Lactobacillus leichmannii as a probe for the quantitation of Vitamin B-12.

by

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B.Sc. (Hons) (Warwick)
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November 1989
DEDICATION

To my husband, Don,
whose love and encouragement has kept me going
and made this all possible.
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<td>A</td>
<td>Absorbance</td>
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<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
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<td>btuB&lt;sub&gt;E&lt;/sub&gt;</td>
<td>btuB gene from <em>Escherichia coli</em></td>
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<td>btuB&lt;sub&gt;S&lt;/sub&gt;</td>
<td>btuB gene from <em>Salmonella typhimurium</em></td>
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<td>°C</td>
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<td>Sodium dodecyl sulphate</td>
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SUMMARY

Initial attempts to isolate the gene encoding Vitamin B$_{12}$ receptor protein (btuB) from *Lactobacillus leichmannii*, resulted in the isolation of a btuB gene from *E. coli*, due to either a cross-over or gene exchange event. Complementation of an *E. coli* btuB mutant was demonstrated and expression analysis, using both in vivo and in vitro systems, revealed the cloned gene to encode a polypeptide with an apparent M$_r$ of 66,400 as determined by PAGE. Nucleotide sequence data confirmed that the cloned gene was identical to the btuB gene from *E. coli*.

The initial 2.0 Kb HindIII genomic DNA fragment from *L. leichmannii*, which exhibited homology to a synthetic oligonucleotide probe (derived from the btuB gene from *E. coli*), was re-cloned into an amplifiable high copy number vector in *E. coli*. The recombinant DNA was found to be stably maintained and had not undergone any physical rearrangements. Nucleotide sequence data revealed three putative open-reading frames, one of which encoded a protein which exhibited a degree of homology to the C-terminus of the Vitamin B$_{12}$ receptor protein (BtuB) from *E. coli*. The functions of the other two open-reading frames remain to be elucidated.

The B$_{12}$ binding protein from *L. leichmannii* has been isolated and purified. It has an M$_r$ of approximately 21,500 as determined by gel filtration. Polyclonal antibodies were raised to it for use in the identification of the desired gene product from the cloned *L. leichmannii* genomic fragment. Cross reactivity was found between the antisera to the B$_{12}$ binding protein from *L. leichmannii* and the B$_{12}$ receptor protein (BtuB) from *E. coli*. The reverse case was found to be true also.

Transformation of *L. leichmannii* was achieved by electroporation. Vectors pSA3, pC194, pCK1, but not pAMβ1, transformed the organism to chloramphenicol resistance, albeit at low frequency.
Preface

"The first major phase in the clinical investigation of Vitamin B$_{12}$ (cobalamin) metabolism and its derangements began with the development and application of methods for the estimation of total B$_{12}$ in tissues and body fluids and the assay of total serum B$_{12}$ has been a routine diagnostic procedure for many years. Most of the information available by such methods has been gained and it is now clear that we are well advanced in the second phase, in which the further development and application of methods for the estimation of individual cobalmins will play an important part".


In 1963 Lindstrand and Ståhlberg, using the technique of chromatography and bioautography, showed that human sera or plasma contained several cobalmins, the chief form being Methylcobalamin. By 1971, Matthews and Linnell had refined this technique, which enabled them to quantify by photometric scanning crimson-stained spots representing the individual cobalmins in blood or tissues (for further details, see Section 1.3).

Considerable interest has recently been generated in the molecular genetics of Lactobacilli, with the speculation that strain improvement can be achieved through recombinant DNA technology. The majority of this work has been biased
towards the organisms used in the Dairy Industry and Plant fermentation. However, these methods could be applied to the development of one of the microbiological assay systems used to detect $B_{12}$ deficiency; the one that utilises the gram positive organism, *Lactobacillus leichmannii* (see Section 1.8).

With the vast amount of information available in the literature regarding the gene encoding the Vitamin $B_{12}$ receptor protein in *Escherichia coli* and *Salmonella typhimurium*, this investigation focuses on the binding of cobalamin in *Lactobacillus leichmannii* with the aim of genetically engineering the organism to recognise different serum cobalamin analogues. The final objective being to undertake site-directed mutagenesis of the structural gene encoding the binding/receptor protein, assess the change in specificity and efficiency and re-introduce the modified gene into *L. leichmannii*. This will facilitate the differential assessment of the various analogues of $B_{12}$ in serum.

With this in mind, this thesis focuses on the application of molecular biology to the exploitation of the lactic acid bacteria and their component macromolecular systems in Vitamin $B_{12}$ assays.
Chapter 1 - Introduction

1.1 General Introduction to Vitamin B₁₂.

Vitamin B₁₂ is a large, complex, water soluble compound with a number of functional groups susceptible to a variety of chemical modifications, leading to a tremendous number of derivatives.

The structure of the Vitamin B₁₂ molecule is shown in Figure 1.1. This molecule (cobalamin) consists of two major parts; the core, which is almost identical to the haem of haemoglobin, other than that the attached metal is cobalt, not iron (and one of the α methane bridges is missing); and a nucleotide, which is set nearly at right angles to the core (corrin) portion.

The corrin nucleus is the central structure of all the corrinoids, the latter being cobalt-containing cyclic structures in the human body, in foods of animal origin (meat, poultry, eggs, fish and milk) and in bacteria, which make not only Vitamin B₁₂, but also various analogs thereof. To use it as a vitamin, the human cell must see it as depicted in Figure 1.1 i.e. as a cobalamin with no alterations except in the R-adduct.

Cyanocobalamin, one of the most widely known forms of Vitamin B₁₂, is obtained by cyanide treatment of all other naturally occurring forms. Among the β-ligands known, the
Figure 1.1 The Structural Formula of Vitamin B$_{12}$.
(taken from Herbert, 1988).

- R
  - CN  cyanocobalamin
  - OH  hydroxocobalamin
  - H$_2$O  aquacobalamin
  - NO$_2$  nitritocobalamin
  - CH$_3$  methylocobalamin
  5'-deoxyadenosyl  5'-deoxyadenosylcobalamin
  (coenzyme B$_{12}$)
cyanide has the highest affinity for cobalt, since the attachment of CN does not require reduction of the cobalt. Basic data of some selected corrinoids can be found in Table 1.1.

Methylcobalamin and adenosylcobalamin are photolabile compounds. From the practical point of view, the light sensitivity of MeCbl and AdoCbl is rather undesired, since many experiments with these compounds, such as estimation, purification and the work with enzyme systems have to be done in the dark or in dimmed red light. However, they are far less photolabile as solids. Prolonged irradiation by sunlight inactivates irreversibly all forms of cobalamin.

All eukaryotic animal cells and most prokaryotic cells require Vitamin B₁₂ coenzymes to sustain activity and viability. Although more than ten B₁₂-dependent enzymes or enzyme systems have been isolated from bacteria, only two of them have been found in man. These two enzymes require for activity two different forms of Vitamin B₁₂: 5'-deoxyadenosyl cobalamin (Ado Cbl) for methylmalonyl CoA-mutase and methylcobalamin (MeCbl) for homocysteine-transferase. Each cobalamin coenzyme is formed by a unique and complex reaction sequence that results in the formation of a covalent carbon-cobalt bond between the central cobalt nucleus of the cobalamin molecule and the ligand, either a methyl group or 5'-deoxy-5'-adenosyl moiety, which confers coenzyme specificity.
Table 1.1 Characterisation of a limited selection of corrinoids.

<table>
<thead>
<tr>
<th>Systematic name abbreviation</th>
<th>Common name</th>
<th>Occurrence and biological activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN-Cbl</td>
<td>Cyanocobalamin</td>
<td>Artificial product obtained from other cobalams by addition of KCN. Organisms convert it quickly to</td>
</tr>
<tr>
<td></td>
<td>Vitamin B₁₂</td>
<td>other forms, mainly aq-Cbl, CH₃-Cbl and Ado Cbl (Ellenbogen, 1974). Active in man, animals and microorganisms.</td>
</tr>
<tr>
<td></td>
<td>Factor II</td>
<td>Not active in enzyme systems.</td>
</tr>
<tr>
<td>Systematic name abbreviation</td>
<td>Common name</td>
<td>Occurrence and biological activity</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-----------------</td>
<td>-----------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>aq-Cbl</td>
<td>Aquacobalamin</td>
<td>Produced by microbes, occurs in man, animals, sewage sludge, soil and plant roots.</td>
</tr>
<tr>
<td></td>
<td>Vitamin B$_{12}$</td>
<td></td>
</tr>
<tr>
<td>OH-Cbl</td>
<td>Hydroxocobalamin</td>
<td>Is absorbed by human and animal guts, mucosa (Glass et al., 1962). Active in man, animals and microbes. Not active in enzyme systems in vitro.</td>
</tr>
<tr>
<td></td>
<td>Vitamin B$_{12}$</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Systematic name abbreviation</th>
<th>Common name</th>
<th>Occurrence and biological activity</th>
<th>Structure and formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ado-Cbl</td>
<td>Vitamin B$_{12}$</td>
<td>Present in man, animals and microbes. Obtained by chemical and enzymatic synthesis from OH-Cbl or aq-Cbl. Absorbed by man somewhat less than OH-Cbl. Active in man, animals and microbes (Coates et al., 1962). Co-factor for a number of enzymes.</td>
<td>C$<em>{72}$H$</em>{101}$O$<em>{17}$N$</em>{18}$PCo</td>
</tr>
<tr>
<td></td>
<td>coenzyme</td>
<td></td>
<td>M$_{r}$ of 1579.6</td>
</tr>
<tr>
<td></td>
<td>CoB$_{12}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Finally, a word about terminology, since some authors use the more familiar 'Vitamin B₁₂', whereas others refer to the vitamin as 'cobalamin'. According to the current recommendations (IUPAC-IUB Commission on Biochemical Nomenclature, 1975), the general term 'cobalamin' should be used except when a specific compound, such as methylcobalamin is meant.

1.2 Medical aspects of Vitamin B₁₂.

Vitamin B₁₂ was applied in medicine in the early twenties, when two American physicians Minot and Murphy obtained positive results in curing pernicious anaemia with a liver diet. This disease was reported as early as 1824. It remained fatal, however, until this discovery.

Liver contains an appreciable concentration of Vitamin B₁₂ and this served for the next two decades as the main source of the then unknown curing factor, also known as the 'extrinsic factor'.

Vitamin B₁₂ was obtained in pure crystalline state in 1948 and its structure was elucidated in the following years. The production of Vitamin B₁₂ on an industrial scale in the early fifties enabled its world-wide application in medicine to treat pernicious anaemia.

<table>
<thead>
<tr>
<th>Systematic name</th>
<th>Common name</th>
<th>Occurrence and biological activity</th>
<th>Structure and formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>methylcobalamin</td>
<td>vitamin B₁₂</td>
<td>Present in man and animals (Lindstrand, 1964).</td>
<td>C₆₃H₉₂O₁₄N₁₄P₂Co</td>
</tr>
</tbody>
</table>


1.3 Vitamin B$_{12}$ deficiency and its detection.

A deficiency of Vitamin B$_{12}$ strikes two systems:

(i) Red blood cells fail to mature, megaloblasts collect in the bone marrow, the patient becomes anaemic and anaemia can progress to death;

(ii) The nervous system commonly suffers damage to the spinal cord, peripheral nerves and the white matter of the brain. Patients may suffer numbness and tingling, stabbing sensations, poor coordination of the legs and fingers, weakness, paralysis, convulsions and many other symptoms secondary to neurological damage. It is thus essential that the deficiency is quickly detected.

Assays of serum cobalamin (Cbl) came into use in the 1950's, although for a number of years they were performed only by specialised laboratories. For a time, serum Cbl levels were measured only by microbiological assays using either;

Lactobacillus leichmannii (Rosenthal & Sarrett, 1952)

or

Euglena gracilis (Ross, 1950)

or less frequently,

Ochromonas malhamensis (Ford, 1953)
or a cobalamin/methionine auxotrophic mutant of

*Escherichia coli* (Davies & Mingioli, 1950).

The assay procedure using *O. malhamensis* has been considered the most specific, since this organism is supported almost exclusively by cobalamins, whereas cobalamins and certain other corrins support the growth of the three other organisms.

Vitamin B_{12} normally present in blood is mainly combined as a labile complex with the α globulin fraction of serum protein (Pitney et al, 1954). In all methods of estimation, it is necessary to extract the substance, this being easily performed by boiling.

Ross (1952) devised a method of estimating the B_{12} content of human body fluids by measuring turbimetrically, after seven days incubation, the growth of *E. gracilis v. bacillaris*. The extract was prepared by boiling at pH 3.6, when protein precipitation does not occur. However, alkali production by the *Euglena* cells caused a rise in pH of the culture with consequent protein precipitation during incubation. To overcome this, Hutner et al, 1956 produced a richer and better buffered culture medium and by using a more rapidly growing strain of *Euglena gracilis*, obtained satisfactory results in 5-6 days. In 1964, Anderson described a modified *Euglena* method, for the assay of B_{12} in
serum. Sub-normal serum $B_{12}$ concentrations could be detected visually as early as 24 hours after the start of the assay. For diagnostic purposes, the assay can be read at 3 days, provided that appropriate dilutions are used, conditions are optimal and growth is measured in a 1 cm cell.

There are several advantages of using the *Euglena* assay, in that it is very sensitive, highly specific and because whole serum is often used, preliminary extraction is unnecessary and direct measurements of both bound and free $B_{12}$ can be made. However, various factors such as medium, light and inoculum can affect growth in aqueous and serum solutions differently and are an important cause of variations between batches and laboratories. Thus, growth conditions need careful control and the technique can be tedious and time consuming due to elaborate procedures required for cleaning of glassware.

In 1955, Spray reported an improved method for the rapid estimation of Vitamin $B_{12}$ in serum. In Rosenthal and Sarett's original method, the assays were read by titrating the acid produced by *L. leichmannii* after 3 days' incubation. In 1954, Girdwood had modified the original method by using turbidity measurements to assess bacterial growth, but he did not give sufficient data to allow a critical assessment to be made of the validity of the results by turbidimetric modification. Spray's study led to the development of an improved culture medium and a more
reliable method of extracting vitamin B₁₂ from serum. This method gave rather higher results than were obtained using *Euglena* and was not as specific, but had the advantage of being able to provide a result in 24 hours. Variations of this basic method have been used by many laboratories (Matthews, 1962; Raven et al, 1972).

*L. leichmannii* has a nutritional requirement for a cobalamin or cobamide, which can, however, be replaced by a deoxyribonucleoside (Kitay et al, 1950). As shown in Table 1.2, any of the five conventional deoxyribonucleosides can replace cobalamin in the culture medium, whereas ribonucleosides and pentoses have no such effect.

Since *L. leichmannii* lacks an absolute Cbl requirement, it is theoretically possible that unknown samples submitted for Cbl analysis could give falsely high results if the sample contained one or more deoxyribonucleosides. Studies by Beck (1983) on human serum samples have shown that deoxyribonucleosides do not occur in quantities sufficient to influence the growth of *L. leichmannii*.

Although it has always been recognized that the microbiological techniques are complex and require the use of standard sera, they are precise and reproducible (Mollin et al, 1976) and are consistently able to diagnose vitamin B₁₂ deficiency. They are, in addition, particularly valuable in the diagnosis of vitamin B₁₂ neuropathy.
Table 1.2  Nutritional requirement of *Lactobacillus leichmannii* (ATCC 7830) for Vitamin B<sub>12</sub> (cyanocobalamin) of a deoxyribonucleoside.
(taken from Beck, 1983).

<table>
<thead>
<tr>
<th>Additions to assay medium</th>
<th>Turbidity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Cyanocobalamin</td>
<td>127</td>
</tr>
<tr>
<td>Thymidine</td>
<td>105</td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td>119</td>
</tr>
<tr>
<td>Deoxyadenosine</td>
<td>92</td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td>100</td>
</tr>
<tr>
<td>Deoxyguanosine</td>
<td>112</td>
</tr>
<tr>
<td>Deoxyribose</td>
<td>0</td>
</tr>
<tr>
<td>Cytidine</td>
<td>0</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0</td>
</tr>
<tr>
<td>Uridine</td>
<td>0</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0</td>
</tr>
</tbody>
</table>

*<sup>a</sup> Turbidity was determined in a Klett colorimeter (Filter 66) after cultivation for 8 hours. Final concentrations of additions were: cyanocobalamin 0.5 ng ml<sup>-1</sup>, deoxyribonucleosides and ribonucleosides 2.5 μg ml<sup>-1</sup> and deoxyribose 5.0 μg ml<sup>-1</sup>. All cultures contained optimal concentrations of free purines and uracil.*
However, the traditional microbiological assay procedure has been replaced increasingly by radioisotope dilution (RID) assays, especially in the form of commercial kits. RID assays involve extraction of bound cobalamin from a sample of serum, its conversion to cyanocobalamin and after mixing with a known quantity of radioactive cobalamin, its association with a cobalamin ligand with high affinity for the vitamin (Mollin et al, 1976; Gottlieb et al, 1965). This introduction provided an alternative test with the apparent advantages of being convenient, easy to perform, whilst providing rapid results. They did, however, throw up unexpected problems. The results with the RID assays tended to be higher than those with microbiological assays (Mollin et al, 1976) and more recent work (Hall, 1977; Mahood, 1977; Cooper & Whitehead, 1978; Kolhouse et al, 1978 & Mollin et al, 1980) has cast doubts on their reliability in distinguishing untreated pernicious anaemia from normal.

Kolhouse and colleagues (1978) showed that higher results were obtained when the assay employed a B12 binding protein, present in all body fluids, termed R-binder, as compared to an assay using gastric intrinsic factor (IF) which was purified to remove R-binder. Indirect evidence suggested that the R-binder detected B12 analogues in addition to those detected with an IF assay. Since then, it has been shown that the forms of B12 detected in an IF-based assay are identical to those measured by microbiological assay (Chanarin & Muir, 1982).
Muir and Chanarin (1983) investigated the competitive
binding of cobalamin analogues to the B_{12} binding proteins
and sought to determine their carrier proteins in normal
serum. They found that both binding agents were equally
effective in removing \[^{57}\text{Co}]B_{12}\) from aqueous solution. IF
was more effective than R-binder in removing \[^{57}\text{Co}]B_{12}\)
added to a serum extract. They found that IF bound both
types of cobalamin, though the binding was less avid for the
microbiologically inactive analogues, than for
microbiologically active analogues.

Oxley (1984) supported this work when he reported the
findings of the Ligand Assay Resource Committee (LARC), when
they decided to determine whether the presence of R proteins
in assay kits was capable of introducing significant errors
in the Vitamin B_{12} assays used. The data provided strong
empirical support for the hypothesis that radioligand assays
yield higher results than bioassays because the former
measure both active and inactive cobalamins.

Fish and Dawson (1983) assessed fourteen commercial kits as
serum B_{12} assays. The analytical performance and clinical
correlations were used to rank the methods employed for the
extraction of the vitamin from its binders and for
separation of 'bound' from 'unbound' counts. The
introduction of extraction without boiling and the
separation by attachment of the B_{12} binder to a solid matrix
have not been shown to be generally better or worse than
conventional methods.
As mentioned previously, Matthews and Linnell (1971) developed a technique of chromatography and bioautography. Cobalamins are extracted from the sample with hot ethanol. After freeze-drying, the extract is desalted by solvent extraction, concentrated in water and the cobalamins separated by thin layer chromatography on cellulose/silica gel. Cobalamins on the chromatogram (5-50 pg per spot) are detected bioautographically by incubating the plate in contact with a layer of agar medium inoculated with a cobalamin-sensitive strain of *Escherichia coli* and a growth indicator. Crimzon zones appear on the bioautogram, which are quantified by transmitted light in a scanning densitometer, and compared with appropriate standards (see Figure 1.2).

In simple cobalamin deficiency, due, for example, to pernicious anaemia, there is a striking and disproportionate reduction in the plasma MeCbl, which occurs while the total plasma Cbl is still within normal limits and may therefore be a better indicator of cobalamin status than the 'serum B\textsubscript{12}' level alone (Linnell and Matthews, 1984).

Herbert concluded in 1985 that the key to reliable results appeared not to reside in a particular assay, but rather in determining for each assay, its own range of results in participants determined clinically and morphologically normal versus participants with deficient Vitamin B\textsubscript{12} (with B\textsubscript{12} deficiency defined independently of a serum B\textsubscript{12} assay). When laboratory assay results differ from clinical
Figure 1.2 Plasma cobalamin separated by two-dimensional chromatography and bioautography.
(taken from Linnell & Matthews, 1984).

(a) Normal subject (total Cbl 460 pg ml\(^{-1}\)).

(b) Patient with untreated pernicious anaemia. MeCbl is disproportionately reduced (total Cbl 80 pg ml\(^{-1}\)).

(c) Patient with untreated tropical ataxic neuropathy. CNCbl is abnormally increased and accounts for 24% of the total Cbl (total Cbl 635 pg ml\(^{-1}\)).

(d) Child with untreated homocystinuria and methylmalonic aciduria due to congenitally impaired synthesis of both cobalamin coenzymes; note the disproportionate reduction in plasma MeCbl (total Cbl 1150 pg ml\(^{-1}\)).
judgement, further evaluation is the appropriate course. There is no 'gold standard' for human serum Vitamin B\textsubscript{12} assays.

During the last decade, dietary supplements containing vitamins have become more popular and there is a need for a fast and sensitive method for the routine analysis of Vitamin B\textsubscript{12} in these products and other foods. Microbiological assays using \textit{L. leichmannii} or other microorganisms are the most widely used methods for measuring Vitamin B\textsubscript{12} concentration in food. However, this technique is rather time consuming and for some time, RID methods have been commonly used for the determination of B\textsubscript{12} in serum (Pratt & Woldring, 1982).

