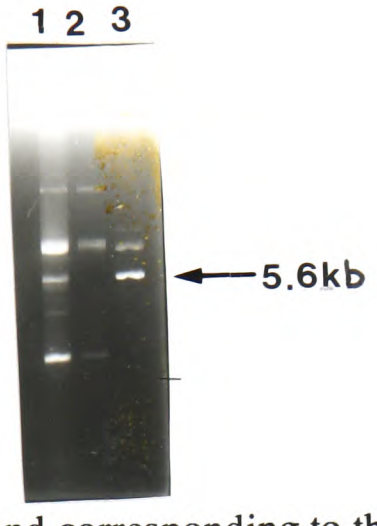
The plasmid vector pTG262 was considered to have greater potential for use as a cloning vector than pNZ12 due to the multi-cloning site which contains unique sites for ten restriction enzymes. Like pNZ12, pTG262 is a shuttle vector, capable of replication in *E. coli* as well as LAB (pNZ12 and pTG262 have the same Gram-positive replicon).

CONFIRMATION OF TRANSFORMATION USING pTG262

Before any cloning experiments could be undertaken, it was necessary to achieve successful transformation of the experimental strains with pTG262. Electroporation experiments as detailed in Chapter Four were performed and Figure 5.2 shows an agarose gel of plasmid DNA from a proposed transformant. A band corresponding to the expected size of pTG262 was visible within the transformants but not in Lb WT plasmid preparations.

Figure 5.2. Plasmid profile of *Lb* 85 indicating presence of pTG262.

Lane 1, *Lb* 85 pTG262; lane 2, *Lb* 85 WT; lane 3, *E. coli* pTG262.



Size refers to pTG262 and a band corresponding to this is evident in Lane 1.

USE OF pTG262 AS A CLONING VECTOR

The cellulase-encoding fragments of pWM95 and pWM191 were both isolated by restriction enzyme digestion followed by the freeze-squeeze method of purification of specific bands from agarose gels. An *Eco*RI-*Hind*III double digestion resulted in fragments of 2.4 kb and 3.4 kb from pWM95 and pWM191 respectively. In both cases, the vector (pUC18) fragment was 2.8 kb in size.

Ligations were set up using these cellulase-encoding fragments and the corresponding digestions of pTG262. The resulting constructs were transformed into *E. coli* MC1022 using the calcium chloride method for producing competent cells. Recombinant plasmids were identified as white colonies on IPTG/X-gal/chloramphenicol plates. After the initial screening, several isolates of the expected pTG262-pWM95 construct were isolated and this new plasmid was termed pA4 (Figure 2.3). No isolates of the expected pTG262-pWM191 recombinant plasmid were obtained from the initial screening.

The structural integrity of pA4 was confirmed by restriction endonuclease analysis of plasmid DNA from *E. coli* MC1022 transformants. As indicated in Figure 5.3, a banding pattern distinct from pTG262 but also containing the expected fragments derived from pWM95 was obtained. The small (1kb) fragment generated from pWM95 after digestion with *Sst*I is particularly useful in confirming the presence of the cellulase-encoding fragment.

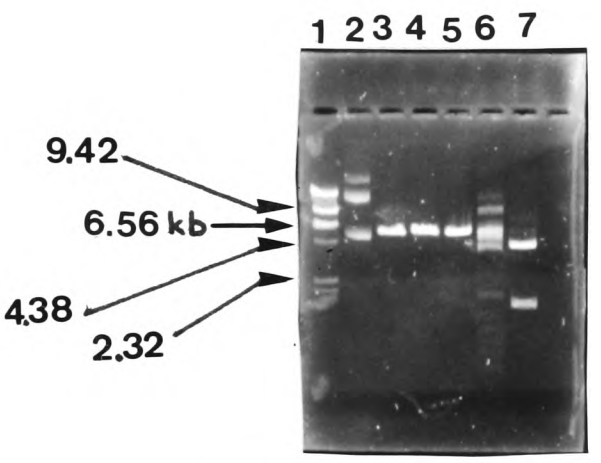
Following purification of pA4 on caesium chloride gradients, transformation into the experimental Lb strains and into *L. lactis* subsp. *lactis* MG1363 was performed by electroporation. Figure 5.4 represents agarose gel electrophoresis of plasmid DNA from *L. lactis* subsp. *lactis* MG1363 after restriction and confirms the presence of intact pA4 within this strain.

Plasmid DNA analysis of transformants from the experimental Lb strains is presented in Figure 5.5. A plasmid band which may correspond to the construct size was identifiable from this preparation along with a much stronger band which was slightly smaller.

Figure 5.3a. Restriction enzyme digests of pWM95.

Lane 1, λ *Hind*III markers; lane 2, undigested pWM95; lane 3, *Eco*RI; lane 4, *Hind*III; lane 5, *Bst*I; lane 6, *Sal*I; lane 7, *Sst*I.

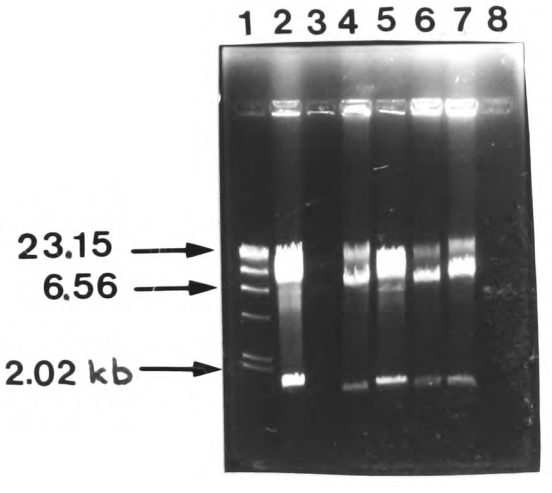
(The *Sal*I digest is incomplete). As pUC18 only contains one *Sal*I site and one *Sst*I site, the extra bands mean that the cellulase-encoding fragment also has sites for these enzymes and the presence of the smaller *Sst*I fragment in particular was taken as evidence for the presence of the cellulase-encoding fragment of pWM95 during the construction of new plasmids.



Sizes refer to λ *HindIII* markers.

Figure 5.3b. Restriction enzyme (*Sst*I) digests of pA4 from *E. coli* MC1022 to confirm structural integrity.

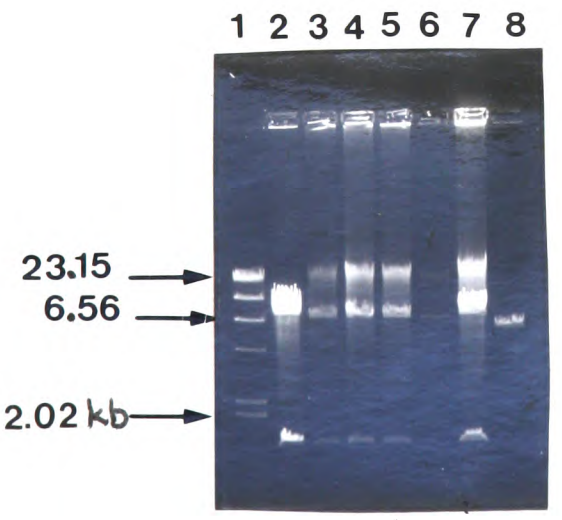
Lane 1, λ*Hind*III markers; Lanes 2 - 7 pA4; lane 8, pTG262.



Sizes refer to λ*Hind*III markers.
Small *Sst*I fragment (approx. 1.9kb) from pWM95 is evident in Lane 1-8, confirming the structural integrity of pA4.

Figure 5.4. Restriction enzyme (*Sst*I) digests of pA4 isolated from *Lactococcus lactis* subsp. *lactis* 1363.

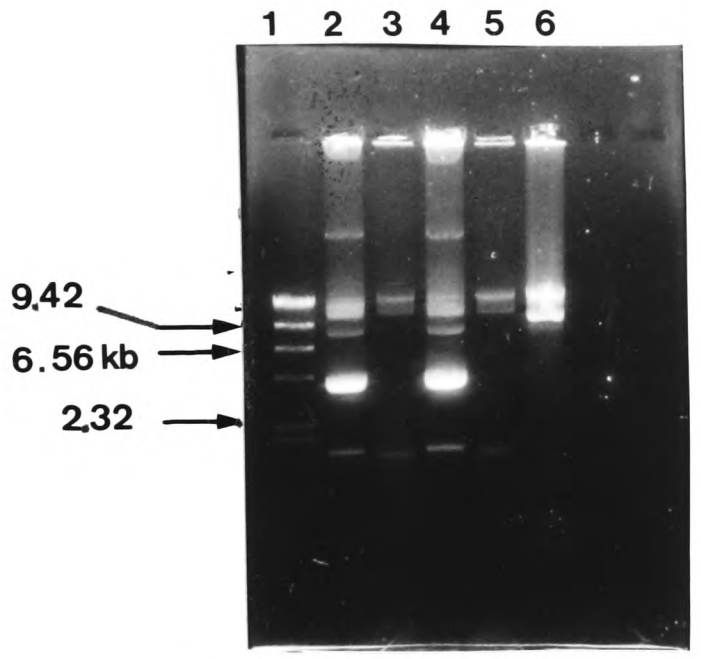
Lane 1, λ*Hind*III markers; lane 2, pA4 from *E. coli* MC1022; lane 3 - 7, pA4; lane 8, pTG262 from *E. coli* MC1022.



Sizes refer to λ*Hind*III markers.
As with Figure 5.3, the small *Sst*I fragment confirms the structural integrity of pA4 within *L. lactis*.

Figure 5.5. Plasmid profiles of *Lactobacillus* strains transformed with pA4.

Lane 1, λ*Hind*III markers; lane 2, *Lb* 85 pA4; lane 3, *Lb* 85 WT; lane 4, *Lb* Lp pA4; lane 5, *Lb* Lp WT; lane 6, pA4 from *E. coli*.



Sizes refer to λ*Hind*III markers. No band corresponding to the size of pA4 is evident with the Lb transformants (Lanes 2 & 4). There is, however, an unidentified band of approximately 4kb.

Neither of these bands was visible from *Lb* WT plasmid DNA preparations.

To clarify which of these bands, if either, was pA4, a hybridisation experiment was carried out. Plasmid DNA from *Lb* (WT), *Lb* transformed with pTG262 and *Lb* transformed with pA4 was hybridised to a nitro-cellulose membrane. This was then probed with non-radioactively labelled, linear pTG262 as detailed in Chapter Two. The resulting hybridisation pattern and the original plasmid DNA pattern are presented in Figure 5.6a and 5.6b. The pTG262 transformant of *Lb* 85 had an identical band pattern to that of the original pTG262 from *E. coli* confirming that successful transformation has taken place. Differences in the amount of DNA loaded on to the gel and also in the purity of the DNA preparations may explain any slight disparity in the alignment of the plasmid bands in lanes two (*E. coli* pTG262) and four (*Lb* 85 pTG262).