Österdahl and Johansson reported the development of a rapid and sensitive method for the determination of Vitamin B\textsubscript{12} in dietary supplements (1988a), using a commercially available RID kit (Becton Dickinson Immunodiagnositcs). They also compared two RID kits for measuring Vitamin B\textsubscript{12} in gruel (1988b).

1.4 Non-enzymatic Vitamin B\textsubscript{12} binding proteins.

The absorption and transport of Vitamin B\textsubscript{12} in man and animals is exclusively mediated by proteins. That Vitamin B\textsubscript{12} in serum is bound to protein (Rosenthal & Sarrett, 1952; Pitney et al, 1954) has been known since shortly after the isolation of the vitamin. In 1968, Herbert suggested that
ingested or injected Vitamin B₁₂ attaches both to α- and β-globulin binding proteins, but that which is attached to β-globulin is delivered to tissues within twenty four hours, whereas that attached to α-globulin is retained by the serum. While both B₁₂-binding globulins may deliver the vitamin to reticulocytes and the liver (Retief et al., 1967) - and presumably other tissues as well - Herbert's studies suggest that the B₁₂-binding β-globulin is primarily a transport protein (Retief et al., 1967), whereas the B₁₂ binding α-globulin functions mainly to conserve the vitamin. B₁₂ binding α-globulin has a greater affinity for B₁₂ and retains the vitamin more tenaciously than does the B₁₂ binding β-globulin (Retief et al., 1966).

These binding proteins were named differently by different authors and it was not until 1979 when the name transcobalamin was commonly accepted.

Transcobalamin II (TCII) is a protein present in trace amounts in human plasma that binds B₁₂; it is characterised by a molecular size of slightly less than 40,000, a fast electrophoretic mobility and an immunologic specificity that separates it from all other known B₁₂ transport proteins (Hall, 1969). The other B₁₂ transport protein of human plasma known at the time, then referred to as the B₁₂ binding protein, but now known as TCI, did not take up much of the recently absorbed B₁₂. This led to the concept of two transport proteins carrying B₁₂ in the plasma for separate purposes (Hall & Finkler, 1965). In 1971, Hall and
Finkler presented evidence to suggest an active transport role for TCII by transferring $B_{12}$ rapidly from the blood to tissue in vivo.

Cobalamin transport in man is very complex and the reader is referred to a review (Sennett et al, 1981).

Cobalamin uptake and transport has been observed in a variety of bacterial species, but has been studied in detail only in *Escherichia coli*. Although exogenous cobalamins are not essential for growth of wild-type *E. coli*, a process exists that facilitates uptake of this group of compounds.

1.5 *Vitamin B$_{12}$* transport in *E. coli*.

The uptake systems for Vitamin $B_{12}$ and numerous iron-siderophore complexes in *E. coli* are unusual in that they employ specific receptor proteins in the outer membrane as an essential component of the high-affinity active transport process (Neilands, 1982). The uptake of cyanocobalamin (CN-Cbl) by cells of *E. coli* is biphasic consisting of an initial rapid phase of $B_{12}$ binding to specific receptors on the outer membrane of the cell envelope which is essentially independent of the energy metabolism of the cell, followed by a slower energy dependent secondary phase in which the $B_{12}$ is transferred from these receptors into the interior of the cell.
In *E. coli* strains used, White et al (1973) showed that the competitive inhibition of CN-[\(^{60}\text{Co}\)] B\(_{12}\) uptake was obtained with unlabelled cyanocobalamin, methylcobalamin, deoxyadenosyl cobalamin and cyanocobinamide. The \(K_i\) values indicated that the initial B\(_{12}\)-binding sites can recognize each of these corrinoids equally well. The only corrinoid tested which was apparently not recognized by the initial B\(_{12}\)-binding sites was cyanocobalamin 5'-phosphate. White and colleagues (1973) suggested that perhaps the B\(_{12}\) receptors on the outer membrane of the *E. coli* cell envelope serve only as a trap to sequester trace amounts of B\(_{12}\) from the growth medium. Such a trapping function may well be the primary role of the B\(_{12}\) binding protein found in cell walls of *Lactobacillus leichmannii* (Sasaki, 1972), since the studies of Scherrer and Gerhardt (1971) on the effective pore sizes of cell wall mucopeptide structures in another gram-positive organism, *Bacillus megaterium*, indicate that such matrices are permeable to molecules of B\(_{12}\) size.

However, it is unlikely that the outer membrane of *E. coli* is permeable to Vitamin B\(_{12}\) and therefore the B\(_{12}\) receptor probably also serves to transport B\(_{12}\) across this structure.

Subsequent work led to the proposal of a relatively simple model for uptake of cobalamins by coliforms. According to this model, shown schematically in Figure 1.3, cobalamin uptake proceeds first by the rapid binding of cobalamin to an outer membrane receptor. Di Girolamo and Bradbeer (1971) reported that the B\(_{12}\) binding site on the cell surface has a very high affinity for Vitamin B\(_{12}\). Subsequently, there is
The outer membrane of the bacterial envelope is shown by the dashed line; the inner membrane by the solid line; and the periplasmic space by the space between the two membranes, expanded at left. PCB, periplasmic cobalamin binding protein; AdoCbl, adenosylcobalamin; MeCbl, methylcobalamin. See text for details.
a slower release of cobalamin into the interior of the cell, an event probably requiring two additional steps.

The receptor protein for Vitamin B$_{12}$ (cyanocobalamin) which is located in the outer membrane of E. coli is encoded by the gene $btuB$.

1.5.1 BtuB in E. coli.

The $btuB$ gene product is a protein with a molecular weight of 66,400 daltons, which is located in the outer membrane of E. coli (Heller & Kadner, 1985; Heller et al, 1985). It serves as a high affinity ($K_d = 0.3$ nM) receptor for Vitamin B$_{12}$ (Holroyd & Bradbeer, 1984). After its initial binding to the BtuB protein the cobalamin was thought to be released from the receptor to the periplasmic space in a process for which at least the proton motive force and the $tonB$ gene product (see later) were required (Bradbeer & Woodrow, 1976).

This property, shared by several iron transport systems makes BtuB fundamentally different from the major porin proteins OmpF, OmpC and LamB (Nikaido & Vaara, 1985).

Besides its role in Vitamin B$_{12}$ transport, BtuB is a receptor for phage BF23 (Bradbeer et al, 1976), the E colicins (Di Masi et al, 1973) and in association with OmpF and lipopolysaccharide, for colicin A (Cavard & Lazdunski, 1981; Chai et al, 1982). Whereas the uptake of Vitamin B$_{12}$
by means of the \textit{btuB} encoded \textit{B}_12 receptor is \textit{tonB} dependent, the entry of the \textit{E colicins} and phage \textit{BF23}, which use the same receptor is not (Bassford \textit{et al}, 1976; Bradbeer \textit{et al}, 1976; Davies \& Reeves, 1975). These activities seem to be only transitory \textit{in vivo} and restricted to a particular sub-class of \textit{BtuB} proteins; most probably those which are newly synthesized and not evenly distributed within the outer membrane. However, cells retain full activity for Vitamin \textit{B}_12 uptake (Kadner \& McElhaney, 1980).

In fact, at least twelve different ligands, some of which are closely related bind, to the \textit{BtuB} protein. In doing so, they exhibit competitive binding kinetics, in that interaction with one ligand prevents the binding of a second (Bradbeer \textit{et al}, 1976; Di Masi \textit{et al}, 1973).

Previous studies have suggested that Vitamin \textit{B}_12 is involved in the repression of the \textit{btuB} gene (Kadner, 1978). Growth in the presence of 100 nM \textit{B}_12 reduces the \textit{B}_12 uptake by as much as 90%. \textit{BtuB} is usually found at a low level (200 - 300 copies per cell) when Vitamin \textit{B}_12 is omitted from the culture media (Di Masi \textit{et al}, 1973).

There is also good evidence to suggest that \textit{B}_12, in the presence of the genes encoding methyltransferase, \textit{metH} and \textit{metF}, represses the gene encoding homocysteine transmethylase (\textit{metE}) (Mulligan \textit{et al}, 1982). The methionine (\textit{met}) regulon of \textit{E. coli} consists of eleven genetic elements (Rowbury, 1983). The last step in the
synthesis of methionine in *E. coli* and *S. typhimurium* is the methylation of homocysteine (Rowbury, 1983). This reaction is carried out by either of two transmethylase enzymes. One is a Vitamin B$_{12}$-independent enzyme (metE gene product) and the other is a Vitamin B$_{12}$-dependent enzyme (metH gene product). The methyl group for this reaction is donated by 5-methyltetrahydrofolate, which is produced by the metF gene product.

metE, F and H genes, as well as all other genes of the methionine pathway are negatively regulated by the metJ gene product, with 5-adenosylmethionine as the co-repressor. However, recently Urbanowski et al, 1987 reported the presence of another met regulatory locus (metR) in both *E. coli* and *S. typhimurium*. This genetic data suggests that metR codes for the transactivator protein for the expression of the metE gene and to a lesser extent, the metH gene that codes for the B$_{12}$ dependent methyltransferase.

Until recently, the nature of the regulation of btuB by repression was not understood and no regulatory gene had been identified. It was thought that the putative repressor was produced in appreciable amounts and was not titrated out by multiple copies of its target.

Lundrigan et al, 1987 obtained mutations which defined a new gene btuR, required for repression of btuB. It appears to encode a repressor of btuB transcription, but has no apparent role in the biosynthesis of methionine or in the
transport of Vitamin B₁₂. The gene is not located near any of the genes of methionine or Vitamin B₁₂ transport. Cellular levels of the BtuR repressor and the location of the operator site on the cloned btuB region are presently under investigation.

Earlier this year Lundrigan and Kadner published results to suggest that BtuR does not in fact regulate btuB expression directly, but that it may be involved in adenosylcobalamin metabolism (Lundrigan & Kadner, 1989).

Very little is known about the structure of BtuB, the location of the ligand-binding sites and other functions, or its orientation in the outer membrane. BtuB has been purified by several techniques. However, purification is complicated by the existence of complexes between the receptor and porin proteins (Imajoh et al, 1982).

The low level of production of the receptor poses another problem for biochemical investigations (Di Masi et al, 1973).

What is known, is that BtuB has a relatively polar composition but lacks substantial regions of hydrophobic character long enough to span a membrane in the α-helical conformation. Genetic approaches may help identify regions of BtuB, which are important in substrate binding and transport or in targeting the protein to the outer membrane.
Several groups have indeed been working in this area in recent years.

Gudmundsdottir et al, 1988 reported that several receptor domains are involved in substrate binding and energy coupling, since none of the mutations they constructed (linker insertions in btuB) affected the entry of only one type of ligand.

It has also been suggested that the protein has a binding site for calcium, since the binding and transport of CN-Cbl, appears to be calcium dependent (Bradbeer et al, 1986).

In 1982, Hunter and Glass reported studies using information suppression of btuB nonsense mutants which allowed the study of the effect of known, single amino acid substitutions on receptor function. They reported that ligand uptake was largely unaffected by such amino acid changes. The few instances in which certain substitutions destroyed sensitivity to two lethal agents (phage BF23 and colicin E3) without affecting B12 uptake suggested a common region on the btuB receptor involved in the binding of these proteinaceous agents.

In 1985, Heller et al, showed that twelve amino acids could be removed from the C-terminus by a PstI deletion of their cloned btuB gene and yet it was still capable of imparting sensitivity to phage BF23 despite being defective in Vitamin B12-binding activity.
Moir and colleagues (1987) found that altering the C-terminus of the btuB gene product prevented its function as a receptor, as did Heller and his colleagues. However, it did not prevent receptor export and this data argues strongly that at least 216 C-terminus residues are not required for transfer to the outer membrane. However, this region may be necessary for correct localisation in the envelope.

It was mentioned earlier that the transport of Vitamin B$_{12}$ was a biphasic process. Its transport across the cytoplasmic membrane of *E. coli* requires the products of *btuC* and *btuD*, two genes in the *btuCED* operon.

1.5.2 *BtuCED* operon in *E. coli*.

The *btuCED* genes appear to be organised as an operon with internal promoters. This type of genetic organisation suggests that the three genes are involved in a common metabolic process and that *btuE* might be the periplasmic CN-Cbl binding protein. However, Rioux and Kadner (1989a) have examined the role of the *btuE* gene and despite its genetic location in the transport operon, the *btuE* product plays no essential role in Vitamin B$_{12}$ transport. Although transport is not dependent on BtuE, uptake may still be dependent on this periplasmic binding protein. If so, this situation would represent a novel type of genetic organisation for this type of transport system.
The \textit{btuC} gene, has been found to influence Vitamin B$_{12}$ uptake or utilisation. The \textit{btuC} function is required for the growth response to Vitamin B$_{12}$ when the outer membrane transport process (\textit{btuB} and \textit{tonB} function) is defective. However, even in a wild-type strain, \textit{btuC} is required for proper transport of Vitamin B$_{12}$ (DeVeaux & Kadner, 1985).

In contrast to the genes \textit{btuB} and \textit{tonB}, \textit{btuC} appears to have no known function apart from its involvement in cobalamin transport. Mutants in this locus were first isolated and characterised by Di Girolamo \textit{et al.}, in 1971, who showed that such strains, which were also \textit{metE}, required increased concentrations of cobalamin in the medium for growth in the absence of added methionine and displayed several changes in cobalamin uptake. These changes included greater exchangeability of cellular cobalamin with exogenous cobalamin, a reduced energy-dependent phase of cobalamin transport and reduced conversion into coenzyme forms of the cobalamin taken up.

Reynolds and her colleagues concluded from their results that the proton motive force and the \textit{tonB} gene product are involved in the release of cobalamin from the outer membrane receptor and that the \textit{btuC} gene product is necessary for cobalamin transport across the inner membrane.

Mutations in \textit{btuD} confer a less extreme deficiency in Vitamin B$_{12}$ utilisation than do \textit{btuC} mutations, although transport was strongly impaired (DeVeaux & Kadner, 1985).
The btuCED region may encode a transport system for passage of Vitamin $\text{B}_{12}$ across the cytoplasmic membrane. This system bears similarities to periplasmic binding protein-dependent transport systems, although the putative periplasmic component is not required for its function.

The products of $\text{btuC}$, $\text{btuD}$ and $\text{btuE}$ were identified in maxi cells as polypeptides with relative molecular masses ($M_r$) of 26,000, 29,000 and 22,000 respectively (DeVeaux et al, 1986), approximating to the sizes predicted from the nucleotide sequence (Friedrich et al, 1986).

Fractionation of maxicells indicated that both BtuC and BtuD are membrane associated. A substantial portion of BtuE was released by procedures that preferentially extract periplasmic proteins. However, no obvious signal sequence was present at the amino-terminus of the predicted polypeptide.

Previous evidence showed that a periplasmic $M_r$ 22,000 polypeptide was released by osmotic shock and bound Vitamin $\text{B}_{12}$ with high affinity, but no direct evidence was presented for its role in transport (Taylor et al, 1972; Bradbeer et al, 1978).

Recent work by Rioux and Kadner (1989a) showed that non-polar, in-frame deletions in $\text{btuE}$ showed that the absence of the $\text{btuE}$ product had no significant effect on the binding,
transport or utilisation of CN-Cbl or its derivatives, whether the outer membrane BtuB/TonB system was active or not. The Vitamin B$_{12}$ binding activity that is still present at wild-type levels in osmotic shock fluids of btuE mutants is likely to be encoded outside btuCED.

They also found an error in the published sequence of btuC. A C residue should be inserted at position 1012 of the sequence described by Friedrich et al, 1986 (see Figure 1.4). This results in extension of the gene from 292 codons ending at nucleotide 1031 to 327 codons ending at nucleotide 1133, to give an $M_r$ of 35,000.

1.6 Vitamin B$_{12}$ transport in S. typhimurium.

The enteric bacteria Salmonella typhimurium and E. coli possess two enzymes that are known to use Vitamin B$_{12}$ as a cofactor; tetrahydropteroyl-glutamate methyltransferase and ethanolamine ammonia lyase (Cauthen et al, 1966; Chang & Chang, 1975; Foster et al, 1964). The first is a methionine biosynthetic enzyme (encoded by the metH gene) for which a cobalamin-independent alternative (encoded by the metE gene) exists (Childs & Smith, 1969). The second is required only if the cells are using ethanolamine as a carbon or nitrogen source.

The in vivo activities of these enzymes depend upon the cells being provided with an exogenous source of the vitamin. This nutritional requirement for a preformed
Figure 1.4  DNA sequence of btuCED.
(from Friedrich et al, 1986).

The non-coding strand is presented and the predicted polypeptide sequence of the open reading frames is shown in one-letter code above the DNA sequence. The numbering begins at the SmaI site located at the 3' end of himA. Some regions of local dyad symmetry are underlined with arrows. Lines are drawn over potential promoter sequences, and putative Shine-Dalgarno sequences are enclosed in boxes.
corrinoid ring has led to the generally held belief that enteric bacteria are unable to synthesise cobalamin de novo. However, Jeter et al, 1984 reported that *S. typhimurium* synthesised cobalamin de novo under anaerobic culture conditions.

The process of Vitamin B12 transport in *Salmonella typhimurium* is thought to be similar to that in *E. coli*. Rough strains of *S. typhimurium* are susceptible to phage BF23 and the *E. coli* colicins (Guterman et al, 1975). Resistance to these lethal agents results from mutations in *bfe* (susceptibility to phage BF23 and the *E. coli* colicins), which is located at the position on the *S. typhimurium* chromosome map analogous to that of the *E. coli* *btuB* gene. In the latest version of the *S. typhimurium* genetic map (Sanderson & Roth, 1988), the *bfe* locus was renamed *btuB*.

Rioux and Kadner (1989b), investigated the involvement of an outer membrane transport component for Vitamin B12 uptake in *S. typhimurium* analogous to the *btuB* product in *E. coli*. An Mr 60,000 protein in the *S. typhimurium* outer membrane was repressed by growth with Vitamin B12 and was eliminated in a *btuB* mutant. The *btuB* product thus appears to play the same role in Vitamin B12 transport by *S. typhimurium* as does the *E. coli* *btuB* product.

They also reported that *S. typhimurium* has also a low-affinity transport system, which allows entry of cobalamins but not of cobinamide, which suggests that it differs in
substrate specificity from BtuB. Cobalamin binding was of low affinity and showed no saturation even at 100 nM CN-Cbl. This new system appears to mediate transport only across the outer membrane, since cobalamin uptake still requires all of the components involved in passage across the cytoplasmic membrane, and it is unlikely that the new transport system is tonB dependent.

The low affinity and capacity for CN-Cbl suggests that some other nutrient may be the primary substrate of this transport system and that cobalamins enter by being poor analogues of this substrate. A likely substrate could be an iron siderophore, since the uptake of iron siderophores requires an outer membrane transport component, similar to that of Vitamin B$_{12}$.

The uptake and transport of iron bears many resemblances to that of Vitamin B$_{12}$ and therefore it is necessary at this point to include some detailed information on the transport of iron in *E. coli*, in order to show the features common to both systems.

1.7 **Iron transport in *E. coli***.

Most substrates taken up by *E. coli* pass through the outer membrane via water-filled pores formed by proteins designated porins (Benz, 1988). However, these pores are inactive for the uptake of iron (III) siderophores or Vitamin B$_{12}$. These compounds bind to specific outer
membrane receptor proteins and are released into the periplasm in a TonB and energy-dependent process.

Schöffler and Braun (1989) proposed a model in which the activity of the FhuA (formerly TonA) receptor is regulated by the energized state of the cytoplasmic membrane and that TonB forms the coupling device between the outer and the cytoplasmic membrane (Hantke & Braun, 1978). According to this model, TonB assumes different conformations in response to the energized state of the cytoplasmic membrane, thereby allosterically regulating the conformation of FhuA.

A similar model was developed for the uptake of Vitamin B$_{12}$ across the outer membrane, which has been mentioned previously (Reynolds et al, 1980). This model gained support by a mutation in the structural gene (btuB) of the Vitamin B$_{12}$ receptor. Vitamin B$_{12}$ was absorbed, but was not transported into cells carrying the btuB 451 mutation. This mutation could be suppressed by two mutations in tonB, suggesting a physical interaction between the receptor and the TonB protein (Heller et al, 1988). The single amino acid replacement in the Vitamin B$_{12}$ receptor was located close to the aminoterminal end in which a consensus sequence called the 'TonB-box' is found in all receptor proteins involved in TonB-dependent transport systems (Coulton et al, 1986; Heller & Kadner, 1985; Lundrigan & Kadner, 1986; Pressler et al, 1988 and Sauer et al, 1987).
The *fhuA* gene encodes an outer membrane protein which is an essential constituent of the ferrichrome transport system. The FhuC, D and B proteins catalyse the transport of ferrichrome and of other Fe$^{3+}$-hydroxamate compounds from the periplasm, through the cytoplasmic membrane, into the cytoplasm (Hantke, 1983). The FhuC protein was found in the cytoplasmic membrane (Fecker & Braun, 1983). The *fhuC* sequence revealed a rather hydrophilic protein displaying a strong homology to ATP-binding proteins (Coulton *et al*., 1987). Such proteins were found in transport systems for certain sugars, peptides and amino acids, which depend on periplasmic binding proteins (Ames, 1986).

This was taken as evidence that transport of ferrichrome across the outer membrane may follow a periplasmic binding protein dependent transport mechanism (PBT). Recently sequences homologous to ATP-binding proteins were also found in the BtuD protein for Vitamin B$_{12}$ transport (Friedrich *et al*., 1986), supporting the hypothesis that outer membrane receptor dependent transport systems follow the PBT mechanisms across the cytoplasmic membrane.