The bands which hybridised to the pTG262 probe in lanes five and six (both *Lb* 85 pA4) were all smaller than the original vector pTG262 (lane one) and hence cannot correspond to pA4 which should have been 2.4 kb larger than the vector. These smaller bands must, however, be derivatives of pTG262 since detectable hybridisation occurred although no further information on the composition of these derivatives could be achieved from this experiment alone.

INVESTIGATION OF pTG262 IN *LACTOBACILLUS* STRAINS .

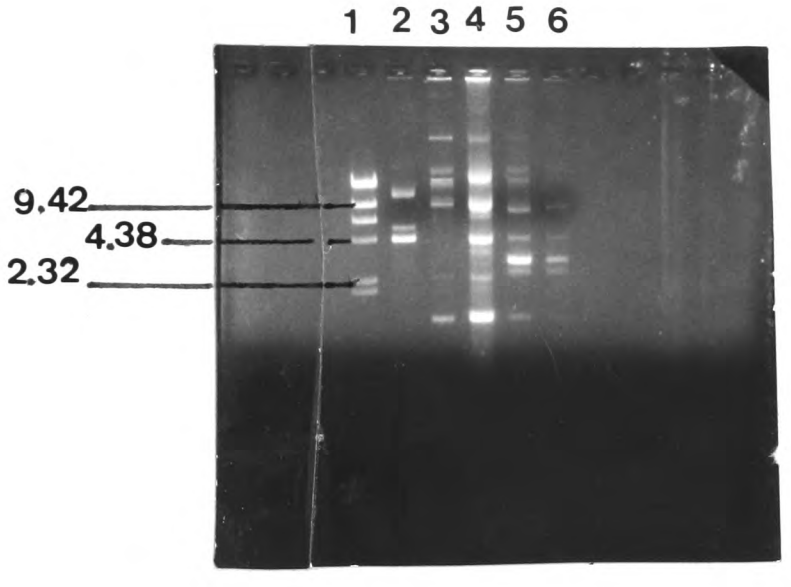
Although the apparent instability of the constructs made with pTG262 effectively precludes its use as cloning vector in the experimental strains, the phenomenon itself was interesting especially since the same constructs seem to be stable within *L. lactis* subsp. *lactis* MG1363 and hence a further investigation into the nature of the possible deletion was instigated.

As previously noted, the presence of the native plasmids limits the use of restriction analysis directly on *Lactobacillus* plasmid DNA and hence pTG262 derivatives from *Lb* strains were retransformed into *E. coli* MC1022 prior to further investigation.

The derivatives of pTG262 harboured within the *Lb* strains apparently varied as indicated

Figure 5.6a. Agarose gel for southern hybridisation and probing with non-radioactively labelled pTG262.

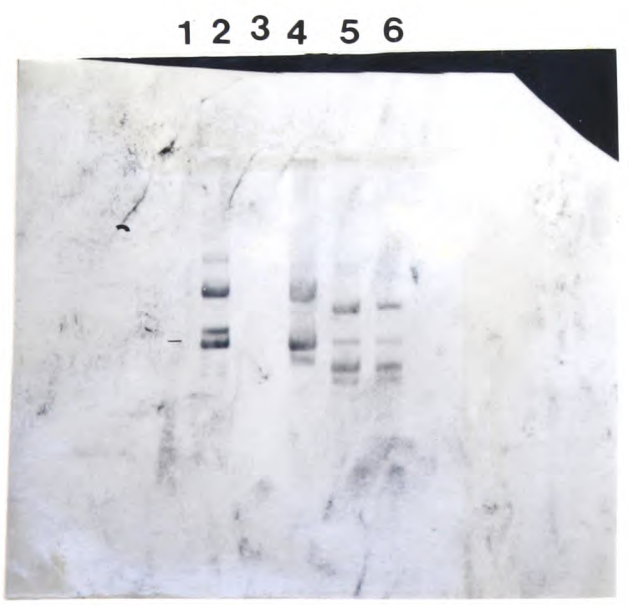
Lane 1, λ*Hind*III markers; lane 2, pTG262 from *E. coli* MC1022; lane 3, *Lb* 85 WT; lane 4, *Lb* 85 pTG262; lane 5, *Lb* 85 pA4; lane 6, *Lb* 85 pA4.



Sizes refer to λ*Hind*III markers.

Figure 5.6b. Hybridisation pattern after probing with non-radioactively labelled pTG262.

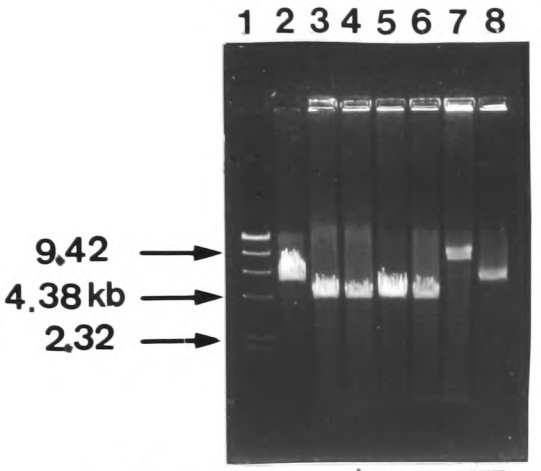
Lanes as for Figure 5.6a.



None of the WT plasmids hybridised with the probe but unidentified bands in Lb 85 pA4 profile did, indicating that they are probably deletion derivatives of pA4.

Figure 5.7. Restriction enzyme (*Bgl*II) digests of pTG262 originally isolated from *Lactobacillus* strains.

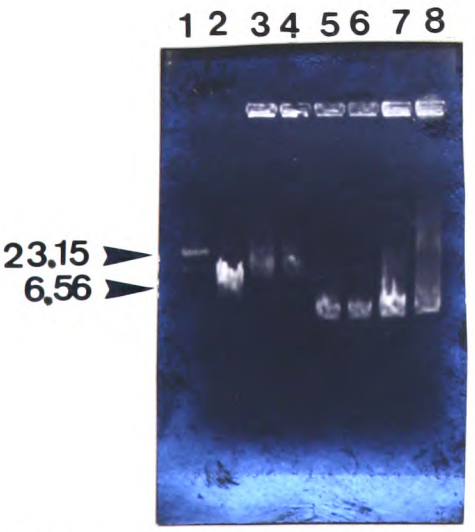
Lane 1, λ*Hind*III markers; lane 2, pTG262 from *E. coli*; lanes 3 - 6, pTG262 from *Lb* 85; lanes 7 & 8, pTG262 from *Lb* 72.



Sizes refer to λ*Hind*III markers.

Figure 5.8. Restriction enzyme (*Bgl*II) digests of pTG262 originally isolated from *Lactobacillus* strains.

Lane 1, λ*Hind*III markers; lane 2 pTG262 from *E. coli*; lanes 3 & 4, pTG262 from *Lb* 72; lanes 5 & 6, pTG262 from *Lb* 65; lanes 7 & 8, pTG262 from *Lb* Lp.



Sizes refer to λ *Hind*III markers.

in Figure 5.7. All of the *Lb* 85 pA4 constructs were approximately two kb smaller than the parent plasmid whereas two different derivatives occurred in the digests of pA4 from *Lb* 72; lane eight appeared to correspond to parental pTG262 whereas lane seven resembled intact pA4. Strains *Lb* 65 and *Lb* Lp, which behave similarly to *Lb* 85 in other respects, exhibited the same apparent deletion as *Lb* 85 (Figure 5.8).

On digestion with *Bgl*II, the pA4 derivative from *Lb* 85, 65 and Lp gave a linear band slightly smaller than intact pTG262 (Figures 5.7 and 5.8). This indicated that the *Bgl*II site within pA4 was still intact. When digested with *Sst*I, however, no indicator fragment from pWM95 was evident, confirming that the insert was no longer present (Figure 5.9).

5.3) pIL VECTORS

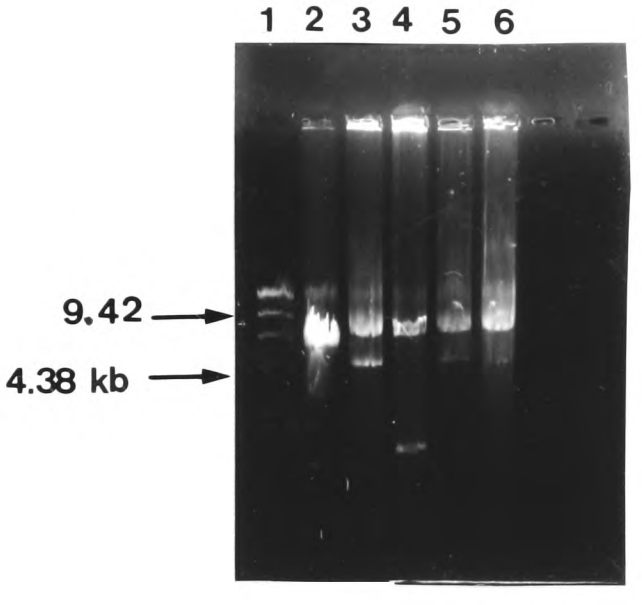
The pIL vectors are a family of plasmids based on pHV1301 which itself is a derivative of pAMß1 (Simon & Chopin, 1988). These vectors have been constructed without the disadvantages of the parental pHV1301 *ie* large in size with few restriction sites. Two pIL vectors were used with this experimental system ; pIL277 which is 4.3 kb in size and pIL253 which is 4.8 kb and also has increased copy number. Both of these plasmids carry only the original erythromycin-resistance determinant from pAMß1 and hence neither can be used in *E. coli* as all laboratory strains of *E. coli* are resistant to high levels of erythromycin. To facilitate the construction of recombinant plasmid based on these vectors and also the availability of workable quantities of DNA, all the initial cloning steps were performed in *B. subtilis* 1280.

EVIDENCE FOR SUCCESSFUL TRANSFORMATION USING pIL VECTORS.

Prior to any cloning experiments, it was necessary to confirm successful transformation into the experimental strains and also maintenance of the plasmids. Electroporation conditions as detailed in Chapter Two were used for plasmid DNA prepared from *L. lactis* subsp. *lactis* IL1403. Figures 5.10 and 5.11 show agarose gel electrophoresis of

Figure 5.9. Restriction enzyme (*Sst*I) digests of pA4-derivative from *Lactobacillus* strains.

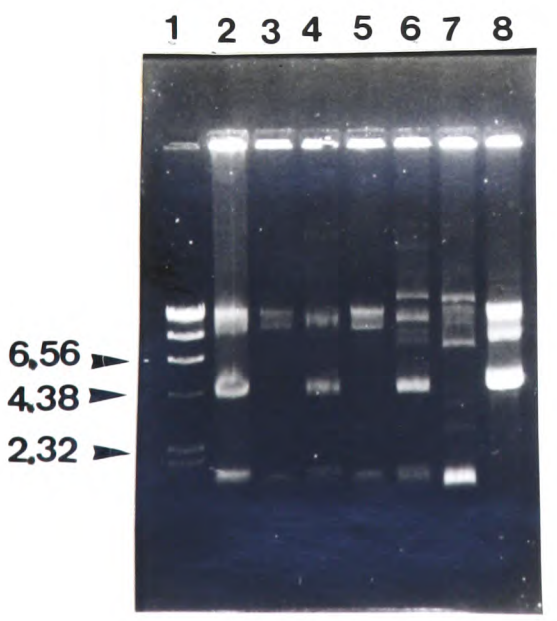
Lane 1, λ*Hind*III markers; lane 2, pTG262 from *E. coli*; lane 3, *Lb* 85 pA4; lane 4, *Lb* 72 pA4; lane 5, *Lb* 65 pA4; lane 6, *Lb* Lp pA4.



Sizes refer to λ *Hind*III markers.
Only Lb 72 (Lane 4) is confirmed as retaining intact pA4.

Figure 5.10. Plasmid profiles of *Lactobacillus* strains after transformation with pIL253.

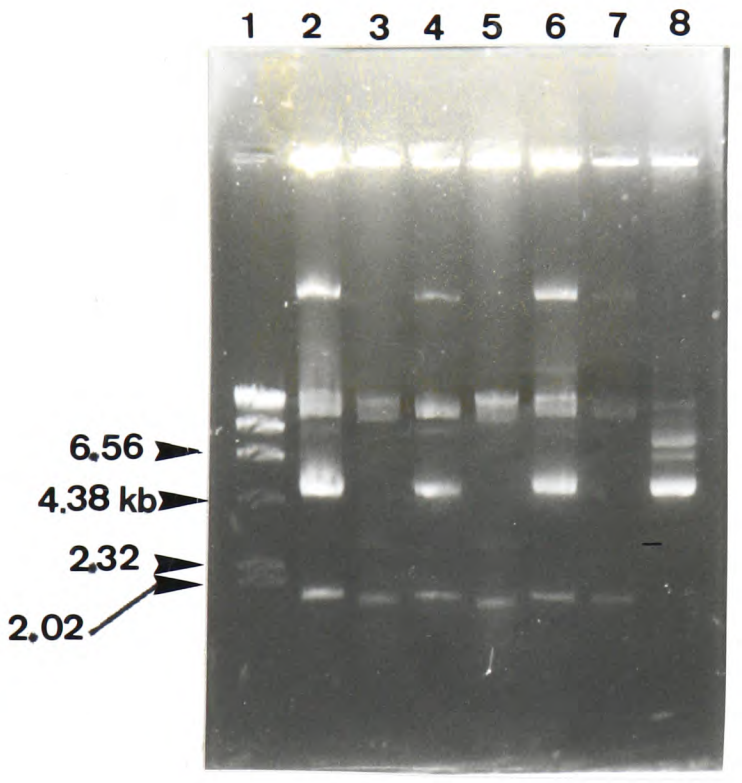
Lane 1, λ*Hind*III markers; lane 2, *Lb* 85 pIL253; lane 3, *Lb* 85 WT; lane 4, *Lb* Lp pIL253; lane 5, *Lb* Lp WT; lane 6, *Lb* 72 pIL253; lane 7, *Lb* 72 WT; lane 8, pIL253 from *L.lactis*.



Sizes refer to λ*Hind*III markers.
All transformants (Lanes 2, 4 & 6) confirm presence of pIL253.

Figure 5.11. Plasmid profiles of *Lactobacillus* strains after transformation with pIL277.

Lane 1, λ*Hind*III markers; lane 2, *Lb* 85 pIL277; lane 3, *Lb* 85 WT; lane 4, *Lb* Lp pIL277; lane 5, *Lb* Lp WT; lane 6, *Lb* 65 pIL277; lane 7, *Lb* 65 WT; lane 8, pIL277 from *L. lactis*.



Sizes refer to λ*Hind*III markers. A band corresponding to pIL277 is evident in all transformants (Lanes 2, 4 &6).

plasmid DNA from *Lb* strains following transformation by pIL253 and pIL277 respectively. A band corresponding in size to each vector was clearly visible within the proposed transformants while no such band appeared in the relevant WT plasmid DNA preparations. Confirmation that this band did indeed represent the vector was obtained by isolation of the band from the gel by the freeze-squeeze technique, followed by restriction endonuclease digestion analysis (Figure 5.12).

USE OF pIL VECTORS AS CLONING VEHICLES.

a) pIL277

The cellulase-encoding fragments of pWM95 and pWM191 were isolated as *Eco*RI-*Pst*I fragment and *Bam*HI-*Pst*I fragments respectively. These fragments were considered to be the most convenient for use with the pIL vectors. Each was ligated to the equivalent digestion of pIL277 and then used to transform *B. subtilis* 1280 protoplasts. Recombinants were selected by erythromycin resistance.

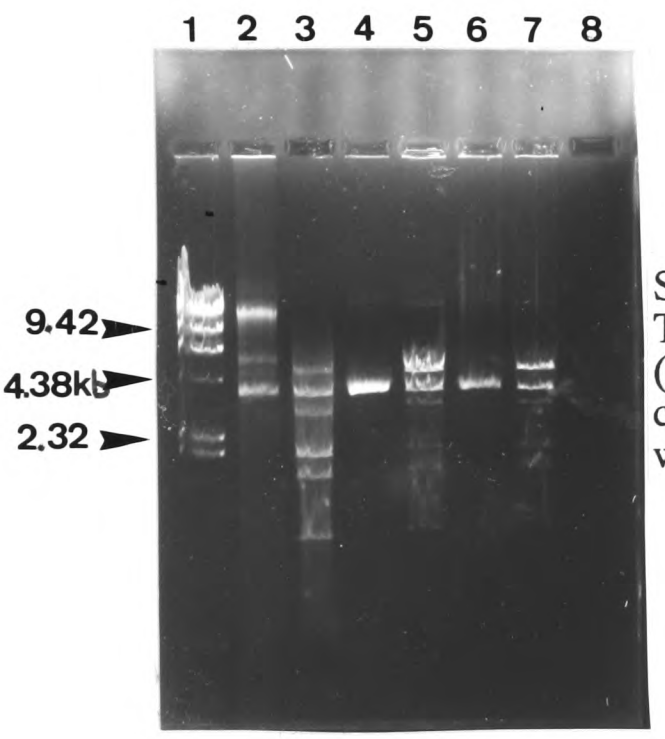
The selection of recombinants based on the pIL vectors is not facilitated by the use of a chromogenic substrate as with pTG262, nor is it possible to use a second antibiotic selection and hence the procedure described above involves the screening of all erythromycin resistant colonies to determine which are due to recombination rather than simply spontaneous mutation or self-ligation of the vector.

The initial set of ligations and cloning experiments resulted in the isolation of pIL277 containing the cellulase-encoding fraction of pWM95. This construct was designated pM1 (Figure 2.8). Restriction endonuclease analysis of pM1 confirmed the presence of the expected sites from both pIL277 and pWM95, including the indicator *Sst*I fragment which confirmed the presence of the cellulase-encoding region. At this point, the ligation of the cellulase positive fraction of pWM191 to pIL277 was unsuccessful.

After confirming the structural integrity of the construct, pM1 was transformed into the experimental *Lactobacillus* and *Pediococcus* strains by electroporation. Figure 5.13 shows the plasmid profiles from these proposed transformants along with the appropriate

Figure 5.12. Restriction enzyme analysis of pIL277 isolated from *Lactobacillus* strains.

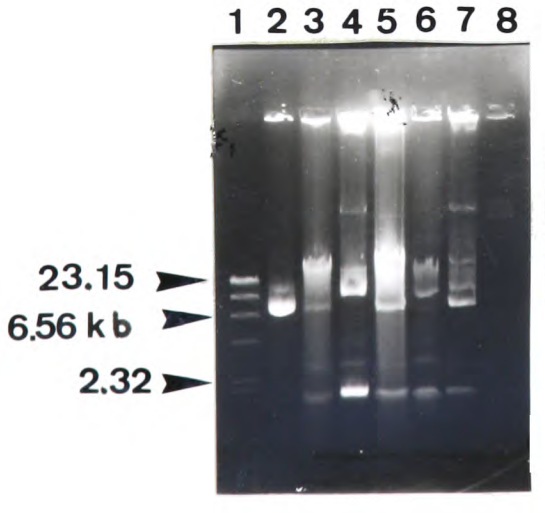
Lane 1, λ*Hind*III markers; lane 2, pIL277 (from *L. lactis*) undigested; lane 3, pIL277 (from *L. lactis*) *Hind*III; lane 4, *Lb* 85 pIL277 undigested; lane 5, *Lb* 85 pIL277 *Hind*III; lane 6, *Lb* Lp pIL277 undigested; lane 8, *Lb* Lp pIL277 *Hind*III.



Sizes refer to λ*Hind*III markers.
The digest of pIL277 from *L. lactis* (Lane 3) matches those from the Lb strains confirming successful transformation with pIL277.

Figure 5.13. Plasmid profiles of *Lactobacillus* and *Pediococcus* strains after transformation with pM1.

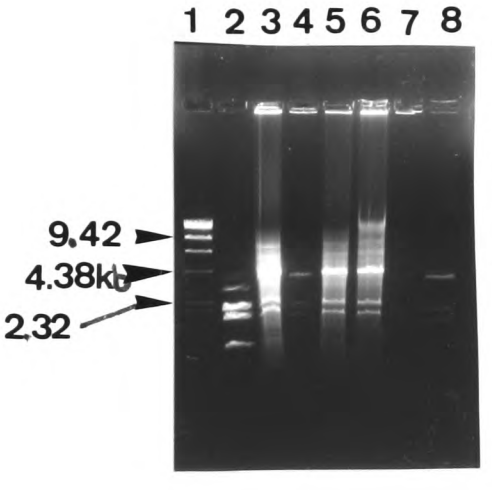
Lane 1, λHindIII markers; lane 2, pM1 from *B. subtilis*; lane 3, *Lb* 85 pM1; lane 4, *Lb* 85 WT; lane 5, *Lb* Lp pM1; lane 6, *Lb* Lp WT; lane 7, *Pd* 37 pM1; lane 8, *Pd* 37 WT.



Sizes refer to λ*Hind*III markers.
A band corresponding to pM1 is evident in all transformant preparations (Lanes 3, 5 & 7).