Periplasmic transport proteins were also identified to be involved in Vitamin B$_{12}$ (Reynolds *et al*., 1980) and Fe$^{3+}$-dicitrate (Pressler *et al*., 1988), strengthening the notion of a PBT mechanism. Furthermore, the Vitamin B$_{12}$ and the Fe$^{3+}$-dicitrate transport systems contain, respectively, one and two very hydrophobic proteins in the cytoplasmic membrane, which are typical of the PBT systems.
The location of FhuD, which is the only candidate for a periplasmic protein of the fhu operon, was investigated by Köster and Brun, 1989. It was released by converting cells to spheroplasts, showing a periplasmic location. Furthermore, FhuD was synthesised in two forms which differed in size by 3KDa. The larger protein is probably the precursor, which is converted to the mature form by cleavage of the signal peptide comprising 30 amino acids. A signal peptide which is typical for proteins which cross the cytoplasmic membrane.

Iron (III) transport into *E. coli* is unique in that the metal ion has to be solubilised by compounds of low molecular weight called siderophores. In addition, essential constituents of the transport systems are receptor proteins in the outer membrane which are highly specific for the iron (III) siderophore complex. How the activity of these receptor proteins is regulated is of general interest. Apparently, they are functionally coupled to the energization of the cytoplasmic membrane and the products of the *tonB* and possibly the *exbB* gene have been implicated in this process (for a recent review, see Braun, 1985).

After lengthy discussion of the genetic approaches used in *E. coli*, for the understanding of *B*₁₂ uptake and transport, the genetics of the lactic acid bacteria will be reviewed, with a view to understanding *B*₁₂ uptake and transport in *Lactobacillus leichmannii*. 
1.8 Genetics of lactic acid bacteria.

The lactic acid bacteria are of considerable economic importance, not only in the dairy industry, but also in the fermentation of meat and vegetables and the ensilation of grass. They consist of both coccoid (lactic streptococci) and rod-shaped bacteria (lactobacilli). There has been speculation on the feasibility of using genetic manipulation to improve these organisms in their capacity to act as starter cultures (Kondo & McKay, 1985; Sandine, 1987). In this respect, there has been much recent progress towards the development of a transformation system and vectors for lactic streptococci (Simon et al, 1986; Sanders & Nicholson, 1987; de Vos, 1987).

In contrast, the bacteria of the genus Lactobacillus appear less amenable to genetic manipulation. The understanding of molecular biology and the improvement of Lactobacillus strains in food fermentation, have been hampered by the absence of an in vitro DNA transfer system.

Since rod lactic acid bacteria are essential for a wide range of dairy fermentations and other industrial production (see Table 1.3), their genetic manipulation is crucial to the improvement of existing technological processes. The reasons for the widespread use of Lactobacilli in the preparation of foods and other fermentation processes is due to the many properties they possess (see Table 1.4).
<table>
<thead>
<tr>
<th>Product</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yoghurt</td>
<td><em>L. bulgaricus</em></td>
</tr>
<tr>
<td>Fermented milks</td>
<td><em>L. acidophilus</em></td>
</tr>
<tr>
<td></td>
<td><em>L. casei</em></td>
</tr>
<tr>
<td></td>
<td><em>L. bulgaricus</em></td>
</tr>
<tr>
<td>Cheeses</td>
<td><em>L. bulgaricus</em></td>
</tr>
<tr>
<td></td>
<td><em>L. helveticus</em></td>
</tr>
<tr>
<td>Soy sauce</td>
<td><em>L. delbrueckii</em></td>
</tr>
<tr>
<td>Crackers</td>
<td><em>L. plantarum</em></td>
</tr>
<tr>
<td>Pickles</td>
<td><em>L. plantarum</em></td>
</tr>
<tr>
<td>Cured ham</td>
<td><em>L. casei</em></td>
</tr>
<tr>
<td></td>
<td><em>L. plantarum</em></td>
</tr>
<tr>
<td>Sausages, meats</td>
<td><em>L. plantarum</em></td>
</tr>
<tr>
<td></td>
<td><em>L. reuteri</em></td>
</tr>
<tr>
<td>Distillery mashes</td>
<td><em>L. casei</em></td>
</tr>
<tr>
<td></td>
<td><em>L. fermentum</em></td>
</tr>
<tr>
<td></td>
<td><em>L. plantarum</em></td>
</tr>
<tr>
<td></td>
<td><em>L. delbrueckii</em></td>
</tr>
<tr>
<td>Product</td>
<td>Organism</td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Feed additives</td>
<td><em>L. acidophilus</em></td>
</tr>
<tr>
<td></td>
<td><em>L. bulgaricus</em></td>
</tr>
<tr>
<td></td>
<td><em>L. lactis</em></td>
</tr>
<tr>
<td>Silage starters</td>
<td><em>L. plantarum</em></td>
</tr>
<tr>
<td>Lactic acid</td>
<td><em>L. delbrueckii</em></td>
</tr>
</tbody>
</table>
Table 1.4 **Advantages of Lactobacillus strains for industrial processes.**
(taken from Chassy, 1985).

<table>
<thead>
<tr>
<th>Advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methods already exist for large-scale cultivation</td>
</tr>
<tr>
<td>Non-pathogenic</td>
</tr>
<tr>
<td>No toxins or toxic products formed</td>
</tr>
<tr>
<td>Aerotolerant</td>
</tr>
<tr>
<td>No aeration required</td>
</tr>
<tr>
<td>Moderately thermophilic (51-54°C)</td>
</tr>
<tr>
<td>Can withstand low pH</td>
</tr>
<tr>
<td>Natural products discourage contamination and spoilage</td>
</tr>
<tr>
<td>Ferment diverse carbohydrate feedstocks (whey, silage, plant juices and hydrolysed starch)</td>
</tr>
<tr>
<td>Rapid and abundant growth</td>
</tr>
<tr>
<td>Cultures are stable and viable</td>
</tr>
<tr>
<td>Non spore-forming</td>
</tr>
</tbody>
</table>
The development of a procedure for protoplast production and regeneration in *Lactobacillus* species could be the first step to obtain protoplast fusion or transformation, as already achieved in group N Streptococci (Gasson, 1980; Kondo & McKay, 1982) and in other gram-positive species (Hopwood, 1981).

1.8.1 Protoplast formation, regeneration and fusion.

Genetic analysis of lactobacilli has been impaired by the lack of a reliable transformation system and by 1984 there were still no published methods completely suitable for the efficient production of protoplasts from the *Lactobacillus* strains used.

Chassy and Giuffrida (1980) had reported that *Lactobacilli* can be lysed by lysozyme in the presence of PEG. PEG used as an osmotic stabilizer caused fusion and aggregation of *Lactobacillus* protoplasts. Mutanolysin, an endo-\(N\)-acetylmuramidase isolated from *Streptomyces globisporus* has also been used to produce protoplasts of *L. casei* (Shimizu-Kadota & Kudo, 1984).

Since then, protoplast formation and regeneration has been reported in *Bacillus thuringiensis* (Temeyer, 1987), *L. reuteri* (Vescovo et al, 1984), *Leuconostoc mesenteroides* (Otts & Day, 1987) and *L. casei* (Lee-Wickner & Chassy, 1984). Transfection has also been reported in *Lactobacillus*
Protoplast fusion is a system of genetic exchange widely used in gram-positive bacteria (Hopwood, 1981) to transfer either chromosomal markers or plasmids (Dancer, 1980; Gasson, 1980). Some examples can be found listed in Table 1.5.

Table 1.5 Protoplast fusion.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces coelicolor</em></td>
<td>Hopwood &amp; Wright, 1978</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Chang &amp; Cohen, 1979</td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td>Vorobjeva et al, 1980</td>
</tr>
<tr>
<td><em>Streptococcus lactis</em></td>
<td>Kondo &amp; McKay, 1982</td>
</tr>
<tr>
<td><em>Clostridium acetobutylicum</em></td>
<td>Reid et al, 1983</td>
</tr>
</tbody>
</table>

More recently, protoplast fusion has been described in *Lactobacillus* species (Cocconcelli et al, 1986; Iwata et al, 1986; Baek et al, 1986a, 1986b).

In 1986, Simon et al, reported the high-efficiency transformation of *S. lactis* protoplasts by plasmid DNA. The
procedure used similar conditions to that used by Chang and Cohen in 1979 for \textit{B. subtilis} i.e. PEG induced DNA uptake by protoplasts.

The following year, Morelli and colleagues reported a method for the transformation of \textit{Lactobacillus} protoplasts by plasmid DNA. The procedure also involved PEG treatment of protoplasts to induce DNA uptake.

Protoplast transformation procedures have no doubt opened the way for the application of recombinant DNA technology to various gram-positive bacteria genera. Unfortunately, these techniques are often neither efficient nor reproducible, presumably because protoplasts are difficult to prepare and to re-generate.

It is clear that the development of a plasmid transformation system is fundamental for further improvements in \textit{Lactobacillus} genetics.

Another approach to strain improvement which has been tried, is by conjugal exchange of genetic information between bacteria.
1.8.2 Conjugal transfer.

The demonstration by Vescovo and his colleagues (1983) that pAM81 conjugation extends to three different Lactobacillus species supported the proposed use (Gasson & Davies, 1980) of this plasmid as a molecular vector in lactic acid bacteria.

pAM81 is 26.5 Kb broad host range plasmid, originally isolated from S. faecalis (Clewell et al, 1974), carrying erythromycin resistance.

It is conjugally transferable to and between a number of other Streptococcus species (Gibson et al, 1979; Le Blanc et al, 1978), including S. lactis (Gasson & Davies, 1980), Staphylococcus aureus (Engel et al, 1980), Clostridium acetobutylicum (Oultram & Young, 1985; Oultram et al, 1987), various species of Bacillus (Orzech & Burke, 1984) including B. subtilis (Oultram & Young, 1985) and various Lactobacillus species (Vescovo et al, 1983; Gibson et al, 1979; West & Warner, 1985; Shrago et al, 1986; Romero & McKay, 1985; Tannock, 1987; Sasaki et al, 1988).

Sasaki et al (1988) reported the conjugal transfer of pAM81 to L. plantarum by a filter mating method. The conjugal transfer frequency of pAM81 from Streptococci to Lactobacilli has been reported to be generally low. They examined various factors affecting the transfer frequency of
this plasmid from \textit{S. faecalis} to \textit{L. plantarum}. They found that frequency depended on the type, pore size and side of the membrane filter used. They also found that the passage of sterilised water through the membrane under reduced pressure after donor and recipient cells were trapped on it, increased the transfer frequency about 10-fold.

Also in 1988, Thompson and Collins reported evidence for the conjugal transfer of plasmid pIP501 from \textit{S. faecalis} into commercially used strains of lactic streptococci and from these strains into two strains of \textit{L. helveticus}, a species used in the manufacture of hard cheeses, notably Emmental. Furthermore, they provided evidence that the plasmid could be transferred between derivatives of one strain of \textit{L. helveticus} and from \textit{L. helveticus} back to \textit{S. faecalis}.

pIP501, a plasmid originally isolated from \textit{S. agalactiae} (Hershfield, 1979) has several useful characteristics which include:-

(i) a potentially broader host range than pAM\textdelta{1}

(ii) it carries two antibiotic resistant markers allowing the scoring of the unselected marker as evidence for plasmid transfer (\textit{Er}^{\text{R}} \text{ and } \textit{Cm}^{\text{R}}).

(iii) it has been the source of DNA used for the construction of potential cloning vectors (Behnke \textit{et al}, 1981).
pIP501 has also been reported by Shrago and Dobrogosz (1988) to transfer conjugally from *S. faecalis* to *L. plantarum*. West and Warner had previously reported transfer of this plasmid to *L. plantarum* in 1985.

The *E. coli*/Streptococcal shuttle plasmids, pVA838 and pSA3 (Dao & Ferretti, 1985) were mobilised from *S. sanguis* to *L. plantarum* by pVA797 (broad host range streptococcal plasmid) by cointegrate formation (Shrago & Dobrogosz, 1988).

In 1987, Trieu-Cuot and colleagues developed a vector strategy that allowed transfer of plasmid DNA by conjugation from *E. coli* to various gram positive bacteria, in which transformation via natural competence had not been previously demonstrated. The prototype vector pAT187 contains the origins of replication of pBR322 and of the broad-host range plasmid pAMβ1, a kanamycin resistance gene known to be expressed in both gram negative and gram positive bacteria, plus the origin of transfer of the IncP plasmid RK2. This shuttle plasmid was mobilised efficiently by the self-transferable IncP plasmid pRK212-1 (co-resident in the *E. coli* donors) to *S. lactis*, *Staphylococcus aureus*, *B. thuringiensis* and *Enterococcus faecalis*. For a summary of the afore mentioned facts, refer to Table 1.6.

The development of a gene transfer system whereby plasmid cloning vectors can be introduced into Lactobacilli has been a priority for investigators of *Lactobacillus* spp. Suitable
<table>
<thead>
<tr>
<th>Organism transferred from</th>
<th>Vector/Plasmid</th>
<th>Organism transferred to</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. avium</em></td>
<td>pAM81</td>
<td><em>L. casei</em></td>
<td>-</td>
<td>Gibson et al, 1979</td>
</tr>
<tr>
<td><em>Streptococcus sp.</em></td>
<td>pAM81</td>
<td><em>L. plantarum</em></td>
<td>-</td>
<td>West &amp; Warner, 1985</td>
</tr>
<tr>
<td></td>
<td>and pIP501</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. sanguis</em></td>
<td>pVA838 &amp; pSA3</td>
<td><em>L. plantarum</em></td>
<td>Mobilised by pVA797 via cointegrate formation</td>
<td>Shrago &amp; Dobrososz, 1988</td>
</tr>
<tr>
<td>Organism transferred from</td>
<td>Vector/Plasmid</td>
<td>Organism transferred to</td>
<td>Comments</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------</td>
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<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td><em>S. lactis</em></td>
<td>pAM51</td>
<td><em>L. reuteri</em></td>
<td></td>
<td>Vescovo et al., 1983</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>L. acidophilus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>L. salivarius</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. lactis</em></td>
<td>pAM61</td>
<td><em>L. reuteri</em></td>
<td></td>
<td>Tannock, 1987</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>L. murinus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>L. fermentum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. faecalis</em></td>
<td>pIP501</td>
<td><em>L. helveticus</em></td>
<td>via commercially used strains of lactic Streptococci</td>
<td>Thompson &amp; Collins, 1988</td>
</tr>
<tr>
<td><em>S. faecalis</em></td>
<td>pIP501</td>
<td><em>L. plantarum</em></td>
<td></td>
<td>Shraro &amp; Dobrososz, 1988</td>
</tr>
<tr>
<td></td>
<td>pVA797</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>pIP501</td>
<td><em>L. helveticus</em></td>
<td></td>
<td>Langella &amp; Chopin, 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>L. delbruckii</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
vectors might be found among those already used successfully with other gram-positive genera. Alternatively vectors based on *Lactobacillus* indigenous plasmids could be constructed.

1.8.3 *Lactobacillus* plasmids.


Lactose metabolism in *L. casei* (Chassy et al, 1978; Hofer, 1977) has been shown to be dependent on the presence of plasmids. The phospho-\(\beta\)-galactoside galactohydrolase gene, one of the lactose metabolic genes has been cloned from the 35 Kb lactose plasmid pLZ64 into *E. coli* (Lee et al, 1982).

N-acetyl-D-glucosamine metabolism also appears to be plasmid determined in two strains of *L. helveticus*, but the biochemical pathway has not been characterised (Smiley & Fryder, 1978).

Plasmids have also been implicated in the multiple antibiotic resistance of isolates of a number of species of *Lactobacilli* (Ishiwa & Iwata, 1980; Morelli et al, 1983; Vescovo et al, 1982).
Axelsson et al. 1988 identified and described the first cloning, to date, of a plasmid-encoded antibiotic resistance gene (Er^T) from Lactobacillus reuteri.

In view of the recent advances in laboratory gene transfer techniques in Lactobacilli, plasmids containing antibiotic resistance markers or constructions thereof, will become useful as cloning vectors for these industrially important bacteria.

This year, studies have been carried out to show that a strain of L. hilgardii, a lactic acid bacterium generally associated with alcoholic fermentation, harbours several cryptic plasmids (Josson et al., 1989). Two of these plasmids have been characterised and one of these, pLAB1000 (3.3 Kb) appeared to replicate in a variety of gram-positive species. They describe the construction of gram-positive cloning vectors based on this replicon. Different shuttle vectors were developed using the pLAB1000 plasmid, which could be stably maintained in Lactobacillus under selective conditions. They are presently developed as promoter-probe vectors or expression secretion vectors for Lactobacillus. These new vectors will enable the expression of different heterologous genes in lactic acid bacteria and used possibly to improve Lactobacillus strains used in silage or dairy fermentations.
Reports on plasmids from gram-positive hosts being able to replicate in gram negative organisms are scarce (Ehrlich, 1977; Kok et al, 1984; del Solar et al, 1987).

Up until recently, the low transformation efficiency of ligated plasmid DNA from gram-positive bacteria has resulted in that most cloning strategies developed in these bacteria usually involve the construction of recombinant plasmid DNA molecules in E. coli, where standard DNA methodology can be applied and their subsequent introduction by transformation in the gram-positive of interest. Table 1.7 shows the Lactobacillus DNA cloned to date.

Returning to the fact that the development of a plasmid transformation system is essential for further improvements in Lactobacillus genetics, an alternative method has recently been investigated and is proving to be very successful. This method has been adapted from the methods of electric field - induced fusion of membranes - termed electrofusion - and the generation of small localised holes in biological membranes - termed electroporation - which were first developed for eukaryotic cells by the group of Zimmermann (for a review see Zimmermann, 1982).

1.8.4 Electroporation.

Electroporation, one of the most versatile tools available for the introduction of DNA into cells, involves the application of a brief, high voltage pulse to a suspension
<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene</th>
<th>Cloned</th>
<th>Nucleotide Sequenced</th>
<th>Expressed in E. coli</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. casei</em></td>
<td>β-D-phosphogalactoside</td>
<td>shot-gun</td>
<td>-</td>
<td>-</td>
<td>Lee et al., 1982</td>
</tr>
<tr>
<td></td>
<td>galactohydrolase gene</td>
<td>clone bank</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>dihydrofolate reductase</td>
<td>using pBR322</td>
<td>-</td>
<td>-</td>
<td>Davies &amp; Gronenborn, 1982</td>
</tr>
<tr>
<td></td>
<td>gene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>dihydrofolate reductase</td>
<td>-</td>
<td>✓</td>
<td>-</td>
<td>Andrews et al., 1985</td>
</tr>
</tbody>
</table>
Table 1.7 continued.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Enzyme/Protein</th>
<th>Expression Method</th>
<th>Location</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. delbruecki</em></td>
<td>gene for malolactic fermentation of wine</td>
<td>-</td>
<td>in E. coli</td>
<td>Williams et al., 1983, 1984</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus 30a</td>
<td>Prohistidine decarboxylase</td>
<td>√</td>
<td>dideoxy sequencing</td>
<td>Vanderslice et al., 1986</td>
<td></td>
</tr>
<tr>
<td><em>L. bulgaricus</em></td>
<td>plasmid encoded β-galactosidase gene from <em>L. casei</em></td>
<td>-</td>
<td>-</td>
<td>Flickinger et al., 1986</td>
<td></td>
</tr>
<tr>
<td><em>L. bulgaricus</em></td>
<td>β-galactosidase gene</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>Schmidt et al., 1989</td>
</tr>
<tr>
<td><em>L. bulgaricus</em></td>
<td>gene encoding serine tRNA with the anticodon CGA</td>
<td>-</td>
<td>expressed &amp; active in E. coli</td>
<td>Hottinger et al., 1987</td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>Thymidylate synthase gene</td>
<td>√</td>
<td>dideoxy sequencing high expression &amp; active in E. coli</td>
<td>Pinter et al., 1988</td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>Factor III&lt;sup&gt;lac&lt;/sup&gt; gene</td>
<td>using dideoxy</td>
<td>-</td>
<td>Alpert &amp; Chassy, 1988</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.7 continued.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene Product</th>
<th>Method</th>
<th>Tech.</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. casei</em></td>
<td>( \beta)-D-phospho-</td>
<td>shotgun</td>
<td>dideoxy</td>
<td>Porter &amp; Chassy,</td>
</tr>
<tr>
<td></td>
<td>galactoside galactohydrolase</td>
<td>cloned</td>
<td>sequencing</td>
<td>1988</td>
</tr>
<tr>
<td><em>L. bulgaricus</em></td>
<td>gene encoding (UCG) tRNA\text{ser}</td>
<td>(\checkmark)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. delbrueckii</em></td>
<td>gene encoding a minor (UCG) tRNA\text{ser}</td>
<td>(\checkmark)</td>
<td>(\checkmark)</td>
<td>-</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>D-2-hydroxyisocaproate dehydrogenase</td>
<td>using</td>
<td>dideoxy expression</td>
<td>Lerch et al, 1989</td>
</tr>
<tr>
<td></td>
<td>pBR322 sequencing in <em>E. coli</em></td>
<td>pBR322</td>
<td>in <em>E. coli</em></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.7 continued.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene Product</th>
<th>Method</th>
<th>Tech.</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. helveticus</em> subsp. jogurti</td>
<td>cryptic plasmid pLJ1</td>
<td>using</td>
<td>dideoxy</td>
<td>Takiguchi et al, 1989</td>
</tr>
<tr>
<td><em>L. bulgaricus</em></td>
<td>DNA fragment containing a 5S RNA gene adjacent to a cluster of five tRNA genes</td>
<td>(\checkmark)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. bulgaricus</em></td>
<td>Hexameric tRNA gene cluster associated with rRNA genes</td>
<td>-</td>
<td>dideoxy sequencing</td>
<td>Pittet &amp; Hottinger, 1989b</td>
</tr>
</tbody>
</table>

\(\checkmark\) and '-' refer to inclusion or exclusion of data, respectively.
of cells plus DNA. The result is transient membrane permeability and the subsequent uptake of DNA. Electroporation has been used to introduce DNA into both eukaryotic and prokaryotic cells.

Since current methods for the transfection and transformation of Lactobacilli using protoplasts are slow, inefficient and inconsistent, Chassy & Flickinger, 1987 evaluated electroporation as a simple alternative.

Compared to most other mechanisms, electroporation is less time-consuming, less tedious, less expensive (and in some cases, the only method available for recovering plasmid-containing transformants).

An optimal voltage and time constant must be found for each cell type (see further details in Chapter 7 of this thesis) and electroporation apparatus, since sub-optimal conditions either fail to introduce DNA or result in excessive cell death due to permanent disruption of the cell membranes.

Aukrust and Nes (1988) reported the transformation of L. plantarum with pTV1. pTV1 contains the pE194 replicon from Staphylococcus aureus and Tn917 from S. faecalis pTV1 was shown to replicate as a high copy number plasmid in L. plantarum and the two encoded antibiotic resistance traits were expressed. There have been no previous reports on transposition in Lactobacilli, although a Lactobacillus
insertion sequence ISL1 has been found in *L. casei* (Shimizu-Kadota et al., 1985; Shimizu-Kadota, 1987).

Luchansky et al. (1988) described the transfer of different plasmids to *L. acidophilus* by electroporation and their efforts to test the efficiency of this technology with other gram-negative genera.

Although the electroporation protocol was not optimized for each of the various genera and strains tested, the results demonstrate that many gram positive bacteria were amenable to plasmid transformation via electroporation, using a common protocol.

Other successes in electroporation by several groups of workers are listed in Table 1.8.

pGK12, used by Luchansky and colleagues is a small 4.4 Kb plasmid carrying CsR (from pC194) and ErR (from pE194). It replicates in a variety of gram-positive hosts as well as *E. coli*, using the origin of replication from pWVO1, a small cryptic plasmid native to *S. cremoris* (Kok et al., 1984).

Efforts to optimise electroporation could address several parameters affecting the physical condition of the recipient cells (growth media, aeration, pH, cell age, integrity of the cell wall), selection of a suitable replicon and vector markers and evaluation of resident plasmid DNA in the recipient.
Table 1.8  List of organisms that have either been transformed or transfected by electroporation.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Vector</th>
<th>Transformation/ Transfection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. lactis</em></td>
<td>-</td>
<td>transformation</td>
<td>Harlander, 1986</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>PL-1</td>
<td>transfection</td>
<td>Chassy &amp; Flickinger, 1987</td>
</tr>
<tr>
<td></td>
<td>(40 kb)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLZ15</td>
<td>transformation</td>
<td>Chassy &amp; Flickinger, 1987</td>
</tr>
<tr>
<td></td>
<td>(28 kb)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pSA3 (Er\textsuperscript{R})</td>
<td>transformation</td>
<td>Chassy &amp; Flickinger, 1987</td>
</tr>
<tr>
<td></td>
<td>pLP825 (Cm\textsuperscript{R})</td>
<td>transformation</td>
<td>Flickinger, 1987</td>
</tr>
<tr>
<td></td>
<td>pNZ12 (Cm\textsuperscript{R})</td>
<td>transformation</td>
<td>Flickinger, 1987</td>
</tr>
<tr>
<td><em>E. carotovora</em></td>
<td>pBR329</td>
<td>transformation</td>
<td>Ito et al, 1988</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>pTV1</td>
<td>transformation</td>
<td>Aukrust &amp; Nes, 1988</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>Tn917</td>
<td>(transposition)</td>
<td>Aukrust &amp; Nes, 1988</td>
</tr>
<tr>
<td>Organism</td>
<td>Vector</td>
<td>Method</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------------</td>
<td>------------</td>
<td>--------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>pUC18</td>
<td>transformation</td>
<td>Calvin &amp; Hanawalt, 1988</td>
</tr>
<tr>
<td><em>S. cremoris</em></td>
<td>pMU1328</td>
<td>transformation</td>
<td>Powell et al., 1988</td>
</tr>
<tr>
<td><em>S. lactis</em> LMO230</td>
<td>pMU1328</td>
<td>transformation</td>
<td>Powell et al., 1988</td>
</tr>
<tr>
<td>(now <em>Lactococcus lactis</em> subsp. lactis)</td>
<td>pMU1329</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pLS1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pAMβ1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>pUC12</td>
<td>transformation</td>
<td>Wilson &amp; Gough, 1988</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>ρ3RF</td>
<td>transfection</td>
<td>Taketo, 1988</td>
</tr>
<tr>
<td></td>
<td>M13mp19RF</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>pSA3</td>
<td>transformation</td>
<td>Taketo, 1988</td>
</tr>
<tr>
<td></td>
<td>pBR322</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>QB phage</td>
<td>transfection</td>
<td>Taketo, 1989</td>
</tr>
<tr>
<td></td>
<td>RNA</td>
<td>of RNA</td>
<td></td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>pC194</td>
<td>transformation</td>
<td>Bone &amp; Ellar, 1989</td>
</tr>
<tr>
<td></td>
<td>PUB110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organism</td>
<td>Vector/Subvector</td>
<td>Transformation</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------</td>
<td>---------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>Leuconostoc paramesenteroides</td>
<td>pAMβ1</td>
<td>transformation</td>
<td>David et al., 1989</td>
</tr>
<tr>
<td>Bordetella</td>
<td>pRK404</td>
<td>transformation</td>
<td>Zealey et al, 1988</td>
</tr>
<tr>
<td></td>
<td>(broad host</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>range cloning</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>vector)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>pAMβ1</td>
<td>transformation</td>
<td>Allen &amp; Blaschek, 1988</td>
</tr>
<tr>
<td></td>
<td>pHR106</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(7.9 kb shuttle</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>vector)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium acetobutylicum</td>
<td>pMTL500E</td>
<td>transformation</td>
<td>Oultram et al, 1988</td>
</tr>
<tr>
<td></td>
<td>(shuttle vector - ErF gene and replication machinery of pAMβ1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactococcus lactis subsp. lactis</td>
<td>pSA3</td>
<td>transformation</td>
<td>McIntyre &amp; Harlander, 1989a</td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>pGB301</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>pGK12</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>(ErF:CmF)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organism</td>
<td>Plasmid</td>
<td>Transformation</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>---------</td>
<td>----------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>pC194</td>
<td></td>
<td>Mahillon et al, 1989</td>
</tr>
<tr>
<td></td>
<td>pE194</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brevibacterium lactofermentum</em></td>
<td>pUL340</td>
<td></td>
<td>Haynes &amp; Britz, 1989</td>
</tr>
<tr>
<td><em>Corynebacterium glutamicum</em></td>
<td>pUL340</td>
<td></td>
<td>Haynes &amp; Britz, 1989</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>pX193</td>
<td></td>
<td>Schurter et al, 1989</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>pKT231</td>
<td></td>
<td>Wirth et al, 1989</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
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</tr>
<tr>
<td><em>Erwinia carotovora</em></td>
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<td></td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>pAMβ1</td>
<td></td>
<td>Luchansky et al, 1988</td>
</tr>
<tr>
<td></td>
<td>pC194</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pGB354&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pGKV1&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
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<td></td>
<td>PSA3</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>pTRK13&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pTV1&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pVA797&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Bacillus,
Enterococcus,
Lactococcus, pGK12 transformation Luchansky et al, 1988
Leuconostoc,
Listeria,
Pediococcus,
Propionibacterium and
Staphylococcus

E. coli TGI M13 DNA transfection Heery & Dunican 1989

Lactococcus pGB301 transformation McIntyre & Harlander, 1989b
lactis subsp. lactis

a 6.2 kb, Cm$^\text{R}$ (Behnke & Gilmore, 1981)
b 4.6 kb, Cm$^\text{R}$Er$^\text{R}$ (van der Vossen et al, 1985)
c 12.5 kb, Cm$^\text{R}$ (Luchansky et al, 1988)
d 12.4 kb, Cm$^\text{R}$Er$^\text{R}$ (Youngman et al, 1983)
e 30.7 kb, Cm$^\text{R}$Tra$^+$ (Evans & Macrina, 1983)

(Tra$^+$ - self transmissible conjugative plasmid)
This section would not be complete without mentioning the classical techniques of microbial genetics, such as chemical mutagenesis.

1.8.5 Classical genetic manipulation of Lactobacilli.

As a genus, *Lactobacillus* sp. are fastidious and require various nutrients, including amino acids, vitamins and nucleotides for growth. Their nutritional requirements do not appear to be the result of an absence of the genes encoding the required enzymes for each pathway, but result from the mutational loss of one or more metabolic steps. Evidence for this phenomenon in *L. casei*, *L. plantarum*, *L. helveticus* and *L. acidophilus* has been reported by Morishita et al, 1974 and 1981. These complicated nutritional requirements of lactic acid bacteria often make classical genetics tedious.

However, Rodriguez-Quinones et al, (1982) reported NTG mutagenesis in *L. plantarum*. They showed that efficiency was good with optimal concentrations 500 \( \mu g \) ml\(^{-1}\) or lower. The frequencies they obtained of induced mutations were of two orders of magnitude higher than spontaneous mutants.

With the view to improving strains, the mutagenesis approach does have short-comings in that it's impossible to derive strains with completely new genes or with mixtures of genes from two or more separate strains.
A classical method which does result in transferring genes between strains is that of phage-mediated transduction. Bacteriophages are common among Lactobacilli and the majority of strains isolated from natural sources appear to be lysogenic for one or more phage (i.e., they harbour phage in a latent state) (Yokokura et al, 1974). However, no laboratory has reported successful transduction of a genetic trait between Lactobacilli. Exploitation of this technique awaits investigation.

1.9 Aims of this project.

Information regarding the molecular genetics in the Lactic acid bacteria is accumulating and therefore, these studies were initiated to genetically engineer strains of Lactobacillus leichmannii, which have increased specificity to different cobalamins.

To achieve this, the gene encoding the Vitamin B$_{12}$ binding protein in L. leichmannii will be cloned and expression studies carried out to identify the gene product. The nucleotide sequence of the gene will be ascertained in order to attempt to alter the specificity of the binding protein, by site-specific mutagenesis. Finally, the modified gene(s) will be replaced into the original organism and the changes in specificity evaluated.
CHAPTER 2

METHODS AND MATERIALS
Chapter 2 : Methods and Materials

2.1 Bacterial strains and plasmids.

The bacterial strains and plasmids used in this study are listed in Tables 2.1 and 2.2 respectively.

2.2 Bacteriophage.

The bacteriophage used in this study are listed in Table 2.3.

2.3 Media.

2.3.1 Lactobacilli media.

Rich media i.e. MRS broth (de Man et al, 1960) was used to grow all Lactobacilli used in this study.

2.3.2 Enterobacteriaceae media.

Rich media i.e. Luria-Bertani broth (LB), as described in Maniatis et al., 1982 was used routinely to grow the enteric organisms used in this study.

2.3.3 Bacilli media.

Rich media i.e. Nutrient broth was used routinely to grow Bacillus subtilis.
Table 2.1  Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>leichmannii 8964</td>
<td></td>
<td>NCIB</td>
<td>White, 1958</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>leichmannii 4797</td>
<td></td>
<td>ATCC</td>
<td>Bergey et al, 1925</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>casei 7473</td>
<td></td>
<td>NCIB</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plantarum</td>
<td></td>
<td>P. Warner</td>
<td>West &amp; Warner, 1985</td>
</tr>
<tr>
<td>NCDO 1752</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>coli DH1</td>
<td>$F^-, recA1, endA1$</td>
<td>D. Cardy</td>
<td>Hanahan, 1983</td>
</tr>
<tr>
<td></td>
<td>$gyrA96\ thi-1, hasDR17$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$(r_k M_k), supE44. \lambda^-$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>coli HB101</td>
<td>$F^-, hasS20 (r_B, M_B)$,</td>
<td>D. Cardy</td>
<td>Boyer &amp; Roulland-Dussoiz, 1969</td>
</tr>
<tr>
<td></td>
<td>$recA8, ara-14, proA2,$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$lacY1, galK2, rpsL20$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$xyl-5, mtl-1, supE44,$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\lambda^-$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain</td>
<td>Genotype</td>
<td>Source</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------------</td>
<td>-------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td><em>Escherichia coli</em> CSH26ΔF6</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, ara, Δ(lac-pro), thi, rpsL, Δ(recA-srl)</td>
<td>D. Cardy</td>
<td>Jones &amp; Holland, 1984</td>
</tr>
<tr>
<td></td>
<td>F&lt;sup&gt;6&lt;/sup&gt;, sup&lt;sup&gt;0&lt;/sup&gt;, λ&lt;sup&gt;−&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> TGI</td>
<td>(pro-lac)&lt;sup&gt;+&lt;/sup&gt;, supE, thi, F&lt;sup&gt;−&lt;/sup&gt;, traO, proAB&lt;sup&gt;+&lt;/sup&gt;, lacI&lt;sup&gt;q&lt;/sup&gt;, lacZ&lt;sup&gt;V15&lt;/sup&gt;</td>
<td>D. Cardy</td>
<td>Carter et al, 1985</td>
</tr>
<tr>
<td><em>Escherichia coli</em> NFB362</td>
<td>MC400, argE3, rpoB, recA6, srl-300 :: Tn10</td>
<td>J-P. Bohin</td>
<td>Aufrère et al, 1986</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Characteristics</td>
<td>Phenotype</td>
<td>Source</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------</td>
<td>------------</td>
<td>--------------</td>
</tr>
<tr>
<td>pBR327</td>
<td>Multicopy cloning vector</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>D. Hodgson</td>
</tr>
<tr>
<td>pBR325</td>
<td>Multicopy cloning vector</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;Tc&lt;sup&gt;r&lt;/sup&gt;Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>D. Cardy</td>
</tr>
<tr>
<td>pBR329</td>
<td>Multicopy cloning vector</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;Tc&lt;sup&gt;r&lt;/sup&gt;Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>D. Hodgson</td>
</tr>
<tr>
<td>PAT153</td>
<td>High copy variant of pBR322</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>C. Oakley</td>
</tr>
<tr>
<td>PACYC184</td>
<td>Stringent cloning vector</td>
<td>Tc&lt;sup&gt;r&lt;/sup&gt;Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>C. Oakley</td>
</tr>
<tr>
<td>pNF48</td>
<td>High copy number vector harbouring btuB gene</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;Sm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>J. P. Bohin</td>
</tr>
<tr>
<td>pSDC1</td>
<td>Derivative of pBR327</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Plasmid</td>
<td>Characteristics</td>
<td>Phenotype</td>
<td>Source</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
<td>-----------</td>
<td>------------</td>
</tr>
<tr>
<td>pSDC2</td>
<td>Derivative of pBR327</td>
<td>Ap&lt;sup&gt;F&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>pSDC3</td>
<td>Derivative of pBR327</td>
<td>Ap&lt;sup&gt;F&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>pAMβ1</td>
<td>Broad host range plasmid</td>
<td>Er&lt;sup&gt;F&lt;/sup&gt;</td>
<td>M. Gasson</td>
</tr>
<tr>
<td>pC194</td>
<td>High copy number plasmid</td>
<td>Cm&lt;sup&gt;F&lt;/sup&gt;</td>
<td>R. Saunders</td>
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<tr>
<td>pSA3</td>
<td>E. coli-Streptococcus Shuttle vector</td>
<td>Tc&lt;sup&gt;F&lt;/sup&gt;</td>
<td>M. Gasson</td>
</tr>
<tr>
<td>pSEC1</td>
<td>Derivative of pBR325</td>
<td>Ap&lt;sup&gt;F&lt;/sup&gt;Cm&lt;sup&gt;F&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>pSEC2</td>
<td>Derivative of pBR325</td>
<td>Ap&lt;sup&gt;F&lt;/sup&gt;Cm&lt;sup&gt;F&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>pBS</td>
<td>phagemid vector</td>
<td>Ap&lt;sup&gt;F&lt;/sup&gt;</td>
<td>I. Garner</td>
</tr>
<tr>
<td>pBS3</td>
<td>Derivative of pBS</td>
<td>Ap&lt;sup&gt;F&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Characteristics</td>
<td>Phenotype</td>
<td>Source</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------</td>
<td>-----------</td>
<td>--------</td>
</tr>
<tr>
<td>pBS7</td>
<td>Derivative of pBS</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>-</td>
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<tr>
<td>pCK1</td>
<td>High copy number plasmid</td>
<td>Cm&lt;sup&gt;r&lt;/sup&gt;Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>M. Gasson</td>
</tr>
<tr>
<td>Phage</td>
<td>Characteristics</td>
<td>Source</td>
<td>References</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------</td>
<td>---------------</td>
<td>----------------</td>
</tr>
<tr>
<td>M13tg130</td>
<td>-</td>
<td>Amersham Int.</td>
<td>Kieny et al, 1983</td>
</tr>
<tr>
<td>M13tg131</td>
<td>-</td>
<td>Amersham Int.</td>
<td>Kieny et al, 1983</td>
</tr>
<tr>
<td>VCS-M13</td>
<td>helper phage</td>
<td>I. Garner</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BF23</td>
<td>T₅-like phage</td>
<td>R. E. Glass</td>
<td>Heller, 1984</td>
</tr>
</tbody>
</table>
2.3.4 Antibiotics.

Antibiotics, where appropriate, were normally prepared as 100 x stocks and used at the following concentrations (unless otherwise stated):

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Stock concentration (mg ml(^{-1}))</th>
<th>Final concentration (µg ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (Ap)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Chloramphenicol (Cm)</td>
<td>35</td>
<td>15</td>
</tr>
<tr>
<td>Kanamycin (Km)</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Streptomycin (Sm)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Tetracycline (Tc)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Erythromycin (Er)</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>

Antibiotic stock solutions were prepared as described in Maniatis et al., 1982.
### 2.3.5 Chemicals used in this study.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>General materials</td>
<td>BDH/Sigma or Fisons</td>
</tr>
<tr>
<td>Restriction enzymes and buffers</td>
<td>Amersham International</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>Amersham International</td>
</tr>
<tr>
<td>T4 polynucleotide kinase</td>
<td>Amersham International</td>
</tr>
<tr>
<td>$^{35}$S-methionine</td>
<td>Amersham International</td>
</tr>
<tr>
<td>$^{32}$P-nucleotides</td>
<td>Amersham International</td>
</tr>
<tr>
<td>Nitrocellulose filters</td>
<td>Schleicher &amp; Schull</td>
</tr>
<tr>
<td>Restriction enzymes and buffers</td>
<td>Boehringer-Mannheim (BRL)</td>
</tr>
<tr>
<td>DNA polymerase I (nick translation)</td>
<td>Boehringer-Mannheim (BRL)</td>
</tr>
<tr>
<td>DNA sequencing materials (acrylamide, bisacrylamide,</td>
<td>BioRad Laboratories</td>
</tr>
<tr>
<td>ammonium persulphate, sodium dodecylsulphate and urea</td>
<td></td>
</tr>
<tr>
<td>X-ray film</td>
<td>Fuji Photo Film Co. Ltd.</td>
</tr>
<tr>
<td>Organic acids and Solvents</td>
<td>May &amp; Baker</td>
</tr>
<tr>
<td>Sephadex G50</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>DNA sequencing nucleotides</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>DNA polymerase I (Klenow)</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>Calf intestinal alkaline phosphatase (CIAP)</td>
<td>BCL</td>
</tr>
</tbody>
</table>
2.4 Growth and maintenance of bacterial cultures.

2.4.1 Organism maintenance.

All *Escherichia coli* strains (with the exception of TG1) were maintained on LB agar plates containing the appropriate antibiotic where required, for up to 6 weeks at 4°C. *E. coli* TG1 was maintained on M9 minimal agar plates containing 0.1 ml, 0.1% (w/v) thiamine litre⁻¹. For longer term storage, an overnight culture was mixed with an equal volume of sterile glycerol in a glass vial and stored at -20°C.

All *Bacillus* strains were maintained on NB agar plates and *Lactobacillus* strains on MRS agar plates, in the same way as for *E. coli*. Long term storage again was the same as for *E. coli*.

2.4.2 Growth conditions.

*E. coli* and *Lactobacillus* strains were grown at 37°C unless otherwise stated in the text. Liquid cultures were routinely propagated in 25 ml universal bottles in a Gallenkamp Orbital Shaker (150 rpm).

*Bacillus* strains were grown at 30°C.
2.4.3 Light microscopy.

All cultures were examined by phase contrast microscopy using an Olympus PM-6 phase contrast microscope operating at x1000 magnification.

2.4.4 Spectrophotometry.

Routine determinations of culture optical density were performed at a wavelength of 600 nm through a 1 cm light path using an LKB Ultrospec II spectrophotometer.

2.5 Chromosomal DNA extraction.

2.5.1. *Escherichia coli*.