Figure 5.14a. Restriction enzyme (*Hind*III) digests of pM1 originally isolated from *Lactobacillus* strains.

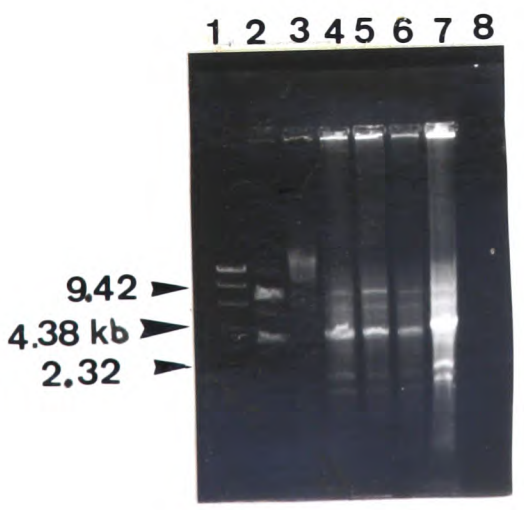
Lane 1, λ *Hind*III markers; lane 2, pIL277; lanes 3 - 7, pM1 from *Lb* 85; lane 8, pM1 from *B. subtilis*.



Sizes refer to λ *Hind*III markers. Digests from Lb strains exactly match those of pM1 from *B. subtilis* confirming its structural integrity within the lactobacilli.

Figure 5.14b. Restriction enzyme (*Hind*III) digests of pM1 originally isolated from *Pediococcus* strains.

Lane 1, λ *Hind*III markers; lane 2, pM1 from *B. subtilis*; lanes 3 & 8, pM1 from *Pd* 1; lanes 4 - 6, pM1 from *Pd* 37.



Sizes refer to λ *Hind*III markers. Digests from Pd strains exactly match those of pM1 from *B. subtilis*, confirming its structural integrity within the pediococci.

WT profiles. A band corresponding to pM1 was visible within all the transformants. Isolated pM1 from these transformants was then used to transform the plasmid-free *L. lactis* subsp. *lactis* strains MG1363 and IL1403. Restriction digests of the plasmid obtained matched those of the original *B. subtilis* 1280 pM1 preparation (Figure 5.14).

b) pIL253

The cellulase-encoding part of pWM191, isolated as a *Bam*HI-*Pst*I fragment, was ligated to the equivalent digest of pIL253. The construct (pM2, Figure 2.9) was transformed into *B. subtilis* 1280 and selected by erythromycin resistance. The structural integrity of pM2 was confirmed by restriction digests (Figure 5.15). pM2 was then transformed into the *Lactobacillus* strains and its presence confirmed by transformation into *B. subtilis* or *L. lactis* and subsequent restriction digests (Figure 5.16).

CELLULASE ACTIVITY

The *Cellulomonas flavigena* fragments of pWM95 and pWM191 both encode endo-ß-1,4-glucanases which can be expressed within *E. coli* TG1 (Akhtar *et al.*, 1988). This has been confirmed within this study by using a congo red plate assay where zones of clearing were visible around the areas of growth of *E. coli* TG1 containing the plasmids but not with WT TG1.

Unfortunately, it proved impossible to determine the presence of cellulase activity from pM1 or pM2 within *B. subtilis* 1280 since the WT strain also exhibited cellulase activity under the congo red plate assay.

Detection of cellulase activity from *Lactobacillus* and *Pediococcus* strains containing pM1 or pM2 was not possible using MRS agar as the basis of the plate assay, perhaps due to acid production from these strains. A similar situation has been reported by Bates *et al.* (1989) for the detection of another cellulase gene within *L. plantarum* and using information thus supplied the assay was subsequently performed using Fastidious Anaerobe Agar (Lab M, England). No such problems were encountered with the use of

M17 agar for the determination of cellulase production from *L. lactis*.

The presence of the constructs pM1 or pM2 did not result in detectable cellulase production from any of the *Lactobacillus* or *Pediococcus* strains, nor from either of the *L. lactis* strains, even though the cellulase-encoding fragment had been confirmed as a constituent of these constructs.

In all cases, detection was by culturing on to CMC-containing agar plates prior to the congo red assay. With *Lactobacillus* spp., cell extract (obtained by a bead-beater) was also used. No cellulase activity was detected in either case.

Confirmation of the coding potential of the cellulase fragment.

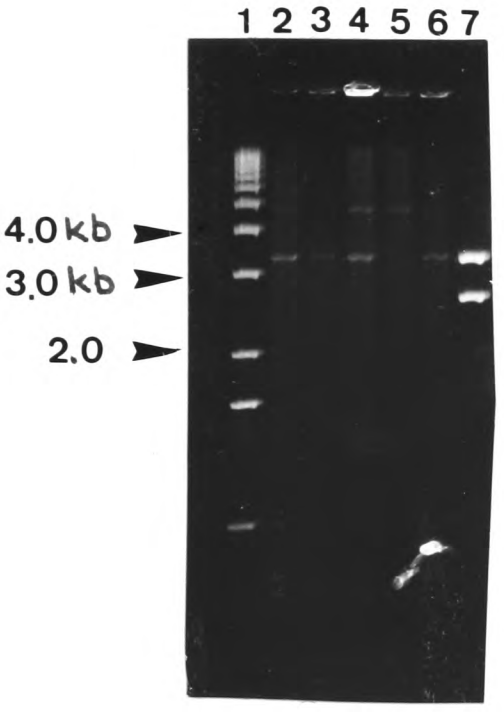
One possible explanation for the lack of cellulase production within the LAB may have been an undetected rearrangement within the DNA which had rendered the coding sequence inoperative.

In order to test this theory, pM1 was transformed into *L. lactis* subsp. *lactis* MG1363 (plasmid free) from *Lactobacillus* 85 and purified from caesium chloride gradients. The cellulase-encoding portion of pM1 was isolated as an *Eco*RI-*Pst*I fragment and ligated to the equivalent digest of pUC18 to recreate pWM95. The integrity of this construct within *E. coli* TG1 was confirmed with restriction analysis and compared to digests of original pWM95 as shown in Figure 5.17. The identical reactions obtained confirmed the recreation of pWM95.

Both isolates of pWM95 resulted in cellulase production on CMC plates under the congo red plate assay (on minimal medium) whereas parent TG1 did not. Hence, it was confirmed that the coding potential of the cellulase fragment of pM1 was intact and that the lack of cellulase production within the LAB was not due to a previously undetected DNA rearrangement.

Figure 5.15. Restriction enzyme (*Sst*I) digests of pM2 to confirm its structural integrity.

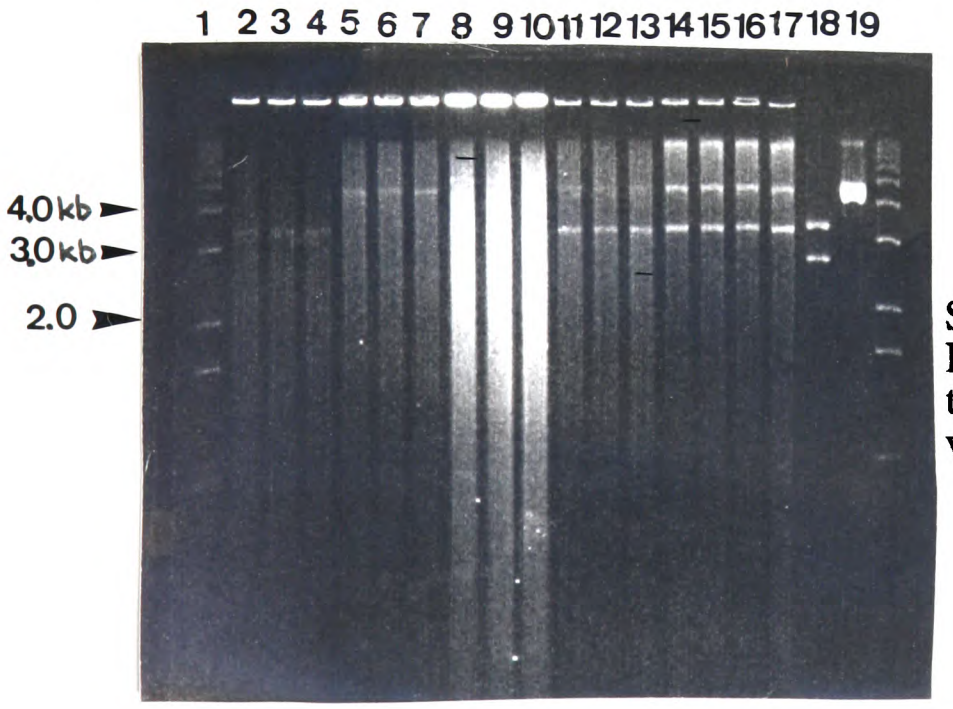
Lane 1, 1kb ladder; lanes 2 -6, pM2 from *B. subtilis*; lane 7, pWM191 from *E. coli*.



Sizes refer to markers. Digests of pM2 from Lb strains match that from *B. subtilis* confirming its integrity.

Figure 5.16. Restriction enzymes digests (*Sst*I) of pM2 from *Lactococcus lactis*.

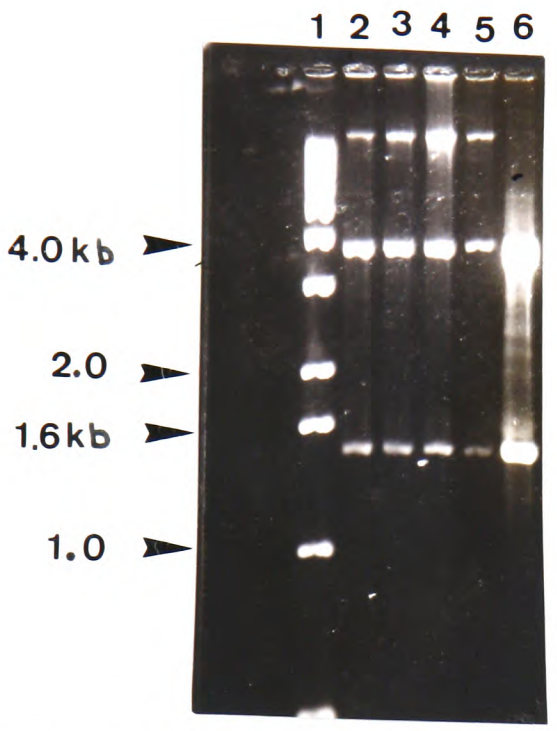
Lane 1, one kb ladder; lanes 2 - 17, pM2 isolates; lane 18, pWM191 *Sst*I; lane 19, pIL253 *Sst*I; lane 20, one kb ladder.



Sizes refer to markers. Digest pattern confirms the integrity of pM2 within *L. lactis*.