Chromosomal DNA extraction was carried out using the technique of Marmur, 1961 with modifications (Oakley & Murrell, 1988). Volumes were scaled down to allow all operations to be carried out in 35 ml polypropylene Oakridge tubes. Cells were harvested and pellet resuspended in 6 ml of T.E. (10 mM Tris-HCl, 1 mM Na-EDTA pH 8.0) and transferred to a 35 ml polypropylene Oakridge tube, to which 3.75 ml 0.25 M EDTA pH 8.0 and 50 mg lysozyme were added and mixed gently. After incubation at 37°C for 15 mins, Proteinase K (250 μl of 20 mg ml⁻¹ stock) was added, followed by 3.25 ml SDS (10% w/v), which was added slowly with mixing and again incubated at 37°C until lysis occurred.
Sodium perchlorate (4 mls of 5M) was added (to aid DNA membrane separation) and placed at 60°C for 15 mins with occasional gentle stirring. An equal volume of TE saturated phenol/chloroform/iso-amylalcohol (25:24:1) was added and mixed gently. To facilitate complete separation of aqueous and organic phases, centrifugation was carried out using 8 x 50 rotor (18,000 rpm, 30 min, 4°C) and upper aqueous layer transferred to fresh Oakridge tube. An equal volume of chloroform was added and mixed gently, then centrifuged again (18,000 rpm, 30 min, 4°C) to separate aqueous and organic phases. Extraction was repeated twice. After final extraction, the aqueous phase was transferred to a polycarbonate 250 ml centrifuge pot. Sodium chloride was added (final concentration 0.1 M) and then two volumes of 100% ethanol. DNA was gently removed and washed with 70% ethanol at room temperature. The washed DNA was dried under vacuum for 30-60 min and then slowly resuspended overnight in 10 ml TE.

RNAse (20 mg ml⁻¹ in TE) was added to final concentration of 100 µg ml⁻¹ and the solution incubated at 37°C for 30 min. Chromosomal DNA was then purified by a Caesium chloride (CsCl) density gradient step.

The RNAse treated DNA solution was made up to 30 ml with TE into which 30 g CsCl was dissolved. Finally 3 ml of Ethidium bromide (10 mg ml⁻¹ stock) were added and the resulting mixture spun using a Beckman L8-70 centrifuge (45,000 rpm, 16 hr, 20°C). The resulting DNA band was extracted and ethidium
bromide removed by repeated iso-amylalcohol extraction and the DNA directly precipitated from CsCl as described by Davies et al., 1980.

2.5.2 Lactobacillus.

A modified method of Vanderslice et al, 1986 was used, whereby cells were harvested by centrifugation (8,000 rpm, 10 min, 4°C), and washed in 0.01 M Tris-HCl pH 8.2 and resuspended in this buffer. An equal volume of 24% w/v PEG 20,000 was added followed by 1/5 volume 0.02 M Tris-HCl, with 300 mg lysozyme dissolved. Incubation at 37°C for 40 mins was followed by centrifugation (20,000 g 15 mins) and pellet resuspended in 0.1 M Tris-HCl, 0.01 M EDTA pH 8.5.Suspension was mixed with 1/10 volume of 10% sarkosyl NL-30 and incubated at 37°C for 15 mins. 1g CsCl ml⁻¹ lysate was added and 400 µl of ethidium bromide (5 mg ml⁻¹ stock) and centrifuged 36,000 rpm, 47 hrs, 20°C. DNA was extracted by standard method (Maniatis et al, 1982).

2.6 Isolation of plasmid DNA.

2.6.1. Lactobacillus.

(a) Small scale (mini-prep).

The method devised by West & Warner, 1985 was used with the following volume modifications: the addition of 600 µl of lysis buffer, followed by the addition of 50 µl of 2M Tris (pH 7.0), in order to achieve neutralisation.
(b) Large scale.

The above method was adapted to lyse larger batch cultures (1 litre). The RNase step and the chloroform/isoamyl-alcohol extraction were omitted.

2.6.2 E. coli.

(a) Large scale.

The alkaline lysis technique of Birnboim and Doly, 1979 was used as described by Maniatis et al, 1982 except that solution II (alkaline SDS solution) was not placed on ice prior to use as the SDS precipitates. Also the inclusion of a centrifugation step (18,000 rpm 30 min) prior to CsCl gradient centrifugation removed material from the subsequent gradient. After removal of ethidium bromide, plasmid DNA was directly precipitated from CsCl as described previously.

(b) Small scale (mini-prep).

A scaled down version of the large scale prep, working with 1.5 ml of culture, instead of one litre, was used. Solution II again was not placed on ice prior to use and also the 70% (v/v) ethanol wash of the DNA pellets was omitted, as this resulted in loss of plasmid.
2.7 General solutions and media.

These solutions are referred to in the text of this chapter and their compositions, and where appropriate, the procedures for their preparation are described below:

**Chloroform (for DNA extraction)**
24:1 (v/v) chloroform: isoamyl alcohol

**Luria-Bertani media (LB)**
(per litre)
10 g Bacto tryptone
5 g Bacto yeast extract
5 g NaCl (pH 7.2)

**Phenol (neutralised, for DNA extraction)**
Neutralised with 1 M Tris-HCl pH 8.0, followed by 0.1 M Tris-HCl pH 8.0 and containing 0.1% (w/v) 8-hydroxyquinoline

**3M Sodium acetate pH 6.3**
3M NaAc brought to pH 6.3 with acetic acid

**20 x SSC**
3M NaCl
0.3 M trisodium citrate

**TE**
10 mM Tris-HCl
pH 8.0, 1 mM EDTA
TBE
0.089 M Tris-borate
0.089 M boric acid
0.002 M EDTA

M9 Salts (x 10)
(per litre)
60 g Na$_2$HPO$_4$ anhydrous
30 g KH$_2$PO$_4$ anhydrous
10 g NH$_4$Cl
5 g NaCl (pH 7.4)

M9 minimal medium
(per litre)
100 ml (x 10) M9 salts
10 ml 10 mM CaCl$_2$
0.1 M MgSO$_4$

K medium
(per litre)
M9 minimal medium
50 ml 20% (w/v) Bacto casamino acids
0.1 ml 0.1% (w/v) thiamine
10 ml 20% (w/v) glucose

Western Transfer buffer (WTB)
(per litre)
14 g glycine
2.42 g Tris
200 ml Methanol

Tris-buffered saline (TBS)
20 mM Tris pH 7.6
0.15 M NaCl
Hershey salts
(per litre)

3.0 g KCl
5.4 g NaCl
1.1 g NH₄Cl
15 mg CaCl₂ 2H₂O
0.2 g MgCl₂ 6H₂O
0.2 mg FeCl₃ 6H₂O
87 mg KH₂PO₄ anhydrous
12.1 g Tris-HCl (pH 7.4)

Hershey medium
(per litre)

Hershey salts
20 ml 20% (w/v) glucose
10 ml 2% (w/v) proline
1 ml 0.1% (w/v) thiamine

2.8 General techniques used for DNA manipulation.

2.8.1 Restriction endonuclease digestion.

Restriction endonucleases and restriction endonuclease buffers (x 10) were obtained, both from Amersham International and BRL, and used according to the manufacturers' instructions.

2.8.2 Dephosphorylation of DNA.

The terminal 5' phosphates were removed from DNA by treatment with calf intestinal phosphatase (CIP). This treatment minimized recircularisation of plasmid DNA and increased the
frequency of recombinant plasmid DNA molecules during ligation and transformation.

The method of D. Hodgson (pers. comm.) was used. To every 10 μl of restriction digestion solution;

- 1.5 μl 0.5 M glycine pH 9.4 (NaOH)
- 1.5 μl 10 mM MgCl₂, 1 mM ZnCl₂
- 0.5 μl CIP (BCL)

were added, mixed and incubated at 37°C for 30 min. The volume was then made up to 100 μl with distilled H₂O, the DNA extracted with an equal volume of phenol (Section 2.8.6), followed by a chloroform extraction and finally ethanol precipitated (Section 2.8.7).

2.8.3 Ligation of DNA.

DNA samples were mixed in appropriate volumes of TE buffer. To subclone fragments, a 4:1 fragment to vector ratio was used with a DNA concentration <50 μg ml⁻¹. For construction of gene libraries and subcloning using 'blunt-end' restriction endonucleases, a ratio of 6:1 fragment to vector was used and the DNA concentration increased to 100 μg ml⁻¹. Ligations were carried out using T4 DNA ligase (Amersham) according to the recommendation of the supplier, at 15°C for a minimum of 18 hours.
2.8.4 Agarose gel electrophoresis.

Horizontal slab gels were prepared by boiling agarose (Sigma) Tris-borate-EDTA (TBE) electrophoresis buffer and cooled slightly before pouring. DNA samples were prepared by adding 0.1 volume of loading buffer (0.25% w/v bromophenol blue, 15% w/v Ficoll-type 400) and they were then loaded into the gel slots. Electrophoresis was carried out with the gels completely submerged in buffer at 100-125 volts or 60 volts overnight. As DNA molecular weight markers, λ+ bacteriophage DNA digested with the restriction endonuclease HindIII was used. This generated fragments of sizes: 23.17, 9.46, 6.75, 4.26, 2.20, 1.92 and 0.58 kb. Restriction fragment sizes were determined with the DNASIZE programme adapted from Schaffer and Sederoff (1981) and run on a BBC Model B microcomputer.

The DNA was stained within the agarose gels with ethidium bromide (0.5 μg ml⁻¹) as described by Maniatis et al, 1982 and then visualised by transillumination with short-wave UV light and photographed using Polaroid Type 655 black and white film.

2.8.5 Quantitation of DNA.

The mini gel method as described by Maniatis et al, 1982 was routinely used to estimate the amount of DNA in a given sample. λ+ (HindIII restricted) DNA was the standard DNA used (50 ng μl⁻¹) for all estimations.
DNA in solution was estimated by using the method described by Schleif & Wensink, 1981.

**2.8.6 Extraction of DNA with phenol/chloroform.**

Phenol/chloroform mix was prepared by dissolving 100 g phenol crystals and 100 mg 8-hydroxyquinoline in 100 ml chloroform and 4 ml iso-amyl alcohol. This was equilibrated by shaking the mixture with two changes of 0.2 volume 1 M Tris pH 8.0 and two changes of 0.2 volume TE, before storage in foil-covered bottles. For the extraction procedure, DNA samples were mixed with an equal volume of phenol mix, to form an emulsion and the phases were separated by a 2 min spin in an MSE microcentaur. The upper aqueous phase was recovered, taking care not to disturb the interface. A further extraction with chloroform/iso-amyl alcohol (24:1) was carried out to remove any remaining phenol. DNA was then recovered by ethanol precipitation.

**2.8.7 Precipitation of DNA.**

To a DNA solution, one fiftieth volume of 5M NaCl and two volumes of 100% ethanol were added, mixed gently and placed at -20°C overnight. DNA was recovered by centrifugation in an MSE microcentaur (5 min, high speed, 4°C). All traces of the supernatant were discarded by use of a vacuum line and the DNA pellet dried under vacuum and resuspended in TE buffer.
For DNA prepared using an I.B.I. electroeluter or during ssDNA preparation for sequencing, 0.5 volume of ammonium acetate (7.5 M pH 7.5) and two volumes of 100% ethanol were added, mixed by vortexing and placed at -20°C overnight and DNA recovered as described above.

2.8.8 Preparation of DNA fragments from agarose gels.

DNA fragments to be eluted were separated by electrophoresis in agarose gels, 0.5% to 2% (w/v) depending on the size of fragment to be isolated. Once separated, the desired fragment was excised from the gel, and placed in an I.B.I. electroeluter. Fragments were eluted according to manufacturer's instructions and then ethanol precipitated as described previously.

2.9 Transformation.

2.9.1 E. coli.

The technique used was essentially that described by Holland, 1983. 1 ml of overnight culture of E. coli was used to inoculate 100 ml of LB in a 250 ml flask. This culture was grown to A600 of 0.5. From this point, all operations were carried out on ice and using ice-cold solutions. 40 ml of culture was added to two universal bottles and left on ice to chill. Cells were pelleted by minimal spins in a multex angled centrifuge and the resulting supernatant carefully removed. Resuspension was in equal volume of 0.1 M MgCl2.
Cell washing was repeated in 20 ml of 0.1 M CaCl₂ (Grade 1 CaCl₂ Sigma) and final resuspension left the cells in 2 ml of 0.1 M CaCl₂. These cells were held on ice for 24 hours (Dagert & Ehrlich, 1979).

200 μl of Competent cells were aliquoted into 1.5 ml Eppendorf tubes and 50 ng DNA added to the cells, mixed gently and then left on ice for 30 min. These were then heat shocked at 42°C for 2 min and returned to ice for 15 min. Two volumes of LB were then added to each tube, incubated for 1 hour at 37°C, to allow expression of genes, dilutions were made and cells plated out onto selective media.

2.9.2 L. leichmannii.

Electroporation, one of the most recent advances for the introduction of DNA into cells (involving the application of brief, high voltage pulse to a suspension of cells and DNA, resulting in a transient membrane permeability and the subsequent uptake of DNA), was investigated. This was due to the fact that current methods of transformation of Lactobacilli were slow, inefficient and inconsistent. Details of the experimentation can be found in Chapter Seven of this thesis.

2.10 Southern transfer of DNA.

This method followed the basic protocol of Southern, 1975, adapted by Maniatis et al, 1982. Denaturation and
neutralisation of DNA in the gel matrix were both carried out for 30 min, at room temperature. The transfer buffer was 20 x SSC instead of 10 x SSC. Before placing the nitrocellulose on it, the neutralised gel was wetted with 2 x SSC to remove excess salt. Usually the transfer of DNA was allowed to proceed overnight. When the filter was removed from the gel, it was soaked in 2 x SSC for a few minutes before drying and baked, under vacuum at 80°C for 2 hours.

2.11 Transfer of bacterial colonies to nitrocellulose and the binding of liberated DNA (colony blots).

The procedure of Grunstein & Hogness, 1975, as described by Maniatis et al, 1982 was used, with the following modifications. Large petri dishes (20 cm x 20 cm) were used containing 500 ml LB agar with the appropriate antibiotic(s). Nitrocellulose was placed onto the LB agar plates and colonies picked onto duplicate plates. Once the plates had been incubated at 37°C overnight, the master plate was stored at 4°C for up to 6 weeks and the nitrocellulose was treated as described by Maniatis et al, 1982.
2.12 Nick translation of DNA.

A modification of the procedure described by Maniatis et al, 1982, was used routinely.

\[
\begin{align*}
\text{X } & \mu \text{l DNA in TE (250 ng)} \\
1 & \mu \text{l 1 mM dATP} \\
1 & \mu \text{l 1 mM dTTP} \\
1 & \mu \text{l 1 mM dCTP} \\
1 & \mu \text{l }^{32}\text{P-dGTP (10 } \mu \text{Ci } \mu \text{l}^{-1}) \\
4 & \mu \text{l 10 x Nick translation buffer} \\
0.8 & \mu \text{l DNA polymerase I} \\
1 & \mu \text{l DNase I (1 } \mu \text{l of 1 mg ml}^{-1} \text{ stock in 50 } \mu \text{l } \text{H}_2\text{O, then 1 } \mu \text{l of above in 50 ml } \text{H}_2\text{O)} \\
Y & \mu \text{l H}_2\text{O}
\end{align*}
\]

X and Y were adjusted to give 20 \mu l final volume.

The above constituents were incubated at 15°C for 3 hours. Reaction was terminated by the addition of 5 \mu l 0.25 M EDTA (pH 8.0) and the nick translated probe separated from unincorporated dNTPs by passage through G50 Sephadex. Labelled DNA was stored at -20°C until required, upon which it was denatured by boiling for 15 min immediately prior to use.

2.13 Oligonucleotide synthesis.

Oligonucleotides were synthesized on an Applied Biosystems synthesizer model 380A (Applied Biosystems, Foster City, CA).
2.13.1 Purification of oligonucleotides.

Purification was achieved by two different methods. The latter being the most convenient.

(a) After deprotection using ammonia and end acid detritylation, sample was freeze dried and resuspended in 100 μl ddw. 10 μl aliquots were electrophoresed through a 42% (w/v) urea-polyacrylamide gel (sequencing gel). All oligonucleotides synthesised for this work were less than 30 bases and therefore a 20% (w/v) polyacrylamide gel was used, being 20 cm in length. Gel was pre-run for 1-1 ½ hours at 700 volts (which drops to 500 volts). An equal volume of 80% (v/v) formamide : 20% (v/v) glycerol, was added to sample. This was then subjected to 90°C for 3 mins and loaded onto gel. The oligo's can be found to run with the dye front according to the table below.

<table>
<thead>
<tr>
<th>% acrylamide</th>
<th>Bromophenol blue</th>
<th>Xylene cyanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>12 mer</td>
<td>55 mer</td>
</tr>
<tr>
<td>12</td>
<td>10 mer</td>
<td>46 mer</td>
</tr>
<tr>
<td>16</td>
<td>9 mer</td>
<td>35 mer</td>
</tr>
<tr>
<td>20</td>
<td>8 mer</td>
<td>28 mer</td>
</tr>
</tbody>
</table>

Gel was run for the required amount of time, wrapped in cling film, and placed on a TLC plate. Oligonucleotide could be
visualised as a band under short wave U.V. Band was cut out and placed in 5 ml of elution buffer (10 mM Mg acetate, 300 mM NH₄ acetate, 0.1% (w/v) SDS and 1 mM EDTA) at 37°C, shaking overnight. DNA solution was carefully extracted and diluted x 10 with ddw. This was then passed through a column of G25 Sephadex with a 0.1 ml layer of DE52 anion exchanger on top. Column was equilibrated with 50 mM NH₄HCO₃ and oligo' eluted with 50 mM NH₄HCO₃/1 M NaCl. A₂₆₀ was measured and fractions containing the oligo' were pooled. Aliquots were freeze dried and resuspended as required.

(b) By use of a Nensorb nucleic acid purification cartridge (DuPont). The method is unique; no salt is required to elute the nucleic acid. Samples containing up to 20 μg of DNA were quickly purified, according to the manufacturer's instructions and recovered in a salt-free liquid, typically 50% methanol in water.

2.13.2 End-labelling.

2 μg of purified oligonucleotide was ethanol precipitated overnight (see Section 2.7.7) and resuspended in 14 μl of water. To this was added 2 μl 10 x kinase buffer (0.7 M Tris-HCl (pH 8.0), 0.1 M MgCl₂, 50 mM DTT), 2 μl of T₄ polynucleotide kinase and 2 μl (20 μCi) of [γ-³²P] ATP. The mixture was incubated for 45 min at 37°C.
2.14 Hybridisation of Southern filters and colony blots.

The procedure described by Maniatis et al., 1982 was essentially followed with the following modifications. Nitrocellulose filters from either Southern or colony blotting were placed dry in heat-sealed Sterilin bags containing pre-hybridisation solution.

(a) For use with $[\gamma^{32}P]$-labelled oligonucleotide.

$$6 \times SSC$$
$$1 \times \text{Denhardts (Denhardt, 1966)}$$
$$200 \mu g \text{ ml}^{-1} \text{ sheared, heat-treated Herring sperm DNA.}$$

Room temperature, 1 hour.

(b) For use with $[\alpha^{32}P]$-labelled DNA. (Nick translated).

$$3 \times SSC$$
$$1 \times \text{Denhardts}$$
$$100 \mu g \text{ ml}^{-1} \text{ sheared, heat-treated Herring sperm DNA.}$$

$$45^\circ C, 1 \text{ hour}$$

After pre-hybridisation, the pre-hybridisation was replaced with fresh solution supplemented with the $^{32}P$-labelled probe.

Hybridisation was carried out for a minimum of 18 hours at the respective temperatures, followed by a variety of stringency washes, employing previously published guide-lines (Marmur &
Doty, 1962; Dove & Davidson, 1962; Bonner et al, 1973) to estimate the percentage DNA homology required for hybridisation. The damp filters were covered in cling-film and examined by autoradiography at -70°C.

2.15 Autoradiography.

Autoradiography was carried out at -70°C for $^{32}P$-labelled material and at room temperature for $^{35}S$-labelled material using Harmer film cassettes (with intensifying screens for $^{32}P$) and Fuji RX X-Ray film. Autoradiograms were developed in Kodak LX-24 developer and fixed in Kodak FX-40 according to the manufacturer's instructions.

2.16 Construction, maintenance and screening of a 
*Lactobacillus leichmannii* limited gene library.

Genomic DNA from *L. leichmannii* was totally digested with the restriction endonuclease HindIII. All DNA fragments in the size range, 1.5 kb to 2.4 kb, were electroeluted from a preparative 0.7% (w/v) agarose gel and ligated into the HindIII site of the appropriate vector, which had been dephosphorylated and transformed in either *E. coli* HB101 or *E. coli* DH1. 1000 of the resulting antibiotic resistant (depending on the vector used) colonies were then colony blotted onto nitrocellulose and onto a master plate. This colony blot was then probed with either [$\alpha^{32}P$]-labelled pNF48 1.5 kb EcoRI insert (see Figure 2.1) or [$\gamma^{32}P$]-labelled oligonucleotide.
2.17 **Expression analysis.**

2.17.1 *In vivo E. coli Maxicell system.*

This procedure was an adaptation of the method proposed by Sancar et al., 1979 to label plasmid-coded proteins *in vivo* with $^{35}$S-methionine. A recA strain carrying the plasmid of interest was grown in 15 ml K-medium (Rupp et al., 1979; Section 2.7) and grown to a cell density of $A_{600} = 0.5 \ (2 \times 10^8 \text{ cells ml}^{-1})$ and placed on ice. 10 ml cells were UV-irradiated (using gentle agitation) with a dosage of approximately 50 Jm$^{-2}$ in a petri dish. The cells were then transferred to a sterile flask and freshly prepared cycloserine was added to a final concentration of 200 $\mu$g ml$^{-1}$. The culture was incubated with shaking for 14-16 hours, but no longer than 16 hours.

Cells were harvested and washed twice in an equal volume of Hershey salts (Section 2.7) and finally resuspended in 5 ml Hershey medium (Section 2.7). Following 1 hour incubation, cells were again harvested and washed once in 10 mM Tris-HCl pH 8.0. SDS-sample buffer (50 $\mu$l of 2 x concentration) was added to the pellet and samples boiled for 5 min to solubilise cell proteins. Samples were stored at -20°C or analysed directly on SDS polyacrylamide gels (Section 2.20).
Figure 2.1  Plasmid pNF48 harbouring the E. coli btuB structural gene.