Figure 5.17. Recreation of pWM95 to confirm coding potential of cellulase-encoding fragment. (Sst I digests)

Lane 1, 1 kb ladder; lane 2 -5, recreated pWM95; lane 6, original pWM95.



Sizes refer to markers. Pattern from original and recreated pWM95 match, confirming successful re-creation.

5.4) pMTL VECTORS

The pMTL vectors (Chambers *et al.*, 1988) are a set of cloning vectors based on almost exactly the same pBR322 backbone as the pUC vectors (Vieira & Messing, 1982). The pMTL vectors differ in the polylinker construction and in being *nic*$^-$ (*ie* unable to be mobilised even in the presence of *trans* acting mobilisation proteins) which allows the lowest categorisation for genetic manipulation experiments. Like the pUC vectors, all the pMTL plasmids contain the *lac*Z gene which enables the identification of recombinants with the chromogenic substrate X-gal.

The plasmid pMTL20 has been used as the basis to create a vector capable of replication and expression in Gram-positive organisms as well as Gram-negatives such as *E. coli* (Oultram *et al.*, 1988). This shuttle vector, known as pMTL500E (Figure 2.10), was made using the erythromycin resistance gene and the Gram-positive origin of replication from pAMß1.

In addition to the original shuttle vector pMTL500E, two other plasmids were also constructed: pMTL500F (Figure 2.11) contains a (Gram-positive) promoter from a clostridial ferrodoxin gene and pCC1 (Figure 2.12) which is pMTL500E with the *Cel*C (endo-ß-1,4-glucanase) gene from *Clostridium thermocellum* (Schwarz *et al.*, 1988).

EVIDENCE FOR SUCCESSFUL TRANSFORMATION WITH THE pMTL PLASMIDS.

Caesium chloride-purified pMTL500F from *E. coli* TG1 was used to transform some of the experimental strains by electroporation. Plasmid DNA from these transformants was used to transform *E. coli* MC1022 to confirm the presence of pMTL500F. Restriction enzyme analysis from the *E. coli* transformants is presented in Figure 5.18.

Repeated attempts to transform the experimental strains with pMTL500E were all unsuccessful, even under conditions where pMTL500F gave a good level ($1x10^2$cfu/µg DNA) of transformants.

Successful transformation of some of the experimental *Lactobacillus* strains was also achieved using pCC1.

Figure 5.19 shows a plasmid band corresponding to pMTL500F and pCC1 in the *Lactobacillus* strains. No similar band was visible from WT plasmid DNA preparations. This successful transformation was confirmed by a hybridisation experiment using linear pMTL500F as the probe. Bands corresponding to pMTL500F and pCC1 were visible within the transformants (Figure 5.20).

Further confirmation was obtained from the transformation of *E. coli* MC1022 with pMTL500F and pCC1 from some of the *Lactobacillus* strains. Figure 5.21 presents agarose gel electrophoresis of restriction enzyme analysis of these *E. coli* transformants. A band pattern identical to the original plasmid was obtained in each case.

CELLULASE ACTIVITY

As previously determined, the WT *Lactobacillus* strains had no cellulase activity under the congo red plate assay. Transformants containing pMTL500F were also cellulase negative but pCC1 transformants were positive, as indicated in Figure 5.22.

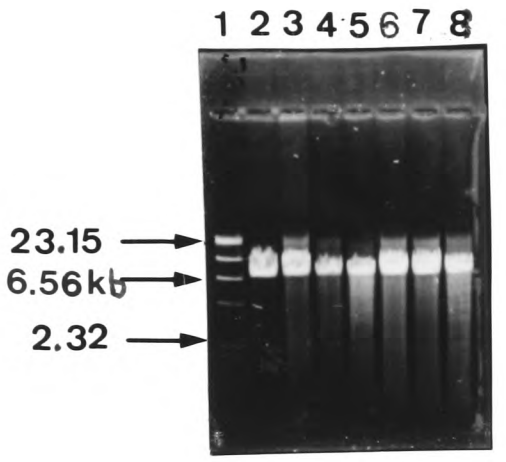
USE OF pMTL500F AS A CLONING VECTOR

pMTL500F contains a Gram-positive promoter sequence and hence can be termed an expression vector *ie* one which can direct the expression of inserted DNA rather than relying on a promoter carried within the insert. The clostridial ferrodoxin promoter carried on pMTL500F may aid the expression of the cellulase genes from *Cellulomonas flavigena* in the experimental *Lactobacillus* strains.

Attempts were made to clone the cellulase-encoding part of pWM95 as a *Kpn*I-*Pst*I fragment to the equivalent digest of pMTL500F. All such cloning experiments were unsuccessful and lack of time precluded further effort.

Figure 5.18. Restriction enzyme (*Bgl*II) digests of pMTL500F from *Lactobacillus* strains.

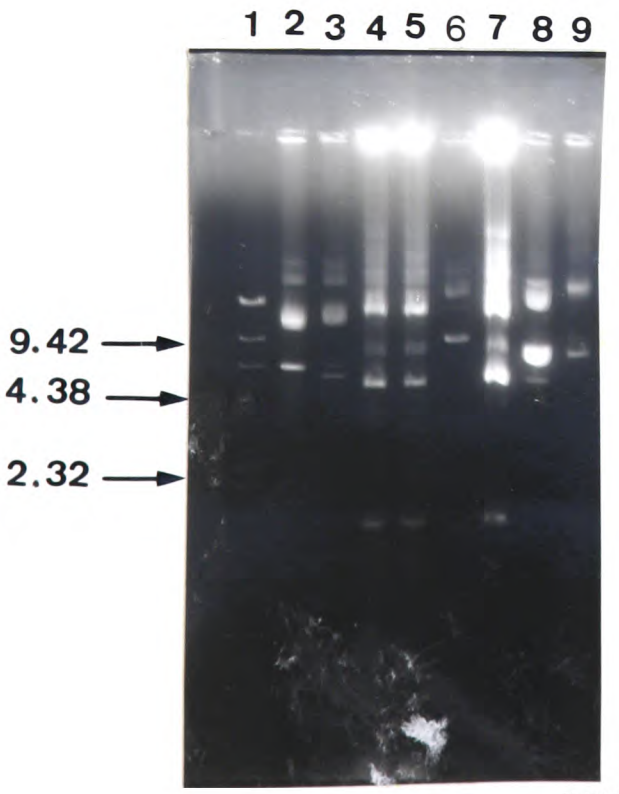
Lane 1, λ*Hind*III markers; lane 2, original pMTL500F; lanes 3, 4 & 7, pMTL500F from *Lb* 85; lanes 5, 6 & 8, pMTL500F from *Lb* Lp.



Sizes refer to λ*Hind*III markers. Digests of pMTL500F from Lb match that from *E. coli* confirming its integrity.

Figure 5.19. Plasmid profiles of *Lactobacillus* strains after transformation with pMTL500F and pCC1.

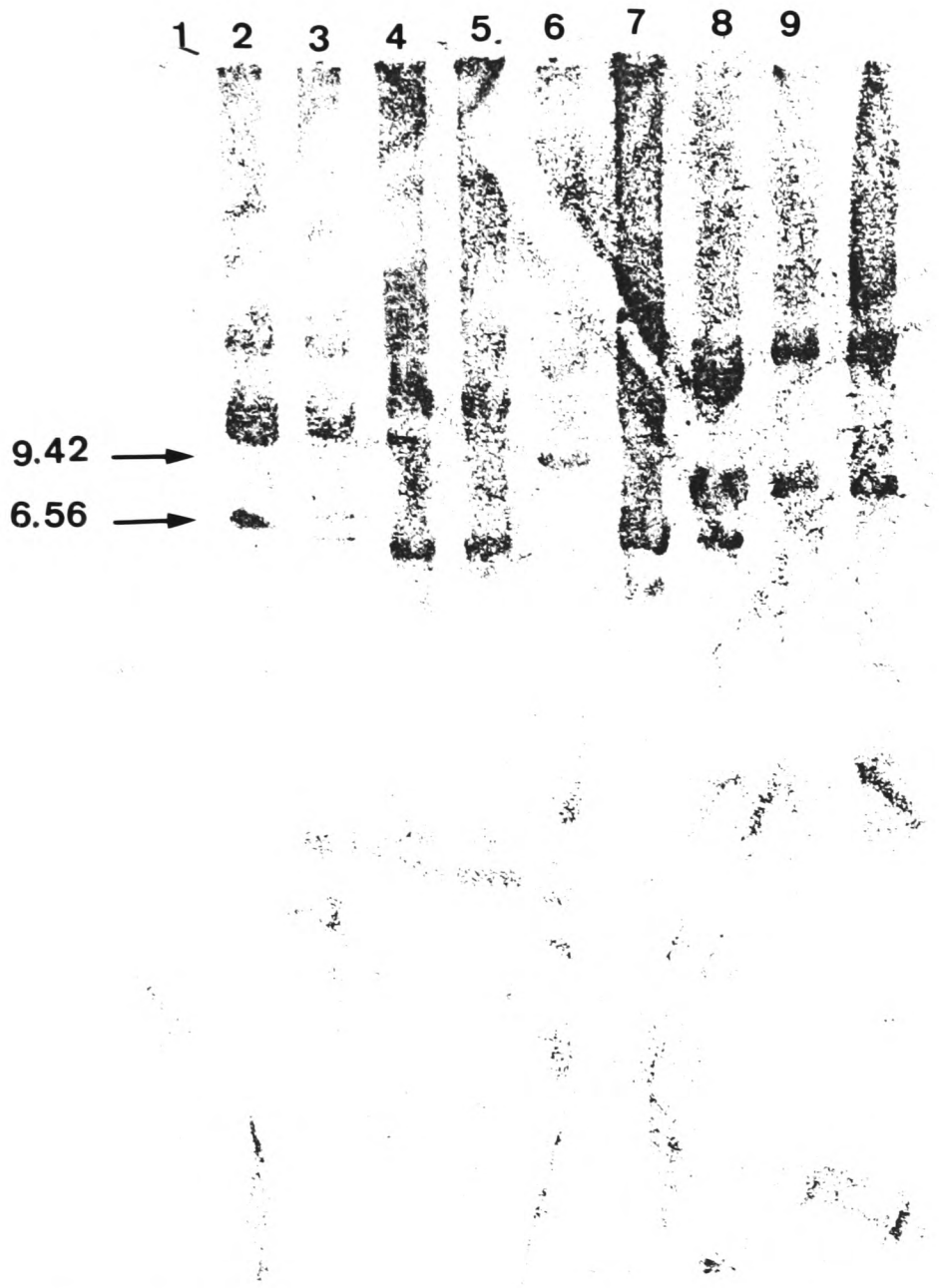
Lane 1, λ*Hind*III markers; lane 2, pMTL500E from *E. coli*; lane 3, pMTL500F from *E. coli*; lane 4, *Lb* 85 pMTL500F; lane 5, *Lb* Lp pMTL500F; lane 6, pCC1 from *E. coli*; lane 7, *Lb* 65 pCC1; lane 8, *Lb* 85 pCC1; lane 9, *Lb* 85 WT.



Sizes refer to λ*Hind*III markers.

Figure 5.20. Hybridisation pattern using labelled pMTL500F.

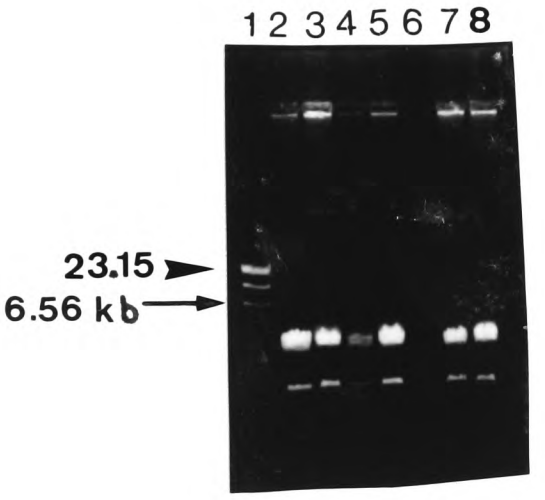
Legend as for Figure 5.19.



The sizes refer to λ*Hind*III markers. Areas of hybridisation correspond to presence of pMTL500F or pCC1.

Figure 5.21. Restriction enzyme (*Bam*HI) digests of pCC1 originally isolated from *Lactobacillus* strains.

Lane 1, *Hind*III markers; lane 2, pCC1 from *E. coli*; lanes 3 & 4, *Lb* 65 pCC1; lanes 5 - 8, *Lb* 85 pCC1.



Sizes refer to λ *Hind*III markers. Digests of pCC1 from Lb stains match that from *E. coli*, confirming its integrity within the lactobacilli.

Figures 5.22a and b. Zones of clearing after staining with congo red indicating cellulase production from *Lactobacillus* strains 85 pCC1 (a) and 65 pCC1 (b).

a) Left = *Lb* 85 pCC1; right = *Lb* 85 WT.

b) Left = *Lb* 65 WT; right = *Lb* 65 pCC1.

a

b

Zones of clearing are visible from both transformants.

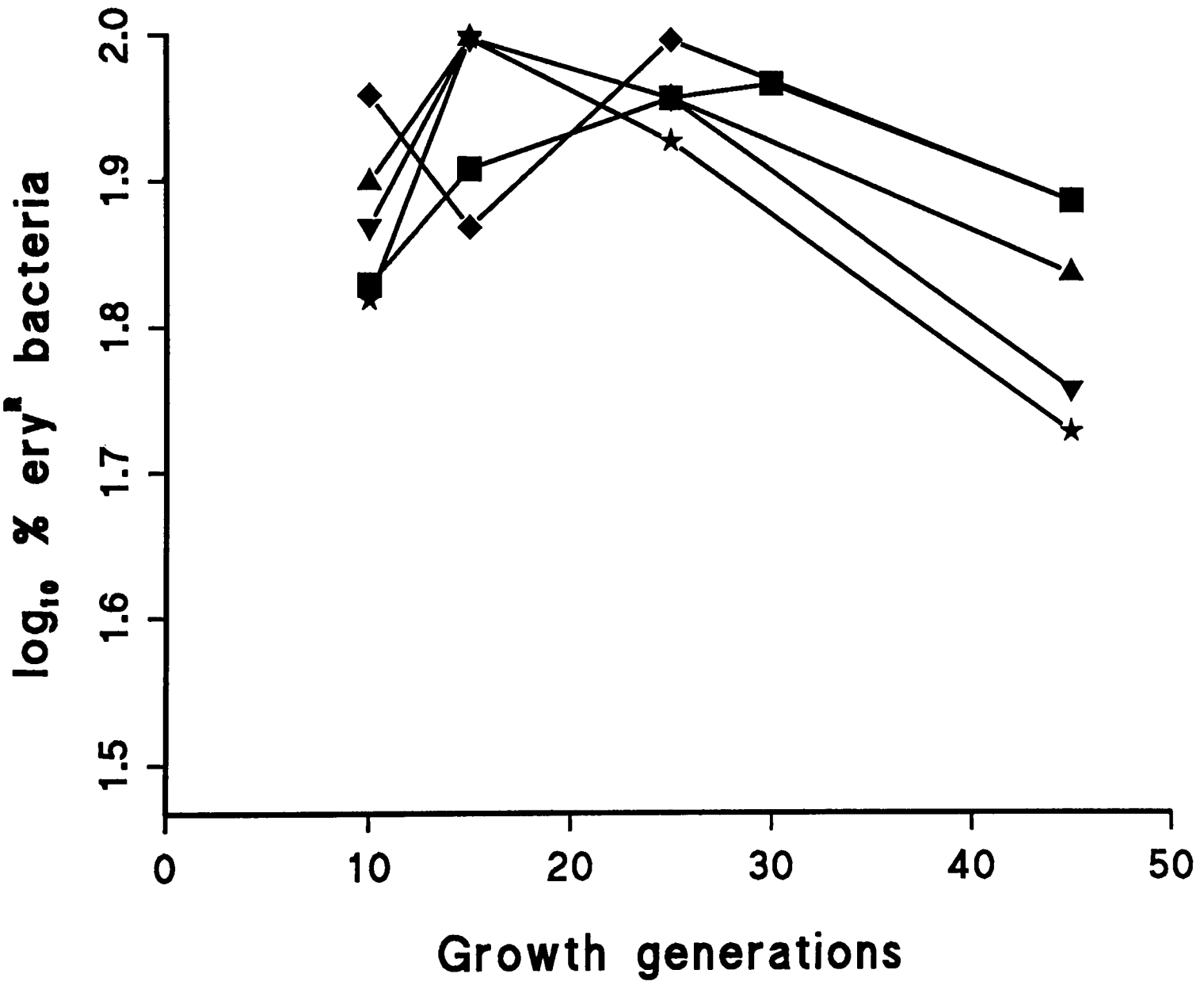
PLASMID STABILITY

All of the plasmids (with the exception of pA4) were stably maintained within the *Lactobacillus* and *Pediococcus* strains under selective pressure.

The stability of some of the plasmids was also investigated in the absence of selective pressure.

Strains were grown through 45 generation by serial dilution, then aliqots plated for viable counts on media with and without erythromycin. The results are shown in Figure 5.23.

Figure 5.23. Plasmid stability in the absence of selective pressure.



CHAPTER SIX

DISCUSSION

ENSILAGE

All the experimental LAB strains used in this study were isolated from a grass silage and hence by their very nature were likely to be good candidates for use as silage additives. Bacterial cultures make up a good proportion of the silage additives currently available; their effectiveness is dependent upon the ability to initiate and maintain fermentation leading to lactic acid production. None of silage additives on the market at present consists of genetically manipulated micro-organisms as there are strict codes of practice and soon-to-be-in-place legislation governing the release of manipulated organisms into the environment. There is a distinct possibility that genetically-manipulated silage bacteria may never be legal and/or that the cost of such preparations may be prohibitive compared with other commercially available additives.

In an assessment of the functions which may improve a silage strain, activities such as enzyme production for increased substrate utilisation, antimicrobial production to inhibit the growth of undesirable organisms, or enhanced LA production to lead to rapid stabilisation of the substrate could all be potentially advantageous.

Classical techniques of strain selection can be as important in the development of new "starter" strains as any benefits which genetic recombination may offer. The existence of amylase-producing strains of *Lactobacillus* have been characterised (Nakamura & Crowell, 1979; Nakamura, 1981) and it may be possible to select such strains for suitability as silage additives.

The production of bacteriocins and bacteriocin-like substances within the LAB has been well documented and indeed one of these products, nisin from *L. lactis* (Mattick & Hirsch, 1947), is now a food-grade substance safe for consumption. Nisin resistance may also have potential as a plasmid marker, hence avoiding the use of antibiotic markers

(von Wright *et al.*, 1990).

Traditional silage additive strains generally possess a rapid fermentation ability, detected by testing and analysis in laboratory silos (Lindgren & Dobrogosz, 1990).

Another prerequisite for suitable silage additive strains is genetic stability so that all functions for which the strain was originally selected are maintained throughout the fermentation. This requirement is particularly important with respect to genetic manipulation; the vector system used must be maintained within silage without any selective pressures. One method of attempting stable inheritance of new traits is integration into the chromosome of the host strain (*eg* Scheirlinck *et al.*, 1989). Full details are discussed below.

ELECTROPORATION

The main requisite for genetic manipulation is the ability to transfer DNA between different organisms. Conjugative transfer is well documented within LAB (*eg* Kempler & McKay, 1979; Chassy & Rokaw, 1981; Shrago *et al.*, 19 ..t there is no natural transformation. The technique of electroporation h ..at potential for facilitating the transfer between many different types of ba ..ince it has a relatively simple methodology and is theoretically not bou ..the strain- and species-dependencies inherent in other techniques.

Although one of the initial reports of electror .. (Chassy & Flickinger, 1987) used a member of the LAB, it was first neces.. demonstrate that the experimental strains isolated from silage could be su.. ole to this form of DNA transfer. The initial electroporation experiments w ..ccessful but the yield of transformants and hence the process of transfer itself ..ound to be rather inefficient. Therefore, an optimisation study was set up to ..pt to achieve an optimal level of transformation and also to increase the total yield of transformants. Screening of many transformants is much more likely to allow detection of recombinants than screening of small numbers.

From the optimisation results, sucrose buffer was found to be the most successful of the buffers tested. Sucrose buffer has a low ionic strength and hence has a higher resistance compared with phosphate buffer, resulting in longer time constants *ie* 2.3ms compared with 1.9ms (using the pulse controller). It is possible that the increase in transformation efficiency with sucrose buffer is related to these longer time constants; perhaps the (theoretically) longer pulse causes greater damage to individual cells or damages a greater proportion of the cell population and hence renders them susceptible to transformation.

The finding that the best buffer contained no divalent ca(calcium or magnesium) was unexpected since it is generally accepted that t nhance the uptake of DNA. Transformation of *E. coli* is dependent upon the pr of calcium ions to facilitate the uptake of DNA (Mandel & Higa, 1970; Dagert lich, 1979). Similarly, DNA uptake in *Streptococcus pneumoniae* is absolutel t on calcium ions (Smith *et al.*, 1981) while, in *B. subtilis*, binding of DNA ccur in the absence of divalent ions but magnesium is essential for DNA ur (Smith *et al.*, 1981). Magnesium ions are also used in the medium for transfo on of *L. lactis* subsp. *lactis* protoplasts (Gasson, 1980) and electroporation o *actis* subsp. *lactis* (Powell *et al.*, 1988) as well as the electroporation of other bacilli (Chassy & Flickinger, 1987). The mechanism of action of these divale ions is unclear; they may shield the phosphate groups of the DNA in such a wa to facilitate the otherwise unlikely association of two phosphate-rich structures, NA and the bacterial cell (Hanahan, 1983).