Key:

- B1  BamHI
- E1  EcoRI
- SI  SalI
- M1  MluI
- P1  PstI
- Z1  SmaI
- Ap  ampicillin resistance gene
- ==  pUC8
2.17.2 DNA-directed *in vitro* transcription-translation (Zubay) coupled system.

The bacterial cell-free coupled transcription-translation system first described by De Vries & Zubay, 1967 and later modified (Zubay, 1973; Collins, 1979), allows the expression *in vitro* of genes contained on a bacterial plasmid or a bacteriophage genome, provided that the relevant control signals are present (e.g. Pribnow box for initiation of transcription, Shine-Dalgarno sequence for translation). The system has been used in this study for the identification of protein products of cloned inserts. Advantages of this system are firstly, incorporation of radioactive label into protein is far more efficient than is possible using *in vivo* methods and secondly, DNA derived from bacteria other than *E. coli* is efficiently expressed (Pratt *et al*, 1981).

The system was used according to the manufacturer's instructions (Amersham International), using $^{35}$S-methionine as the labelled amino acid. Plasmid encoded proteins were identified by fluorography of electrophoresed samples on polyacrylamide gels (Section 2.20).
2.17.3 Gram positive coupled transcription-translation.

The method devised by Thompson et al., 1984 was used.

Cells were harvested and resuspended in S30 buffer (50 mM Hepes-KOH pH 7.5 at 20°C, 10 mM MgCl₂, 60 mM NH₄Cl, 10% (v/v) glycerol; 5 mM β-mercaptoethanol) using 2.5 ml buffer per gram wet weight. French pressed twice 12,000 psi and centrifuged (30 min, 4°C, 15000 rpm). Supernatant was removed and re-centrifuged under similar conditions, at which stage the resultant 30,000 x g supernatant (which gave 1 A₂₆₀ in 5 µl) was designated "S30". This was assayed immediately for activity in coupled transcription-translation. Incubation was carried out at 30°C in 30 µl S30 buffer with the final concentration of MgCl₂ adjusted to 12 mM. Each assay contained 2 A₂₆₀ units of S30, 2 µg plasmid DNA (added last), 8 µl synthesis mix (see below) and 2 µl [³⁵S]-methionine.
Synthesis mix contained:

200 mM Hepes-KOH (pH 8.2) at 20°C
48 mM ammonium acetate
95 mM potassium acetate
7 mM dithiothreitol
5 mM ATP
3.4 mM CTP, UTP, GTP
100 mM phosphoenolpyruvate - Na$_3$
19 amino acids (-methionine) - each at 1.4 mM
7.5 % (w/v) polyethylene glycol 6000
folic acid, Ca$^{++}$ salt, 0.14 mg ml$^{-1}$ (final conc.)

This mix was stored in small aliquots at -70°C, under which conditions it is stable indefinitely.

Assay mixtures were incubated for 20 min at which time unlabelled methionine (250 fold excess) was added and incubation was allowed to proceed for a further 10 min at 30°C to allow completion of all radiolabelled polypeptides.
Samples for analysis were mixed with one third volume of SDS sample buffer, and boiled for 10 min prior to loading on a single percentage linear SDS polyacrylamide gel (Section 2.20).
2.18 DNA Sequencing.

The dideoxynucleotide chain termination method of DNA sequencing (Sanger et al, 1977; Sanger et al, 1980) was employed.

2.18.1 Template preparation.

Template was prepared by either of two methods.

(a) Using M13tg130 and M13tg131 vectors.

Template preparation was essentially as described by Bankier et al, 1986, with the following modifications. E. coli TG1 was used as the host for M13 recombinant propagation. After propagation of phage for 5½ hours, cultures were transferred to 1.5 µl Eppendorf tubes and centrifuged in an MSE Microcentaur (20 min, high speed, room temperature). After careful transfer of supernatants to a fresh Eppendorf tube, 150 µl of PEG (6,000 mwt, 20% w/v) was added to each supernatant, vortexed briefly and left standing at room temperature for a minimum of 10 min. The supernatant/PEG solution was centrifuged to remove PEG. Any residual PEG was removed after a brief 30 sec. spin. The resulting phage pellet was then resuspended in 100 µl TE, allowed to stand at room temperature for 10 min and 50 µl TE saturated phenol added, vortexed well and centrifuged for 5 min at room temperature. 90 µl of the resulting aqueous layer was
removed and placed into a fresh Eppendorf tube containing 7.5M NH₄ acetate (45 µl) and 100% ethanol (200 µl), vortexed and placed at -20°C overnight. After precipitation, the vacuum-dried pellet was redissolved in 20 µl TE and stored at -20°C.

(b) Using Bluescribe M13 plasmid.

pBS(+/−), formerly pBluescribe M13 (+/−) is a 3,204 bp phagemid derived from pUC19 (see Figure 2.2). The vector carries a colEl origin, ampicillin resistance, T3 and T7 promoters flanking the pUC19 polylinker and a lacZ promoter for blue/white color selection or fusion protein induction with IPTG. It also carries an M13 origin of replication allowing single strand DNA rescue, via helper phage infection for single strand sequencing.

A method devised by I. Garner (pers. comm.) was followed. Firstly, clones of bluescribe recombinants were isolated and a stock of helper phage VCS-M13 was produced and titrated on XL-1 blue (kindly donated by I. Garner). Titre should be of the order of $1 \times 10^{11}-10^{12}$ pfu ml⁻¹. A 10 ml overnight recombinant culture was used to inoculate 500 ml of media. Incubation on rotating table at 37°C took place for 2.5 hours (early exponential phase), followed by addition to each flask of $3.5 \times 10^{10}$ pfu VCS-M13 and incubation continued for 4 hours. Cells were harvested (8,000 rpm, 4°C, 10 min) and supernatant transferred to sterile pot. To this was added 20 ml of 20% PEG 6000/2.5 M NaCl and left overnight at 4°C. Precipitated phage particles were pelleted (9,000 rpm, 4°C, 10
The PBS (+/-) phagemids are 3.2 kb colony forming hybrids, derived from the phage f1 and Stratagene's pBS plasmid.

Key:

- ori: origin of replication
- Ap: ampicillin resistance gene
- MCS: multiple cloning site
- E1: EcoRI
- S1: SaeI
- K1: KpnI
- X1: SmaI/XmaI
- B1: BamHI
- Xb1: XbaI
- H11: HincII
- P1: PstI
- Sp1: SphI
- H111: HindIII
- P11: PvuII
- N1: NdeI
min) and supernatant was carefully removed. Visible pellet was resuspended in 20 ml TE and a clearing spin carried out (4°C, 10 min, high speed MSE Microcentaur). Phage particles re-precipitated with 4 ml of 20% PEG 6000/2.5 M NaCl for 30 min and again pelleted. Pellet was resuspended in 200 µl of TE, extracted with 500 µl phenol and centrifuged at full speed in an MSE Microcentaur, 4°C, 10 min. Upper phase was extracted with 500 µl of chloroform/isoamyl alcohol (24:1) and spun as above. Samples were ethanol precipitated, resuspended in 200 µl TE and stored at -20°C.

Yields are much less than the M13 vectors, although usually 5 µl of the above 200 µl is sufficient to sequence as with M13 recombinants.

2.18.2 Dideoxy Sequencing.

This was carried out using the extended method of DNA sequencing with Klenow fragment. Recently, by modifying the standard dideoxy DNA sequencing procedure to separate chain extension and chain termination, more DNA sequencing data is obtained with the large fragment of DNA polymerase I (Klenow). Using this separated reaction strategy, it is possible to read far more from a single set of reactions.

For the template-primer annealing reaction, between 2.5 µl and 5 µl of template DNA was mixed with 1.5 µl sequencing buffer (5), 7.5 ng of primer (range between 17-25 bp) and sterile H₂O up to 10 µl final volume. Annealing was accomplished at 55°C
for 1 hour. All subsequent steps i.e. sequence reactions and denaturing were carried out in microtitre trays. Denaturation was carried out in an oven (Mini/696/Clad) at 80°C for 15 min and placed immediately on ice prior to loading the whole of each reaction mixture on a 6% (w/v) polyacrylamide, Tris-borate-urea sequencing gel.

2.18.3 Buffer gradient acrylamide gel electrophoresis.

The procedure described by Bankier et al., 1986 was followed using either 40 cm x 20 cm or 100 cm x 20 cm vertical sequencing gel apparatus (Raven). The power supply used for electrophoresis was an LKB Model 2103 power pack.

2.18.4 Sequence analysis.

All DNA sequence derived from this work was recorded and analysed using the Microgenie Sequence Analysis Program of Queen & Korn, 1986 and an IBM PCAT computer.

2.19 Outer membrane preparation.

The method described by Achtman et al., 1983 was followed, with modifications to the type of centrifuge used only.
2.20 Polyacrylamide gel electrophoresis (PAGE).

2.20.1 Slab gels.

Proteins were analysed on 4-15% (w/v) non-denaturing exponential gradients or single percentage (w/v) linear, SDS-polyacrylamide gels (Laemmli, 1970). The constitution of buffer and acrylamide solutions are given in Tables 2.4 and 2.5. Ammonium persulphate was always freshly prepared and the gel, once poured, was overlaid with water saturated butan-1-ol. Electrophoresis was carried out using an LKB gel system at a constant current of 8 mA for 16 hours or 30 mA for 3 hours.

2.20.2 Gel staining.

(a) Coomassie blue staining.

Gels were stained for at least an hour in 300 ml, 10% (v/v) glacial acetic acid, 30% (v/v) propan-2-ol, 0.05% (w/v) Coomassie brilliant blue. Destaining was carried out in the above solution, without stain for 30 min and then in 300 ml, 10% (v/v) glacial acetic acid for 2 hours.

(b) Silver stain.

Gels were soaked in 50% methanol for a minimum of 8 hours with three changes. 1.6 g of silver nitrate was dissolved in 8 mls
Table 2.4.1 Stock solutions for non-denaturing PAGE

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide A</td>
<td>25% (w/v) acrylamide</td>
</tr>
<tr>
<td></td>
<td>1.25% (w/v) bisacrylamide</td>
</tr>
<tr>
<td>Acrylamide B</td>
<td>60% (w/v) acrylamide</td>
</tr>
<tr>
<td></td>
<td>0.3% (w/v) bisacrylamide</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>3M Tris-HCl (pH 8.8)</td>
</tr>
<tr>
<td>Ammonium persulphate (Amps)</td>
<td>10% (w/v)</td>
</tr>
<tr>
<td>Temed</td>
<td>used as supplied</td>
</tr>
<tr>
<td>Electrophoresis buffer</td>
<td>0.05 M Tris</td>
</tr>
<tr>
<td></td>
<td>0.26 M Glycine</td>
</tr>
<tr>
<td>Sample buffer (per 100 µl sample)</td>
<td>10 µl 75% (v/v) glycerol</td>
</tr>
<tr>
<td></td>
<td>10 µl 0.1% (w/v) bromophenol blue</td>
</tr>
</tbody>
</table>
Table 2.4.2 Composition of non-denaturing exponential gradient gels.

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>4%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide A</td>
<td>4.6 ml</td>
<td>-</td>
</tr>
<tr>
<td>Acrylamide B</td>
<td>-</td>
<td>7.2 ml</td>
</tr>
<tr>
<td>Resolving buffer</td>
<td>3.75 ml</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>double distilled water</td>
<td>20.4 ml</td>
<td>7.2 ml</td>
</tr>
<tr>
<td>30% (v/v) glycerol</td>
<td>-</td>
<td>10.625 ml</td>
</tr>
<tr>
<td>Temed</td>
<td>17.5 µl</td>
<td>7.5 µl</td>
</tr>
<tr>
<td>Amps</td>
<td>150 µl</td>
<td>75 µl</td>
</tr>
</tbody>
</table>
Table 2.5.1  Stock solutions for SDS-PAGE.

<table>
<thead>
<tr>
<th>Stock Solutions</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolving gel buffer</td>
<td>0.75 M Tris-HCl pH 8.8</td>
</tr>
<tr>
<td></td>
<td>0.2 % (w/v) SDS</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>0.25 M Tris-HCl pH 6.8</td>
</tr>
<tr>
<td></td>
<td>0.2 % (w/v) SDS</td>
</tr>
<tr>
<td>Acrylamide stock</td>
<td>44% (w/v) acrylamide</td>
</tr>
<tr>
<td></td>
<td>0.8% (w/v) bisacrylamide</td>
</tr>
<tr>
<td>Ammonium persulphate (Amps)</td>
<td>1% (w/v)</td>
</tr>
<tr>
<td>TEMED</td>
<td>As supplied by manufacturer</td>
</tr>
<tr>
<td>Electrophoresis buffer</td>
<td>0.025 M Tris</td>
</tr>
<tr>
<td></td>
<td>0.129 M Glycine</td>
</tr>
<tr>
<td></td>
<td>0.1 % (w/v) SDS</td>
</tr>
<tr>
<td>Sample buffer (x2)</td>
<td>0.025 M Tris-HCl pH 6.8</td>
</tr>
<tr>
<td></td>
<td>0.8 % (w/v) SDS</td>
</tr>
<tr>
<td></td>
<td>10 % (v/v) glycerol</td>
</tr>
<tr>
<td></td>
<td>5 % (v/v) β-mercaptoethanol</td>
</tr>
<tr>
<td></td>
<td>Bromophenol blue</td>
</tr>
</tbody>
</table>
Table 2.5.2  Composition of SDS-PAGE resolving gels.

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>15%</th>
<th>11%</th>
<th>8.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolving gel buffer</td>
<td>27.0 ml</td>
<td>27.0 ml</td>
<td>27.0 ml</td>
</tr>
<tr>
<td>Acrylamide stock</td>
<td>18.4 ml</td>
<td>13.6 ml</td>
<td>10.6 ml</td>
</tr>
<tr>
<td>double distilled water</td>
<td>7.2 ml</td>
<td>12.0 ml</td>
<td>15.0 ml</td>
</tr>
<tr>
<td>Amps</td>
<td>1.9 ml</td>
<td>1.9 ml</td>
<td>1.9 ml</td>
</tr>
<tr>
<td>Temed</td>
<td>0.1 ml</td>
<td>0.13 ml</td>
<td>0.13 ml</td>
</tr>
</tbody>
</table>
Table 2.5.3 Composition of SDS-PAGE stacking gels.

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>4.5%</th>
<th>7.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stacking gel buffer</td>
<td>10 ml</td>
<td>10 ml</td>
</tr>
<tr>
<td>Acrylamide stock</td>
<td>3 ml</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>double distilled water</td>
<td>7 ml</td>
<td>6.7 ml</td>
</tr>
<tr>
<td>Amps</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Temed</td>
<td>0.04 ml</td>
<td>0.04 ml</td>
</tr>
</tbody>
</table>
of ddw and slowly added to a solution containing 42 ml 0.36% NaOH and 2.5 ml NH₃. The volume was made up to 200 ml and was used to stain the gel for 15 mins. After two washes with ddw, gel was soaked in developer (2.5 ml 1% citric acid, 0.4 ml formaldehyde, made up to 500 ml with ddw) until the bands appeared. Reaction was stopped with a solution containing 10% (v/v) acetic acid and 45% methanol.

2.20.3 Fluorography.

Fluorography, or impregnation of gels with a fluor increases the sensitivity of X-ray film to [³⁵S]-labelled proteins.

In this study, it was carried out according to the method of Skinner & Griswold, 1983. After electrophoresis, polyacrylamide gels were fixed in glacial acetic acid for 5 mins, then soaked in 20% (w/v) solution of PPO in glacial acetic acid for 1½ hours, with gentle agitation. Gels were then transferred into ddw for 30 mins, with agitation, during which time the PPO precipitated in the gel. Gels were then dried under vacuum at 60°C for 2 hours.

2.20.4 Photography.

Stained gels and autorads were routinely photographed from above using a Pentax SP500 camera with Kodak Panatomic X film (ASA 32).
2.21 **Vitamin B\textsubscript{12} receptor protein purification.**

The method used was essentially that of Sasaki, 1972 with modifications. 4 litres of cells were harvested (8,000 rpm, 4°C, 20 mins) and passed through a French Pressure cell twice (18,000 psi). Cell walls were sedimented at 27,600 x g for 20 mins and washed twice with TSM (10 mM Tris-succinate buffer pH 7.2 containing 5 mM Magnesium acetate). Supernatant and washings from the walls were combined and again subjected to the above centrifugation. Walls were resuspended in the same buffer at a concentration of 25 mg walls per ml in a final volume of 150 ml. 5 mg of Vitamin B\textsubscript{12} (Sigma) was added to the suspension, followed, by a incubation for 20 min with stirring. 5 mg of ribonuclease and 100 mg of lipase was then added, followed again by incubation at 37°C for 24 hours. Walls were spun down as before, washed twice with ddw and resuspended in ddw. HCl was added to give a final concentration of 0.2 M and an approximate concentration of 15 mg ml\textsuperscript{-1} of walls. This was followed by incubation, with stirring, at 37°C. The Vitamin B\textsubscript{12} receptor protein would then be found in the supernatant. The supernatant was neutralised with 10 M NaOH and dialysed against 0.01 M phosphate buffer (pH 7.0), containing 0.1 M NaCl (PBN) at 4°C for 48 hours.

\textit{A\textsubscript{280} and \textit{A\textsubscript{361} values were measured and protein amount determined (Section 2.22). Then the dialysed material was concentrated down to a third of the volume and placed onto a}
CM-cellulose column equilibrated with PBN. The column was eluted first with PBN and then with a linear gradient concentration of phosphate buffer (pH 7.0), containing a constant concentration of 0.1 M NaCl.

2.22 Protein determination.

Protein concentration was assayed using a modified Lowry method described by Herbert et al, 1971, using bovine serum albumin standards. The phenol reagent of Folin-Ciocalteu is reduced by phenols and many other substances, such as proteins to molybdenum blue, which may be determined colorimetrically.

To 0.5 mls of a suitable dilution of protein sample, 0.5 mls of 1 M NaOH was added and the sample boiled for 5 mins to ensure complete solubilisation and then rapidly cooled. 2 mls of 0.5% (w/v) CuSO₄.5H₂O dissolved in 1% (w/v) sodium potassium tartrate were added to 50 mls of 5% (w/v) Na₂CO₃ and 2.5 mls of this freshly prepared solution were added to each protein sample. Samples were left to stand for 10 min at room temperature and 0.5 mls of a 50% (v/v) solution of Folin-Ciocalteu reagent (BDH) rapidly added and mixed well. Samples were left to stand at room temperature for 30 min and A₇₅₀ measured against a blank of ddw treated as described above. Protein concentrations were determined by reference to a plot of protein concentration against absorbance prepared by using standard bovine serum albumin concentrations for each assay. Chromic acid-washed tubes were employed at all times.
2.23 Assay for receptor protein.

The method used was that described by Aufrère et al, 1986.

2.24 Preparation of antisera.

Antisera was raised in a New Zealand White rabbit. A blood sample was taken from the ear before injection; this contained pre-immune serum.

100 \(\mu\)g of protein in 1 ml of phosphate buffer (pH 7.0), containing 0.1 M NaCl was mixed with 1 ml of Freund's complete adjuvant by passing the mixture repeatedly between two glass syringes. The resulting stiff white emulsion was injected sub-cutaneously into the back of the neck. 21 days later, a booster injection of the same protein sample, mixed with 1 ml of Freund's incomplete adjuvant was administered and this was repeated after seven days. One week later, the rabbit was bled. Blood was taken once every two weeks for several weeks. Pre-immune and post-immune sera were stored in aliquots at -20\(^\circ\)C.

2.25 Western transfer of protein.

Transfer of proteins separated by PAGE to nitrocellulose.

The method of Towbin et al, 1979 was used.
A nitrocellulose membrane filter was cut to the size of the polyacrylamide gel, soaked in Western transfer buffer (WTB) and placed on top of the gel. Care was taken to remove air bubbles between the gel and the nitrocellulose. Using a BioRad Transblot tank and power supply according to the manufacturer's instructions, transfer of proteins from gel to nitrocellulose was carried out at 280 mA for 3 hours.

After transfer, the nitrocellulose filter was rinsed in ddw to remove excess salt. Filter was then stained to visualise protein bands with Ponceau S (0.5% (w/v) in 5% (v/v) TCA). Stain was totally removed by washing in Tris buffered saline (TBS). Two 10 min washes in TBS were followed by fresh TBS containing 2% (w/v) dried milk and shaken for 1 hour to allow protein to bind non-specifically to the nitrocellulose. Antiserum at a dilution of 1:500 was added and the filter allowed to shake in this solution overnight at room temperature. The filter was then washed twice in TBS containing 0.1% (v/v) Tween 20.

Detection of antigen-antibody complexes using a horseradish peroxidase colour reaction.

Peroxidase conjugated goat anti-rabbit IgG was used as secondary antibody and chloronaphthol (Sigma) as the colour reaction reagent.
After the washes in TBS as indicated above, filter was transferred to a fresh solution of TBS, containing 0.1% (v/v) Tween 20 and 1:300 secondary antibody and left shaking for 2 hours. After 2 washes in TBS containing 0.1% (v/v) Tween 20 and 2 washes in TBS, filter was transferred to a solution containing 1% (w/v) NaCl, 1% (v/v) 1 M Tris HCl (pH 7.5), 0.05% (w/v) 4-chloro-1-naphthol (Sigma) and 10% (v/v) of methanol.

50 µl hydrogen peroxide (Fisons) was then added to initiate the staining reaction. Before completion of reaction, filter was washed with ddw, air dried and stored in the dark.
Chapter 3. The isolation and cloning of *L. leichmannii* genomic DNA exhibiting homology to the *E. coli* gene (btuB) encoding the Vitamin B$_{12}$ receptor protein.

3.1 Introduction.

In order to identify and isolate a specific DNA fragment encoding the Vitamin B$_{12}$ receptor protein in *Lactobacillus leichmannii*, a heterologous hybridisation approach was employed, rather than a direct ‘shotgun’ approach. The latter method was not chosen, due to the low transformation efficiency of ligated plasmid DNA into protoplasts, as compared to that of CaCl$_2$-treated *E. coli*, together with the fact that it relies upon expression of the gene for its initial isolation.

The cloning strategy used involved the construction of recombinant plasmid DNA molecules in *E. coli* where standard DNA methodologies could be applied.

In addition, the structural gene for the Vitamin B$_{12}$ receptor (btuB) has been cloned from *E. coli* (Heller et al., 1985; Aufrère et al., 1986; Moir et al., 1987), which may be used as a heterologous probe. Consequently there is considerable information available pertinent to the structure and expression of this gene.
3.2 Results and Discussion.

In an attempt to isolate any 'btuB-like' sequences encoding the Vitamin B$_{12}$ receptor protein in *L. leichmannii*, a synthetic oligonucleotide was designed from the *E. coli btuB* gene, to use as a heterologous probe to the *Lactobacillus* genome.

3.2.1 Design and synthesis of the oligonucleotide probe.

An *E. coli* mutant has been described (Kadner & Liggins, 1973) in which Vitamin B$_{12}$ transport was completely defective. Subsequent complementation analysis indicated that the mutation in this strain was in the *btuB* gene. The mutational lesion was determined by nucleotide sequencing and a single amino acid substitution near the amino terminus of *btuB* was identified (Heller & Kadner, 1985). This mutation was associated with a T to C transition at residue 394 - as illustrated in Figure 3.1. This nucleotide change predicts the substitution of proline for leucine at the eighth amino acid residue of the mature polypeptide.

An oligonucleotide probe was designed from this region. Initially, the amino acids around residue 394 were studied with respect to codon usage. The IBM 'Staden' program was used to determine the frequency of codon usage for each amino acid in the entire *btuB* gene (Figure 3.2). The deduced nucleotide sequence of the mixed oligonucleotide
Figure 3.1 Nucleotide sequence of the structural gene for the Vitamin B₁₂ receptor (BtuB) in *Escherichia coli.*
**Figure 3.2 Codon usage of the *Escherichia coli* btuB gene.**

<table>
<thead>
<tr>
<th>Codon</th>
<th>Amino Acid</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTT Phe</td>
<td>11 (1.8)</td>
<td>TCT Ser</td>
</tr>
<tr>
<td>TTC Phe</td>
<td>7 (1.1)</td>
<td>TCC Ser</td>
</tr>
<tr>
<td>TTA Leu</td>
<td>5 (0.8)</td>
<td>TCA Ser</td>
</tr>
<tr>
<td>TTG Leu</td>
<td>8 (1.3)</td>
<td>TCG Ser</td>
</tr>
<tr>
<td>CTT Leu</td>
<td>6 (1.0)</td>
<td>CCT Pro</td>
</tr>
<tr>
<td>CTC Leu</td>
<td>7 (1.1)</td>
<td>CCC Pro</td>
</tr>
<tr>
<td>CTA Leu</td>
<td>0 (0.0)</td>
<td>CCA Pro</td>
</tr>
<tr>
<td>CTG Leu</td>
<td>19 (3.1)</td>
<td>CCG Pro</td>
</tr>
<tr>
<td>ATT Ile</td>
<td>14 (2.3)</td>
<td>ACT Thr</td>
</tr>
<tr>
<td>ATC Ile</td>
<td>9 (1.5)</td>
<td>ACC Thr</td>
</tr>
<tr>
<td>ATA Ile</td>
<td>2 (0.3)</td>
<td>ACA Thr</td>
</tr>
<tr>
<td>ATG Met</td>
<td>3 (0.5)</td>
<td>ACG Thr</td>
</tr>
<tr>
<td>GCT Val</td>
<td>11 (1.8)</td>
<td>GCC Ala</td>
</tr>
<tr>
<td>GTC Val</td>
<td>15 (2.4)</td>
<td>GCA Ala</td>
</tr>
<tr>
<td>GTA Val</td>
<td>8 (0.3)</td>
<td>GCG Ala</td>
</tr>
<tr>
<td>GTG Val</td>
<td>13 (2.1)</td>
<td></td>
</tr>
<tr>
<td>TAT Tyr</td>
<td>38 (6.2)</td>
<td>TGT Cys</td>
</tr>
<tr>
<td>TAC Tyr</td>
<td>11 (1.8)</td>
<td>TGC Cys</td>
</tr>
<tr>
<td>TAA End</td>
<td>0 (0.0)</td>
<td>TAG End</td>
</tr>
<tr>
<td>TGA End</td>
<td>0 (0.0)</td>
<td>TGG Trp</td>
</tr>
<tr>
<td>CAT His</td>
<td>8 (1.3)</td>
<td>CGT Arg</td>
</tr>
<tr>
<td>CAC His</td>
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</tr>
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<td>CAA Gin</td>
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<tr>
<td>GAT Asp</td>
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<td>GAC Asp</td>
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<td>AAG Lys</td>
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<td>AGG Arg</td>
</tr>
<tr>
<td>GAT Asp</td>
<td>3e (5.9)</td>
<td>GGC Gly</td>
</tr>
<tr>
<td>GAC Asp</td>
<td>12 (2.1)</td>
<td>GGC Gly</td>
</tr>
<tr>
<td>GAA Glu</td>
<td>18 (2.0)</td>
<td>GGA Gly</td>
</tr>
<tr>
<td>GAG Glu</td>
<td>6 (1.0)</td>
<td>GGS Gly</td>
</tr>
</tbody>
</table>
probe is presented in Figure 3.3. It is made up of 27 bases with four degeneracies.

Since there is considerable amino acid sequence homology existing within outer membrane proteins, such as ompC, ompF and phoE, the 'diagon' sub-routine of the 'Staden' program was employed to determine the level of homology between the btub gene and the proposed oligonucleotide with these outer membrane proteins. The results obtained are presented in Figure 3.4 and suggest a high probability that the synthetic probe chosen would not hybridise to any genes in L. leichmannii which may encode proteins similar to the outer membrane proteins mentioned above. This correlates well with the work of Heller and Kadner (1985), who also came to the conclusion that there are no strongly conserved sequences common to outer membrane proteins.

The newly synthesised oligonucleotide was purified according to the method detailed in Section 2.13.1. The melting temperature for the mixed oligonucleotide probe was estimated to be 85°C (Lathe, 1985).

L. leichmannii genomic DNA was prepared and digested to completion with a variety of restriction endonucleases, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed at low stringency with the [γ-32P]dATP labelled oligonucleotide. The resulting autoradiograph is presented in Figure 3.5.
Figure 3.3  Design and sequence of the synthetic oligonucleotide.

(a)  4  5  6  7  8  9  10  11  12
    - Ser - Pro - Asp - Thr - Leu - Val - Val - Thr - Ala -

(b)  
    TCG  GGC  CTA  TGA  GAC  CAG  CAC  TGA  TTG

(c)  
    AGC/TCCGATACTTC/GGTC/GGTC/GACTAAC

(a) Amino acid sequence of the N-terminal region of the mature E. coli BtuB polypeptide. (b) Reverse translation to nucleotide triplets of amino acids 4 to 12 and (c) The oligonucleotide probe derived from them.
Figure 3.4  Comparison of the proposed synthetic oligonucleotide with other outer membrane proteins.

Key:-

(a)  btuB ORF vs ompF ORF

(b)  btuB ORF vs mixed oligonucleotide probe

(c)  ompF ORF vs mixed oligonucleotide probe

(d)  btuB ORF vs ompC ORF

(e)  ompC ORF vs mixed oligonucleotide probe

(f)  btuB ORF vs phoE ORF

(g)  phoE ORF vs mixed oligonucleotide probe.
Figure 3.5 Examination of *L. leichmannii* genomic DNA for homology with the synthetic oligonucleotide.

*L. leichmannii* 4797; *L. leichmannii* 8964, *L. casei* 7473 and *E. coli* HB101 genomic DNAs were restricted as indicated, fractionated by agarose gel electrophoresis and Southern blotted onto nitrocellulose. The resulting blot was probed with the $[^{32}P]$dATP labelled 27' mer oligonucleotide at low stringency and subjected to autoradiography.

Key to tracks:

- **A** *E. coli* HB101 genomic DNA restricted with *EcoRI*
- **B** *L. leichmannii* 4797 genomic DNA restricted with *SalI*
- **C** *L. leichmannii* 4797 genomic DNA restricted with *HindIII*
- **D** *L. leichmannii* 4797 genomic DNA restricted with *EcoRI*
- **E** *L. casei* 7473 genomic DNA restricted with *SalI*
- **F** *L. casei* 7473 genomic DNA restricted with *HindIII*
- **G** *L. casei* 7473 genomic DNA restricted with *EcoRI*
- **H** *L. leichmannii* 8964 genomic DNA restricted with *SalI*
- **I** *L. leichmannii* 8964 genomic DNA restricted with *HindIII*
- **J** *L. leichmannii* 8964 genomic DNA restricted with *EcoRI*

Tracks K to T are autoradiographs of corresponding tracks A to J probed with $[^{32}P]$dATP labelled oligonucleotide.
Progressively more stringent washes were employed until the probe was washed off.

3.2.2 Cloning of L. leichmannii DNA exhibiting homology to the synthetic oligonucleotide.

The 2.0 Kb HindIII fragment of L. leichmannii 4797 was chosen for isolation. The cloning strategy adopted (Figure 3.6) involved the construction of a selective gene bank in the HindIII site of pBR327; the resulting library being screened by hybridisation.

Due to possible sequence homology between the synthetic oligonucleotide probe and the resident btuB gene of the surrogate E. coli HB101 host used to construct the gene library, the amplifiable high copy number vector pBR327 (Figure 3.13) was used to optimise the autoradiograph signal of any positive clone over the background signal resulting from this homology.

Screening of the library was carried out with [γ-32p]dATP labelled synthetic oligonucleotide and the resulting autoradiograph (Figure 3.7) shows two clones clearly above background, designated clone 1 and clone 2. Plasmid DNAs from both clones were isolated, restricted with HindIII, fractionated by agarose gel electrophoresis, Southern blotted and probed with the labelled oligonucleotide probe. Both clones harboured a single 2.0 Kb HindIII DNA fragment.
Figure 3.6 Cloning strategy adopted for the isolation of *L. leichmannii* DNA exhibiting homology to the synthetic oligonucleotide.

1. Transform *E. coli* HB101
2. Select Ap^R_1_ colonies
3. Colony blot
4. Probe with ^32_P-labelled oligo or bluB gene
5. Autoradiograph
6. Isolate positive colonies
Figure 3.7 Screening of a selective *L. leichmannii* gene bank for sequences homologous to the synthetic oligonucleotide.

The cloning of the *L. leichmannii* DNA exhibiting homology to the synthetic oligonucleotide was carried out as outlined in Figure 3.6. The resulting colony blot was probed at high stringency. The figure shows two clones (clones 1 and 2) clearly above background.

Keys to controls:

A  pBR327

B  pNF48
which exhibited strong homology to the oligonucleotide probe. These clones were redesignated pSDC1 and pSDC2.

3.2.3 Cloning of *L. leichmannii* DNA exhibiting homology to the *E. coli* btuB gene.

Screening of the *Lactobacillus* limited genomic library was also carried out with a \([\alpha-^{32}\text{P}]\text{dATP}\) labelled 1.5 Kb EcoRI fragment isolated from plasmid pNF48 (Figure 2.1) harbouring the *E. coli* btuB gene. Two putative clones, identical to those above - pSDC1 and pSDC2 - were clearly identified above background.

3.2.4 Characterisation of pSDC1 and pSDC2.

Initially, the clones were mapped with respect to the restriction endonuclease sites in the vector (Figure 3.8). pSDC1 and pSDC2 appeared to produce similar restriction patterns although undigested DNAs appeared different. There appeared to be anomalies with the expected fragment sizes of various restriction enzymes from the 'vector' portion of pSDC1. Therefore, digests were carried out on pBR327 in conjunction with pSDC1 (see Figure 3.9). All digestions concerning the vector alone yielded fragments of the correct size (other than a partial digestion with restriction endonuclease *sphl*).

Numerous double digests were therefore carried out in order to determine the restriction endonuclease sites within the
Figure 3.8  Restriction endonuclease mapping of the
*Leichmannii* clones pSDC1 and pSDC2.

pSDC1 plasmid DNA was digested to completion with a number
of restriction endonucleases and the resulting DNA fragments
were fractionated by agarose gel electrophoresis.

Key to tracks:-

A  pSDC1 DNA restricted with *Hind*III
B  pSDC1 DNA restricted with *Eco*RI
C  pSDC1 DNA restricted with *Bam*HI
D  pSDC1 DNA restricted with *Pst*I
E  pBR327 DNA restricted with *Eco*RI
F  pSDC1 DNA restricted with *Hinc*II
G  pSDC1 DNA restricted with *Ava*I
H  pSDC2 DNA restricted with *Hind*III
I  pSDC2 DNA restricted with *Hinc*II
J  pSDC2 DNA restricted with *Ava*I
K  pSDC1 DNA
Figure 3.9  Restriction endonuclease mapping of the

*L. leichmannii* clone pSDC1.

Keys to tracks:-

A  λ HindIII DNA markers
B  pBR327 DNA
C  pSDC1 DNA
D  pSDC2 DNA
E  pBR327 DNA restricted with HindIII
F  pBR327 DNA restricted with HinclII
G  pBR327 DNA restricted with AvaiI
H  pBR327 DNA restricted with BamHI
I  pBR327 DNA restricted with PstI
J  pSDC1 DNA restricted with HindIII
K  pSDC1 DNA restricted with HinclII
L  pSDC1 DNA restricted with AvaiI
M  pSDC1 DNA restricted with BamHI
N  pSDC1 DNA restricted with PstI
O  pBR327 DNA restricted with ClaiI
P  pBR327 DNA restricted with SphiI
Q  pBR327 DNA restricted with EcoRI
R  pSDC1 DNA restricted with ClaiI
S  pSDC1 DNA restricted with SphiI
T  pSDC1 DNA restricted with EcoRI
insert (Figures 3.10, 3.11 and 3.12). From this data, a restriction map of pSDC1 was determined, as detailed in Figure 3.13.

3.2.5 Expression analysis of the cloned Lactobacillus DNA.

The structural gene encoding the Vitamin B₁₂ receptor protein, is required in its entirety for the purpose of expression and manipulation, initially in E. coli and then for re-introduction into Lactobacillus for analysis. These studies were therefore designed to determine the expression and functionality of the Lactobacillus 'btuB-like' sequence in a heterologous host and to identify the resulting gene product.

3.2.5.1 Complementation analysis.

The E. coli btuB⁻ mutant, L1-431 was transformed with the recombinant pSDC1 and pNF48 (control). Transformants were selected onto LB agar containing ampicillin (100 µg ml⁻¹). Approximately equal numbers of transformants were obtained. To detect whether the recombinant pSDC1 had complemented the btuB lesion due to an intact and functional btuB gene, located within the 2.0 Kb HindIII fragment of pSDC1, the T5-like phage, BF23 was employed, since as previously described, BF23 also uses the BtuB protein as its receptor and therefore plaques will form on plates containing a complemented lesion of btuB. This hypothesis
Figure 3.10 Restriction endonuclease mapping of pSDCl.

pSDCl plasmid DNA was digested to completion with a number of restriction endonucleases and fractionated by agarose gel electrophoresis.

Key to tracks:

A  λ HindIII DNA markers  
B  pSDCl DNA restricted with PstI and PvuII  
C  pSDCl DNA restricted with PstI and EcoRV  
D  pSDCl DNA restricted with PstI and SalI  
E  pSDCl DNA restricted with PstI and AvaI  
F  pSDCl DNA restricted with PstI and HindIII  
G  pSDCl DNA restricted with PstI and HindIII  
H  pSDCl DNA restricted with PstI only  
I  pSDCl DNA restricted with PvuII and EcoRI  
J  pSDCl DNA restricted with EcoRV and SalI
Figure 3.11  Restriction endonuclease mapping of pSDC1.

Key to tracks:-

A  pSDC1 DNA restricted with EcoRV and BglII
B  pSDC1 DNA restricted with EcoRI and EcoRV
C  pSDC1 DNA restricted with EcoRI and HindIII
D  pSDC1 DNA restricted with EcoRI and SacI
E  pSDC1 DNA restricted with EcoRI and SalI
F  pSDC1 DNA restricted with EcoRI and XhoI
G  pSDC1 DNA restricted with HindII and EcoRI
H  pSDC1 DNA restricted with HindII and PvuII
I  pSDC1 DNA restricted with PstI and ClaI
J  pSDC1 DNA restricted with PstI and EcoRI
K  pSDC1 DNA restricted with PstI and HindIII
Figure 3.12 Restriction endonuclease mapping of pSDC1.

Key to tracks:

A  A HindIII DNA markers
B  pSDC1 DNA restricted with EcoRI and PvuII
C  pSDC1 DNA restricted with EcoRI and EcoRV
D  pSDC1 DNA restricted with EcoRI and HindIII
E  pSDC1 DNA restricted with EcoRI
F  pSDC1 DNA restricted with HindIII and PstI
G  pSDC1 DNA restricted with HindIII and PvuII
H  pSDC1 DNA restricted with HindIII and EcoRI
I  pSDC1 DNA restricted with HindIII
J  pSDC1 DNA restricted with PstI and HindIII
K  pSDC1 DNA restricted with PstI and HindIII
L  pSDC1 DNA restricted with PstI and EcoRI
M  pSDC1 DNA restricted with PstI
Figure 3.13 Restriction endonuclease map of pSDC1 with the published map of pBR327 for comparison.

Key:

- **Ea**  
- **HIII**  
- **BI**  
- **EV**  
- **PII**  
- **PI**  
- **SI**  
- **HII**  
- **AI**  
- **XI**  
- **Cl**  
- **SpI**
held true for *E. coli* L1-431 transformed with pSDCl, as did *E. coli* L1-431 transformed with pWF48.

The 2.0 Kb *HindIII* insert of pSDCl was subsequently recloned in the opposite orientation in pBR327, by restriction of pSDCl with *HindIII*, followed by religation and transformation into *E. coli* HB101. The resultant plasmid was designated pSDC3 and the orientation of the insert confirmed by restriction analysis. Subsequent transformation and failure of complementation of the *btuB* lesion in L1-431 with pSDC3 revealed that the cloned *btuB* gene was not expressed from a regulatory region contained within the cloned DNA fragment but was possibly expressed from a foreign (vector) promoter.

### 3.2.5.2 *In vivo* expression analysis.

In order to identify any polypeptides encoded by the 2.0 Kb *Lactobacillus* *HindIII* fragment, it was necessary to attempt to express the gene located on plasmid pSDCl and/or pSDC2 in an *E. coli* gene expression system. The two *in vivo* gene expression systems applicable to recombinant plasmids in common use are, minicells (Clarke-Curtiss & Curtiss, 1983) and maxicells (Sancar et al., 1979). The uses and relative merits of these systems are discussed by Stoker et al. (1984).
The maxicell system was chosen because it is versatile, amenable to use with large numbers of samples and with careful handling, can produce good results.

The use of maxicells relies on the deficiency in DNA repair observed in UV-sensitive strains, such as those which carry a recA mutation. The recA strain carrying the plasmid of interest is UV-irradiated and incubated overnight to allow the breakdown of UV-damaged chromosomal DNA, whilst leaving undamaged plasmid DNA molecules intact. Plasmid-coded proteins can then be preferentially labelled with $^{35}\text{S}$-methionine and samples analysed by SDS-PAGE.

The plasmids pSDC1, pNF48, pSDC2 and pBR327 were transformed into the E. coli maxicell strain CSH26AF6 (Stoker et al., 1984). The resulting transformants were allowed to synthesise proteins in the presence of $^{35}\text{S}$-methionine as described in Chapter 2 (Section 2.17.1). The de novo synthesised plasmid encoded proteins were electrophoresed on an 11% SDS-denaturing polyacrylamide gel and visualised by fluorography. The resulting fluorograph (Figure 3.14) clearly shows the pattern of proteins synthesised by the E. coli maxicells harbouring pBR327 and additional proteins encoded by pSDC1 and pSDC2.

1.2.5.1 In vitro expression analysis.

The specific matching of a cloned gene with a previously unidentified polypeptide product and whether that gene has
Figure 3.14 *In vivo* translation products of pSDC1 and pSDC2.

The fluorograph shows $^{35}$S-methionine labelled polypeptide products of pSDC1 and pSDC2 using the *E. coli* maxicell system.

**Key to tracks:**

A pNF48 encoded polypeptides
B pSDC1 encoded polypeptides
C pSDC2 encoded polypeptides
D CSH26AF6 control
E pBR327 encoded polypeptides
been cloned together with its own promoter, is frequently difficult to establish using in vivo systems. However, in vitro systems circumvent these problems. Two further advantages of the in vitro system, first described by De Vries and Zubay (1967) are that, incorporation of radioactive label into protein is far more efficient than is possible using in vivo methods and also DNA derived from bacteria other than E. coli is efficiently expressed (Pratt et al., 1981).

Following the in vitro transcription-translation reactions using the E. coli cell free coupled transcription-translation system (Section 2.17.2) and 35S-methionine as the labelled amino acid, the resulting proteins were electrophoresed on an 11% SDS-denaturing polyacrylamide gel and fluorographed. The resulting fluorograph (Figure 3.15) shows that when either pSDC1 or pSDC2 were used as templates, they directed the synthesis of a single major polypeptide with an $M_r$ of 66,400, in addition to vector (pBR327) specific polypeptides.