The data generated from the experiments using the pulse controller, the 0.2cm inter-electrode distance cuvettes and increased voltages, all indicated a connection between the level of survival immediately after the pulse and the transformation efficiency; the TE increased as the survival level dropped. In contrast, increases in resistance (and hence time constant) also led to increases in levels of cell death but corresponded to a decrease in TE. It would appear that the time constant is the critical factor; increases in pulse

length may push the level of cell death beyond that which is optimal for transformation.

Varying the level of DNA used to perform the transformation experiments led to the generation of a DNA saturation curve consistent with those achieved during classical transformation experiments (Hayes, 1968). DNA saturation is a function of the binding of the DNA to the cell or of the uptake of the DNA by the cell. This suggests that the mechanisms of DNA uptake are the same for electroporation as for other transformation methods. There is some published evidence to suggest that uptake of DNA during electroporation does not involve prior binding to the cell surface (Dower *et al.*, 1988). More recent published reports suggest the opposite (Xie *et al.*, 1990; T[illegible] 1990) and indeed these experiments have shown that transformation eff[illegible]y is increased dramatically by prior DNA binding (all of these reports used st[illegible] *E. coli*).

While recent studies into the mechanisms of elect[illegible]ation have been able to demonstrate the occurrence of transient pores in a[illegible]n erythrocyte) cell surface by rapid freezing electron microscopy, they have [illegible]ggested that uptake of DNA through these pores is unlikely because of the re[illegible] small surface area that the pores occupy (Tsong, 1990).

The indication from t[illegible]erimental results that freezing of cell culture prior to electroporation gr[illegible]duced the efficiency was mildly surprising since glycerol is a recognised cryopr[illegible]ctant and it has also been shown in Chapter Four that this reduction in transformation was not simply due to a loss of viability caused by freezing. With *L. lactis* LM0230, McIntyre & Harlander (1989b) found that while freezing and storage in 10% glycerol at -20°C and -60°C did cause a transformation drop, freeze-drying at -60°C did not. No investigation of the benefits or otherwise of freeze-drying were undertaken in the present study. Storage at -85°C for several months has not been found to be detrimental to *Lactobacillus curvatus* (Gaier *et al.*, 1990) while no reduction in the number of transformants after freezing has also been recorded for *L. plantarum* CCM

1904 (Bringel & Hubert, 1990). From the differences in the published methods it would seem that the effect of freezing on transformation is strain dependent. The major disadvantage caused by the inability to freeze cultures to be used for electroporation is the lack of a common source from which a standard level of transformants may be gained.

Following the buffer changes at the beginning of the optimisation, the most successful procedures for increasing the TE were the use of the cell wall weakening agents, especially glycine. Other experimental parameters such as field strength and time constant exhibited a relationship between the level of cell damage and transformation, both of which increased until an optimal cell damage was reached. The use of glycine in particular also reflected this relationship; maximal levels of cell damage were reached at concentrations of glycine over 2%.

A review of the published reports on electroporation of the LAB demonstrates that while many species and strains react similarly, there is still a substantial degree of variation. This was also evident from the data presented here where all of the *Pediococcus* strains were transformed much less efficiently than the *Lactobacillus* strains. Despite this disadvantage, transformation was still successful and both species were proved to be potential candidates for further cloning work.

VECTORS

Successful transformation of the LAB within this study was achieved with a variety of vectors. Not all of the vectors were considered to be suitable for cloning. The main advantage of pNZ12 (de Vos, 1987) is its broad host range and along with a range of other vectors, some based on the same replicon, has proved to be successful in other cloning studies *eg* de Vos & Gasson, 1989; de Vos *et al.*, 1989.

Both pTG262 and the pIL vectors were recognised to have greater potential for cloning

within the present study than pNZ12 because of the range of unique restriction enzyme sites within each of these plasmids allowing a greater choice for cloning.

The structural instability of the cellulase-encoding constructs within pTG262 was apparently caused by loss of the insert and probably also the multicloning site. The loss of the multicloning site from this vector has been a problem in previous work (M. Gasson, personal communication). As a result of this instability, emphasis was switched to the pIL vectors (Simon & Chopin, 1988).

Despite evidence (presented in Chapter Five) that the cellulase-encoding constructs based on the pIL vectors had been successfully created and were transferred and maintained intact within the experimental Lb strains, no evidence of cellulase production was detected.

The pMTL vectors (Chambers *et al.*, 1988) are a range of vectors based on the same replicon as the pUC vectors (Vieira & Messing, 1982) which have been constructed specifically for M13 sequencing. A Gram-positive to Gram-negative shuttle vector, pMTL500E, has been created from pMTL20 combined with the erythromycin-resistance gene and the replication origin of pAMß1 and has been shown to be successfully maintained within a Gram-positive host, *Clostridium acetobutylicum* (Oultram *et al.*, 1988).

Plasmid pCC1, kindly loaned by NP Minton, consists of the *cel*C gene of *Clostridium thermocellum* within pMTL500E. The inclusion of pMTL500E allowed the construction of another set of cellulase-encoding constructs, in this case recombinants detected by the insertional inactivation of the *lac*Z' gene. In addition, pCC1 provided a positive control system for the detection of cellulase production from the experimental strains.

CELLULASE PRODUCTION

The detection of cellulase activity was carried out by the congo red plate assay of Teather & Wood (1982). The substrate within this assay is a soluble form of cellulose, carboxymethylcellulose (CMC) and therefore strains which exhibit cellulase activity by

this assay are, strictly speaking, exhibiting CMC-ase activity. The dye congo red forms a pink complex with the ß1,4-glucan of the substrate and hence the plate assay is only useful in the detection of ß1,4-glucanases.

The detection of zones of clearing under the congo red assay is reliant on either secretion of the enzyme or leakage through the cell wall. Since the *Cellulomonas* cellulase gene products are apparently secreted by *E. coli* (Akhtar *et al.*, 1988) and *cel*C from *Clostridium thermocellum* also produces zones of clearing under the congo red plate assay (Schwarz *et al.*, 1987), it was not anticipated that detection by the same assay would cause any problems in the LAB used here.

CELLULASE-ENCODING GENES

1) *Cellulomonas flavigena* DNA

The genus *Cellulomonas* comprises Gram-positive, slender rod-shaped bacteria most of which can grow anaerobically, ferment glucose to acid and are cellulolytic. *Cellulomonas flavigena* is the type species (Bergey's Manual of Systematic Bacteriology, Volume Two First Edition, 1986).

The cellulase-encoding genes of some *Cellulomonas* species have been studied quite extensively *eg* two endoglucanase genes, the latter named *cen*A (Whittle *et al.*, 1982; Wong *et al.*, 1986) and a cellobiohydrolase, *cex* (O'Neill *et al.*, 1986) have all been cloned from *Cellulomonas fimi.* Genes from *Cellulomonas uda* have also been studied (Nakamura *et al.*, 1986).

Many cellulolytic bacteria produce a range of cellulase-degrading enzymes rather than a single enzyme and *Cellulomonas* spp. are no exception (Knowles *et al.*, 1987). The two main cellulase-encoding genes from *Cellulomonas fimi* (one endoglucanase and one exoglucanase) have both been expressed in *E. coli* under the control of Gram-negative promoters, thus achieving growth of *E. coli* on cellulose as a substrate (Din *et al.*, 1990).

The cellulase genes of *Cellulomonas flavigena* were originally regarded as suitable for cloning into *Lactobacillus* since they come from a Gram-positive organism which is

capable of anaerobic growth (production in silage would be under anaerobic conditions). Their use was made absolutely necessary due to a lack of alternatives at the time.

The cellulase-encoding genes contained within pWM95 and pWM191 were both ß1-4, endoglucanases isolated from *Cellulomonas flavigena* from a soil sample from Pakistan (Akhtar *et al.*, 1988). Both of these plasmids have been demonstrated to transform *E. coli* TG1 to cellulase production using the congo red assay.

The cellulase-encoding fragment of pWM95 is a 2.4kb fragment of *C. flavigena* chromosomal DNA while that within pWM191 is 3.4kb in size; radio-labelled probe analysis has suggested that these two inserts may encode different genes since there was little hybridisation homology between them (Akhtar *et al.*, 1988). Sequence analysis of the fragment within pWM95 has revealed two open reading frames (ORFs), both in the same orientation. One of these, ORF A has been identified as encoding CMC-ase activity from deletion studies and was found to show homology upstream of the initiation site to the *cex* gene (exoglucanase-encoding) and the *cen*A gene (endoglucanase-encoding) both from *Cellulomonas fimi*. ORF A was also shown to exhibit strong homology (45% at the DNA level) throughout its length to the *cel*B gene of *Clostridium thermocellum* (A. Kelly, personal communication). The 3' end of ORF B has also been shown to resemble the 3' end of *cex* (66% homology at DNA level) and of *cen*A (50% homology at DNA level). Both ORF's contain putative active sites (A. Kelly, personal communication).

In an interesting analogy between the cellulase enzymes from *Cellulomonas flavigena* and *Clostridium thermocellum*, it is worth noting that there is very little homology between the different cellulase genes from *C. thermocellum* and at least one *Cellulomonas* spp. has been found to make cellulosomes (Lamed & Bayer, 1988).

At the time of writing, no further information on the cellulase encoding gene(s) of pWM191 is available. No sequence information which may have allowed localisation of the cellulase genes to smaller parts of the inserts has been produced. This is quite a distinct disadvantage since further "pruning" of non-encoding parts of the insert may

have enhanced the expression of the cellulase gene(s) within another vector system. Precise positioning of the *Cellulomonas* genes with a *Lactobacillus/Pediococcus* promoter to aid expression was also precluded due to the lack of sequence information. The lack of sequence information also prevents an investigation of the orientation of the cellulase genes within the vectors.

The cellulase-positive plasmids pWM95 and pWM191 are based on pUC18 (Yanisch-Perron *et al.*, 1985) and hence were selected on the basis of insertional inactivation of the *lac*Z gene. The pUC series of vectors are expression vectors which have been designed to include control signals for efficient transcription of cloned sequences (Balbas *et al.*, 1986). The pIL vectors are cloning vectors, designed to carry foreign pieces of DNA between different strains of bacteria and contain none of the control signals present within the pUC vectors. Hence, expression of an insert of foreign DNA within the pIL vectors is more heavily reliant on its own expression signals and their recognition by the host strain's enzymes. It is unlikely that this difference would explain the apparent failure of the *Cellulomonas* cellulase-encoding sequences to express within the LAB used here since the *E. coli lac*Z expression signals would not be expected to function in *Lactobacillus*.

The plasmid pMTL500F has been designed as an expression vector and contains a Gram-positive promoter from the *Clostridium pasteurianum* ferredoxin gene (Graves *et al.*, 1985). Insertion of foreign DNA adjacent to this promoter reduces the requirement for the insert to be expressed only from its own promoter sites. It is probable that the insertion of the *Cellulomonas flavigena* cellulase-encoding genes into pMTL500F would have failed to lead to cellulase production from the LAB strains had such a experiment been successful. The lack of precise sequence data on the *Cellulomonas* genes would have remained a major problem here since there is no information on the position of inherent terminator signals.

A second major difference between the genera *Lactobacillus* and *Cellulomonas* is the mole percentage G + C ratio within the DNA. *Lactobacillus plantarum* DNA has a G + C

content of 44-46% while *Cellulomonas flavigena* DNA contains a level of 72.5-74.8%, while ORF A of pWM95 has 76% G + C (Bergey's Manual of Systematic Bacteriology, Volume Two and A. Kelly, personal communication). This means that control signals such as promoters within the *Cellulomonas* DNA are not likely to be recognised by *Lactobacillus* enzymes because of their different codon usage. This explanation is also plausible for *Lactococcus lactis* with a G + C ratio 38.6% and *Pediococcus pentosaceus* with a ratio of 35-39%, even though none of the cellulase constructs from *Cellulomonas* was ever successfully transformed into the latter genus. It is also possible that the marginally more comparable ratios between *Cellulomonas* and *E. coli* (48-52%) aided the original expression of the cellulase genes within the pUC vectors.

The lack of cellulase activity from pM3 suggests that the differences in the cellulase-encoding sequence present too great a disadvantage for the host enzymes to overcome.

2) *Clostridium thermocellum* DNA

Clostridium thermocellum is a thermophilic anaerobe which produces large amounts of cellulolytic enzymes. Many of these enzymes are produced as part of a multi-subunit complex known as a cellulosome (Lamed *et al.*, 1983). All three types of cellulase enzyme are produced by *C. thermocellum* and several have been cloned and characterised *eg* Hazelwood *et al.*, 1988. The number of endoglucanases produced by this one organism is particularly striking (Hazelwood *et al.*, 1988).

Enzymes originating from *C. thermocellum* may be considered apt for use within LAB for a number of reasons. As well as being from Gram-positive organisms, these enzymes are produced in their native state under anaerobic and thermophilic conditions, both of which are characteristic of silage fermentations. This suitability has been utilised by some recent authors (*eg* Scheirlinck *et al.*, 1989; Bates *et al.*, 1989; Scheirlinck *et al.*, 1990) and will be more fully discussed below.

Plasmid pCC1 consists of pMTL500E with the *cel*C gene from *C. thermocellum* (Petre *et al.*, 1986). The nucleotide sequence of this endoglucanase gene has been elucidated

(Schwarz *et al.*, 1988). The cellulase gene was cloned as a 1.6kb fragment carrying the indigenous promoter (cloned adjacent to the *lac*Z sequence) but with no terminator sequences (N. Minton, personal communication). Endoglucanase (cellulase) activity derived from pCC1 has been previously demonstrated in *E. coli* and *B. subtilis* (N. Minton, personal communication).

The successful detection of cellulase activity from pCC1 in *Lactobacillus plantarum* 85 under the congo red plate assay in effect acts as a positive control for demonstrating cellulase activity from the experimental strains.

The presence of the native *C. thermocellum* promoter within pCC1 is very significant; it aids expression in the foreign host. The very detection of activity indicates that the clostridial DNA control signals are recognised within *L. plantarum*. It is worth noting that the G + C content of *C. thermocellum* is 38-39%, a value much more consistent with that of *Lactobacillus plantarum* DNA (44-46%).

Five other *C. thermocellum* endoglucanase genes have been expressed within *L. plantarum*: *cel*C (Bates *et al.*, 1989); *cel*A (Scheirlinck *et al.*, 1989); *cel*D, *cel*F and *cel*G (Scheirlinck *et al.*, 1990). The report presented in this thesis is, however, the first time that demonstrable cellulase (CMC-ase) activity has been detected from the *cel*C gene from *C. thermocellum* within *L. plantarum*.

Although the *cel*C gene from *C. thermocellum* was cloned into *L. plantarum* 80 by Scheirlinck *et al.*, (1989) no enzyme activity was detected.

RELEVANT PUBLISHED REPORTS

A number of recent published reports have also dealt with the expression of cellulase-encoding genes and other related enzymes within *Lactobacillus* spp.

Reflecting the current interest in producing silage strains which express activities theoretically allowing them to utilise a greater proportion of the substrate available, most of these reports use commercial silage strains *eg* Bates *et al.*, 1989; Scheirlinck *et al.*, 1989; Jones & Warner, 1990; Scheirlinck *et al.*, 1990.

Detection of the following enzyme activities have been reported: alpha-amylase from *Bacillus stearothermophilus* and *cel*A (cellulase) from *Clostridium thermocellum* in *L. plantarum* (Scheirlinck *et al.*, 1989); *cel*E (cellulase) gene product from *C. thermocellum* in *L. plantarum* (Bates *et al.*, 1989); products of *cel*A, *cel*D, *cel*F, *cel*G, *xyn*Z (xylanase) from *C. thermocellum*, one endoglucanase and one xylanase from *Clostridium acetobutylicum* and also an endoglucanase from *Butyrivibrio fibriosolvens* all in *L. plantarum* (Scheirlinck *et al.*, 1990); a *B. subtilis* endoglucanase gene in *L. acidophilus* (Baik & Pack, 1990); alpha-amylase from *Bacillus amyloliquefaciens* in *L. plantarum* (Jones & Warner, 1990).

All of these foreign enzyme activities originated from Gram-positive hosts and expression within *Lactobacillus* spp. was achieved from their indigenous promoter sequences. As a point of reference, the mol percentage G + C levels of the host organisms is as follows: *Bacillus stearothermophilus* 43.5-62.2%; *C. acetobutylicum* 28-29%; *Butyrivibrio fibriosolvens* 41%; *Bacillus subtilis* 41.5-47.5%; *Bacillus amyloliquefaciens* 43.5-44.9%.

The stability of some of the recombinant plasmids involved in these published reports was a problem in three of these studies (Bates *et al.*, 1989; Scheirlinck *et al.*, 1989; Baik & Pack, 1990) although the former two used the same vector, pSA3 (Dao & Ferretti, 1985). The other unstable vector, pCK98 (Lee & Pack, 1987), is, like pSA3, based on a small, naturally occurring Gram-positive plasmid.

Many such small native plasmids which have been utilised to form vectors replicate via a single stranded DNA intermediate, probably by rolling-circle replication (Gruss & Ehrlich, 1989). This mechanism results in a higher degree of both homologous and illegitimate recombination which in effect leads to a high level of segregational instability (Gruss & Ehrlich, 1989). Such vectors, therefore, seem inappropriate for use within a silage fermentation where no selective pressure can be maintained. The plasmid pSH71, on which both pNZ12 and pTG262 are based, also replicates by rolling-circle replication

(M Gasson, personal communication).

Vectors derived from the large broad-host range plasmid pAMß1 do not replicate by a single stranded intermediate and hence are not as segregationally unstable (Jannière *et al.*, 1990). Such vectors include the pIL family (Simon & Chopin, 1988). A published report which used one of the pIL vectors containing an alpha-amylase insert (Jones & Warner, 1990) found no loss of plasmid after repeated subculturing in the absence of antibiotic but in the presence of starch.

The stability in the absence of selective pressure of some of the plasmids used in this study was investigated by recording the level of antibiotic resistance remaining after growth for a given number of generations in MRS broth and the results were presented in Figure 5.23. All three (pM1, pMTL500F and pCC1) of the plasmids tested were found to be relatively stable. Both of the vectors on which these plasmids are based (pIL277 and pMTL500E) have Gram-positive replicons based on pAMß1 and hence are expected to be segregationally stable. While the stability of the two vectors based on other plasmids (pNZ12 and pTG262) was not investigated to the same degree, the loss of the cellulase-encoding fragment from pA4 while under still under selective pressure does give some indication of the difference.

INTEGRATION

The instability of some of the vectors used for cloning foreign DNA in LAB can be overcome by achieving integration of the recombinant molecule into the host's chromosome.

Chromosomal integration has been previously demonstrated within *L. lactis* subsp. *lactis* (Leenhouts *et al.*, 1989; Chopin *et al.*, 1989). Both of these reports recorded integration by a Campbell-like mechanism where a duplication of the homologous chromosomal insert occurs.

This method has been utilised by Scheirlinck *et al.* (1989) to achieve the expression of the *cel*A gene from *C. thermocellum*. A suicide vector system, consisting of *L. plantarum*

chromosomal fragments within a vector which did not replicate in lactobacilli was used, although this requires a *Lactobacillus* strain which could be transformed at high frequencies.

Making use of one of the unstable vectors (carrying a chromosomal fragment for recombination) precludes the requirement for a suicide system and hence the need for a highly transformable strain. The plasmid pSA3, with or without a chromosomal insert has been demonstrated to integrate into the chromosome of *L. plantarum* (Rixon *et al.*, 1990). In both these reports of integration, the inserted DNA was found to be stably maintained even in the absence of selective pressure. A second major advantage of integration is the amplification of the gene under selective pressure; tandemly repeated units within the chromosome lead to an increase in gene product.

CONCLUSIONS

1) This work has shown that it is possible to use classical genetical analysis techniques on environmental isolates of pediococci and lactobacilli as well as laboratory strains.

2) The relatively new technique of electroporation has been demonstrated to result in efficient and reproducible transformation of the strains under study.

3) Although no cellulase activity was detected from the cloned *Cellulomonas flavigena* genes, cellulase activity was detectable from the cloned *cel*C gene of *Clostridium thermocellum* in some of the *Lactobacillus* strains. There have been no previous published examples of demonstrable activity from this gene within lactic acid bacteria.

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ACKNOWLEDGEMENTS

I would like to take this opportunity to thank Prof. B. Moseley, Dr. M Gasson & Dr. G Reid for all their help and support, Dr. N Minton for the provision of the pMTL plasmids, Dr. A Kelly for the pWM plasmids and related information and everyone in Edinburgh and Norwich for their encouragement. This work was supported by a DAFS studentship. Finally, I would like to dedicate the thesis to my husband, Stuart Mauchline, for his patience and understanding during the write-up.

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