The polypeptide encoded by pSDC1 and pSDC2 had an identical $M_r$ to that produced by pNF48 which harbours the E. coli btuB gene. This polypeptide was thus proposed to be the product of the L. leichmannii btuB gene.

After the success of gene expression from circular DNA, pSDC1 was digested to produce linear templates for analysis in the Zubays in vitro system. pSDC1 was digested with HindIII initially which resulted in the loss of the
Figure 3.13 In vitro translation products of p8DC1 and p8DC2.

The fluorograph shows the various $^{35}$S-methionine labelled plasmid encoded products. The Mr 30,000 polypeptide corresponds to β-lactamase of the vector pBR327.

Key to tracks:

A pNF48 encoded products
B pBR327 encoded products
C pSDC1 encoded products
D pSDC2 encoded products
Mr-66,400 protein, as produced by pNF48 (positive control) and pSDC1. This implies that the 2.0 Kb HindIII insert did not in fact possess its own promoter, but possibly the 66,400 polypeptide was the result of read-through from vector sequences. This data correlates well with the complementation data. To test this hypothesis the following digests of pSDC1 were made; PvuII (to release two linear fragments) and PstI (to release one linear fragment, inactivating the ampicillin gene in the process). The resulting autoradiograph is shown in Figure 3.16. It can be seen that a protein with an Mr of 66,400 was expressed, using the PvuII restricted DNA as the template. This suggests that a functional promoter sequence exists possibly between the HindIII site of the initial insert and the newly formed PvuII site in pSDC1 (see Figure 3.13). The PstI restricted DNA used as the template produced a spurious result. There was no expression of the Mr-66,400 protein, although there was expression of an Mr-30,000 protein - another protein which had the same molecular mass as β-lactamase and normally co-migrates on a polyacrylamide gel with β-lactamase.

The 2.45 Kb PvuII fragment was therefore sub-cloned into pBR329, which has a unique PvuII site. Putative sub-clones were subjected to plasmid analysis and digested with both HindIII and PvuII in order to determine the orientation of the insert (Figure 3.17). All putative sub-clones exhibited homology to the [α-32P]dATP labelled 1.5 Kb EcoRI fragment from pNF48. Clones were designated pSDC41 and pSDC42, in accordance with pSDC1 and pSDC3 respectively.
Figure 3.14  *In vitro translation products of linear templates of pSDC1.*

The fluorograph shows the various $^{35}$S-methionine labelled template encoded products. The $M_r$ 30,000 polypeptide corresponds to $\beta$-lactamase of the vector pBR327.

Key to tracks:

A  pSDC1 encoded products

B  pSDC1/PstI encoded products

C  pSDC1/PvuII encoded products
Figure 3.17 Plasmid analysis of putative sub-clones of p8DC1.

Nineteen putative clones were subjected to plasmid analysis, restricted with both PvuII and HindIII (separately) and fractionated on an agarose gel.

Key:

a tracks - DNA digested with PvuII

b tracks - DNA digested with HindIII
After further restriction analysis (data not shown) of pSDC41, in order to correlate with the data produced from analysis of pSDC1, the final restriction map is presented in Figure 3.18. It was this construct, pSDC41, which was used in subsequent studies.

It appears that from the work carried out and detailed in this chapter that *L. leichmannii* DNA can be expressed in a heterologous host.

To complement these expression studies, the nucleotide sequencing of this gene was initiated to enable comparative analysis with the *E. coli* *btuB* gene.

### 3.2.6 Nucleotide sequence of the *L. leichmannii btuB* gene (*btuB*).

The restriction map of the *btuB* region previously determined (Figure 3.18) was used as a basis for the sequencing strategy outlined in Figure 3.19. The specific restriction fragments required for sequencing were all derived from the recombinant pSDC41. Each specific restriction fragment was prepared by digestion of pSDC41 with the appropriate restriction enzymes, fractionated on a 2.5% preparative agarose gel and sub-cloned in both possible orientations into bacteriophage M13 strains *tg* 130 and *tg* 131. *E. coli* strain TG1 was used as host for the M13 *tg* phages. Each restriction fragment was sequenced by the
Figure 3.18 Restriction endonuclease map of pSDC41 with published map of pBR322 for comparison.

Key:

- PII
- PI
- EI
- BI
- PI
- HIII
- EV
- SI
- PstI
- EcoRI
- Glu
- HindIII
- EcoRV
- BamHI
- AvaI
- SphI

pSDC41
6900bp
Figure 3.19 Sequencing strategy for the \textit{L. leichmannii} B gene region.

Key:

\begin{itemize}
  \item HIII HindIII
  \item HII HincII
  \item PI PstI
  \item EV EcoRV
  \item SI Sali
\end{itemize}
dideoxynucleotide chain termination procedure (Sanger et al., 1977).

Nucleotide sequence data was generated initially from the 5' and 3' ends of the 2.45 Kb PvuII DNA insert. When this sequence data was compared for homology with the nucleotide sequence of the *E. coli* *btuB* gene, the sequences were found to be 100% homologous. By comparing the restriction endonuclease sites it could be seen that these also appeared to be identical. Therefore, it appeared that the cloned *btuB* sequences were in fact those belonging to *E. coli* and not from *L. leichmannii*.

### 3.3 Summary and Overview.

It was thought that the structural gene for the Vitamin B$_{12}$ receptor (*btuB*) had been cloned from *L. leichmannii* by heterologous hybridisation, utilising an internal *btuB* gene fragment from *E. coli* and a synthetic oligonucleotide, derived from the *E. coli* *btuB* gene, as gene probes.

A heterologous hybridisation approach was used to isolate the putative *btuB* gene from *L. leichmannii* in these studies, as it did not rely upon expression of the cloned gene for its initial isolation, unlike a direct 'shotgun' approach, in which gene libraries are either conjugated or transformed into *E. coli* or *Lactobacillus* mutants.
The 2.0 Kb HindIII DNA fragment (thought to have been cloned from *L. leichmannii*) was shown to complement the *btuB* lesion in *E. coli* L1-431, only as part of the recombinant pSDCl, indicating that the fragment did not contain its own promoter sequences. From the *in vitro* transcription and translation studies, together with the *in vivo* maxicells studies, it was proposed that the M₉=66,400 polypeptide produced was the functional product of the *L. leichmannii btuB* gene. *in vitro* transcription and translation studies, together with complementation studies also revealed the direction of transcription of the proposed *btuB₉* gene to be in the orientation presented in Figure 3.19.

The fact that the cloned *btuB* sequence was that of *E. coli* and not *L. leichmannii* suggests some sort of recombination event had occurred. The probability of this event occurring had been markedly reduced by the use of a recA⁻ strain of *E. coli*, HB101. It is unlikely for an event such as described in this chapter to have occurred, unless the host strain HB101 was indeed no longer recA⁻.

There were in fact initial cloning problems with the putative clones clumping in liquid culture. After numerous attempts to propagate the positive clones, several were found that could grow ‘normally’ in liquid culture. The clumping of cells may be due to the synthesis of a detrimental gene product cloned within the recombinant. Rearrangement of these recombinants may have occurred which allowed the cells to grow ‘normally’. This rearrangement is
reflected in the results obtained from restriction analysis. Unfortunately, the restriction map of the btuB gene from E. coli was not referred to until after the nucleotide sequence data had been generated. Had this been done, several weeks' work would have been redirected.

Another important point gained from the literature was that, although Salmonella typhimurium had been shown to possess a gene [formerly known as bfe (Mojica & Garcia, 1976) and later renamed btuB, since it was found to be at the analogous map position as the E. coli homolog (Sanderson & Roth, 1988)], which encodes for a Vitamin B\textsubscript{12} receptor protein of similar size to E. coli; the Vitamin B\textsubscript{12} binding protein in Lactobacillus is in fact reported to be in the region of a third the size; $M_r$-15,000 (Sasaki & Kitahara, 1964a) and later, $M_r$-21,500 (Sasaki, 1972). This information leads to the conclusion that the btuB gene cloned was indeed isolated from E. coli and not L. leichmannii.

With no (or very little) information available on genes encoding Vitamin B\textsubscript{12} receptor proteins in other organisms, particularly Gram-positive organisms, it was necessary to attempt to re-clone the gene encoding the Vitamin B\textsubscript{12} binding protein in L. leichmannii.
CHAPTER 4
CHAPTER 4 - CLONING OF THE STRUCTURAL GENE FOR THE VITAMIN 
B\textsubscript{12} RECEPTOR PROTEIN FROM LACTOBACILLUS LEICHHANNII

4.1 Introduction.

Initial cloning studies were based on the presumption that the Vitamin B\textsubscript{12} receptor protein in Lactobacillus leichmannii was of a similar size to that found in E. coli and S. typhimurium. However, evidence for this could not be found in the literature.

It was over twenty five years ago that Kitahara and Sasaki (1963) presented evidence to show that Vitamin B\textsubscript{12} requiring Lactobacilli characteristically take up and preserve large amounts of B\textsubscript{12} in vivo, from the surrounding media. The cellular site of B\textsubscript{12} accumulation was shown to be located in the cell wall, where a B\textsubscript{12}-binding principle exists (Sasaki & Kitahara, 1963a; 1963b; 1964b). Initial attempts at isolation and purification of the B\textsubscript{12} polypeptide complex revealed a molecular weight of 15,000 (Sasaki & Kitahara, 1964a). Several years later, Sasaki (1972) obtained a value nearer 21,500 daltons with microheterogeneity on a sephadex G-75 column, after cation-exchange chromatography of the complex.

Thus, it can be seen that the Vitamin B\textsubscript{12} receptor protein found in Lactobacillus spp. is only a third of that found in E. coli and S. typhimurium. Presumably however, there will be conserved domains for the binding of the Vitamin.
4.2 Results and Discussion.

The 2.0 Kb HindIII genomic DNA fragment of *L. leichmannii* previously shown to contain homologous sequences to the synthetic oligonucleotide probe (see Figure 3.5) was chosen for isolation. The cloning strategy adopted was similar to that described previously, as detailed in Figure 3.6, involving the construction of a selective gene bank in the HindIII site of an amplifiable high copy number vector pBR325 (Bolivar, 1978). Other vectors, pACYC184 (Chang & Cohen, 1978) and pAT153 (Twigg & Sherratt, 1980) were tried, but failed to produce any positive clones. The resulting gene library was screened by hybridisation using a $[\gamma^{32}\text{P}]$ labelled oligonucleotide probe, as before. The resulting autoradiograph (Figure 4.1) shows one clone very clearly above background, designated clone 1. Subsequent plasmid and hybridisation analysis of the clone revealed that it harboured a 2.0 Kb HindIII insert. This clone, chosen for further analysis was redesignated pSEC1. pSEC1 was mapped with respect to several restriction endonucleases (Figures 4.2, 4.3 and summarised in 4.4).

In order to verify that the cloned 2.0 Kb HindIII fragment was derived from *L. leichmannii* genomic DNA and that no rearrangements had occurred during the cloning procedure, *L. leichmannii* genomic DNA was digested to completion with a number of restriction endonucleases, fractionated by agarose gel electrophoresis, Southern blotted and then probed at
Figure 4.1  
Screening a selective *L. leichmannii* gene bank for sequences homologous to the synthetic oligonucleotide.

The cloning of the *L. leichmannii* DNA showing homology to the oligonucleotide probe was as described in the text. The resulting colony blot was probed at high stringency. The Figure shows one clone very clearly above background.

Key to controls.

A  pNF48  
B  pBR325
Restriction endonuclease mapping of the \textit{L. leichmannii} recombinant pSEC1.

pSEC1 plasmid DNA was digested to completion with a number of restriction endonucleases and the resulting DNA fragments were fractionated by agarose gel electrophoresis.

Key to tracks:

1. \(\lambda\) DNA restricted with \textit{HindIII}
2. pSEC1 DNA restricted with \textit{HindIII}
3. pSEC1 DNA restricted with \textit{EcoRI}
4. pSEC1 DNA restricted with \textit{EcoRV}
5. pSEC1 DNA restricted with \textit{PvuII}
6. pSEC1 DNA restricted with \textit{PstI}
7. pSEC1 DNA restricted with \textit{HincII}
8. pSEC1 DNA restricted with \textit{BamHI}
9. pSEC1 DNA restricted with \textit{ClaI}
10. pSEC1 DNA restricted with \textit{BgIII}
11. \(\lambda\) DNA restricted with \textit{HindIII}
12. pSEC1 DNA restricted with \textit{NcoI}
13. pSEC1 DNA restricted with \textit{KpnI}
14. pSEC1 DNA restricted with \textit{AvaI}
15. pSEC1 DNA restricted with \textit{SalI}
16. pSEC1 DNA restricted with \textit{SmaI}
17. pSEC1 DNA restricted with \textit{PvuI}
18. pSEC1 DNA restricted with \textit{MluI}
19. pSEC1 DNA restricted with \textit{XhoI}
20. pSEC1 DNA restricted with \textit{Sau3A}
Figure 4.3 Restriction endonuclease mapping of the *L. leichmannii* recombinant pSEC1.

pSEC1 plasmid DNA was digested to completion with a number of restriction endonucleases and the resulting DNA fragments were fractionated by agarose gel electrophoresis.

Key to tracks:

1. λ DNA restricted with HindIII
2. pSEC1 DNA restricted with HindIII and MluI
3. pSEC1 DNA restricted with HindIII and AvaI
4. pSEC1 DNA restricted with HindIII and NcoI
5. pSEC1 DNA restricted with HindIII and BglII
6. pSEC1 DNA restricted with HindIII and PvuII
7. pSEC1 DNA restricted with EcoRV and AvaI
8. pSEC1 DNA restricted with EcoRV and NcoI
9. pSEC1 DNA restricted with EcoRV and BglII
10. pSEC1 DNA restricted with EcoRV and PvuII
11. pSEC1 DNA restricted with BglII and NcoI
12. pSEC1 DNA restricted with BglII and PvuII
13. pSEC1 DNA restricted with MluI and AvaI
14. pSEC1 DNA restricted with MluI and BglII
The 2.0 Kb HindIII fragment within pSEC1 has mapped with respect to a number of restriction endonucleases.

Key:

- E1: EcoRI
- Nl: NcoI
- PII: PvuII
- Cl: Clal
- HIII: HindIII
- EV: EcoRV
- BI: BamHI
- SI: SalI
- AI: AavI
- PI: PstI
- BII: BgII
- XI: XhoI
- MI: MluI
- NII: NcoII

Origin of replication
Inverted repeat
Tetracycline resistance gene
Ampicillin resistance gene
Chloramphenicol resistance gene

None of the following sites were present:

- PstI
- EcoRI
- KpnI
- NcoI
- XhoI
- SmaI
- BamHI
- Clal
- SalI
- AavI
- PvuII
high stringency with \(\alpha^{32P}\)-labelled pSEC1 2.0 Kb \textit{HindIII} insert. The resulting autoradiograph banding pattern (Figure 4.5) was compared to the restriction endonuclease map (Figure 4.4). The banding pattern of the restricted genomic DNA probed with the pSEC1 insert correlated with the cloned DNA, indicating that the pSEC1 insert was derived from \textit{L. leichmannii} DNA and had not undergone any physical rearrangement, as was found in the initial cloning studies, detailed in Chapter 3 of this thesis.

4.1 \textit{Summary and Overview.}

This chapter details the successful isolation and cloning of a 2.0 Kb \textit{HindIII} genomic DNA fragment of \textit{L. leichmannii} exhibiting homology to the synthetic oligonucleotide probe (described in Chapter 3), into a \textit{recA}\textsuperscript{−} strain of \textit{E. coli} DH1.

There are many examples of genes cloned into \textit{E. coli} from Gram-positive organisms including \textit{Lactobacillus} genes. Examples of the latter have already been listed in Table 1.7. Examples of genes from other Gram-positive organisms cloned into \textit{E. coli} include; two chloramphenicol acetyltransferase genes from \textit{Clostridium butyricum} (Dubbert et al., 1988); \(\alpha\)-amylase gene from \textit{Clostridium acetobutylicum} (Verhasselt et al., 1989); the gene for phospho-\(\delta\)-galactosidase from \textit{Staphylococcus aureus} (Breidt & Stewart, 1987); the tagatose 1,6-bisphosphate aldolase gene from \textit{Streptococcus lactis} (Yu et al., 1988) and the arginine
Figure 4.5 Verification of the origin and continuity of the *L. leichmannii* recombinant pSEC1.

*L. leichmannii* genomic DNA was restricted as indicated, fractionated by agarose gel electrophoresis and transferred to a nitrocellulose filter. This filter was then probed with the pSEC1 2.0 Kb HindIII insert at high stringency (assuming an appropriate 5% bp mismatch).

Key to tracks:-

A DNA restricted with HindIII
B *L. leichmannii* genomic DNA restricted with HindIII
C *L. leichmannii* genomic DNA restricted with PvuII
D *L. leichmannii* genomic DNA restricted with HincII
E *L. leichmannii* genomic DNA restricted with EcoRV
F *L. leichmannii* genomic DNA restricted with BglII
G *L. leichmannii* genomic DNA restricted with AvaI

Tracks H to M are autoradiographs of corresponding tracks B to G probed with pSEC1 2.0 Kb HindIII insert.
repressor gene from *Bacillus subtilis* (Smith et al., 1986),
to name just a few.

DNA from Gram-positive organisms has, over the years, been
preferentially cloned in *E. coli*, as opposed to a Gram-
positive cloning system such as *Bacillus subtilis*, because
direct cloning of DNA sequences within plasmids which can
propagate in *B. subtilis* (Ehrlich, 1977; Gryzcan et al.,
1978) is very inefficient when compared to the *E. coli*
system. The central problem as recognized by Canosi et al.
(1978) and confirmed subsequently by Gryzcan et al. (1980),
seems to be that *B. subtilis* requires plasmid multimers for
efficient transformation of competent recipient cells.
Therefore, the cloning system of choice is that of *E. coli*,
which is the most extensively studied and best understood,
with well developed methodologies.
Chapter 5  Nucleotide sequence of the 2.0 Kb *L. leichmannii* genomic DNA insert of pSEC1.

5.1 Summary.

The 2.0 Kb genomic DNA fragment from *L. leichmannii* has been sequenced. It has been shown to possess three putative open reading frames; ORF 1, ORF 2 and ORF 3 (with predicted Mr values of 8,602, 17,388-23,013 and 17,792 respectively), the first of which bears homology to the Vitamin B_{12} receptor protein (BtuB) of *E. coli*. This first open reading frame is thought to be present only in part and that the remainder could be found on a larger genomic DNA fragment from *L. leichmannii*. This, however, remains to be elucidated.

5.2 Introduction.

To-date there is only a limited amount of information available on the molecular structure of genetic material from Lactobacilli, and none at all from *L. leichmannii*. The complete nucleotide sequence of the cloned *Lactobacillus* genomic DNA was determined, in order to locate the gene encoding the Vitamin B_{12} receptor protein in *L. leichmannii*, and also to enable comparative analyses to be made, with respect to promoter structure, preferred codon usage and derived amino acid sequences, between the *Lactobacillus* gene (encoded within the cloned 2.0 Kb insert) and,
a) other *Lactobacillus* genes and
b) genes encoding a Vitamin B₁₂ receptor protein from other organisms.

### 5.3 Results and Discussion.

#### 5.3.1 Nucleotide sequence of the 2.0 Kb *Lactobacillus* DNA insert of pSECl.

The 2.0 Kb *L. leichmannii* genomic DNA insert of pSECl was prepared for sequencing by the digestion of pSECl with *HindIII*, fractionated on an agarose gel and sub-cloned in both possible orientations into the *HindIII* site of a phagemid vector derived from pUC19 - PBS (+/-). The vector possessed ampicillin resistance, *lacZ* for blue/white colour selection and an M13 origin of replication allowing single stranded DNA rescue via helper phage infection for single strand sequencing. These details are illustrated in Figure 2.2, along with several other features, which can be found in Chapter 2 of this thesis.

Ten putative sub-clones were chosen for plasmid analysis, digested with *HindIII*, fractionated on an agarose gel and were all found to contain 2.0 Kb DNA inserts (Figure 5.1). In order to determine whether the fragment had been cloned in both possible orientations, the recombinants were digested with both *EcoRV* and *BglIII*, fractionated on an agarose gel and the orientation determined by the sizes of the resulting fragments. A 0.6 Kb fragment would result
Figure 5.1  Construction of pBS recombinants for nucleotide sequence analysis.

pSEC1 plasmid DNA was restricted with HindIII to release the 2.0 Kb DNA insert, and subsequently sub-cloned into the pBS (+/-) vector. Ten putative sub-clones were subjected to plasmid analysis, digested with HindIII and fractionated on an agarose gel.
from one orientation (a) and a 1.5 Kb fragment from the other (b), shown in Figure 5.2. Clones 3 and 7 were chosen for further study and were redesignated pBS3 and pBS7.

Recovery of single stranded DNA from cells containing pBS recombinants was as described in Methods and Materials (Section 2.18.1), using E. coli TG1 as the host and VCS-M13 as the helper phage.

The sequencing strategy, outlined in Figure 5.3 involved the synthesis of oligonucleotide primers in both directions. Each section of the insert was sequenced by the dideoxynucleotide chain termination procedure (Sanger et al., 1977).

*L. leichmannii* DNA (2,000 bp), which contained sequences homologous to the synthetic oligonucleotide, derived from the *btuB* gene of *E. coli*, was sequenced (see Figure 5.4). The DNA sequence contained at least two putative open reading frames (ORFs) - the first being of two possible lengths, depending upon the position of the putative promoter region. Thus, ORF 2a consisted of 471 bp (nucleotide position 450 to 920) and encoded a polypeptide of 157 amino acid residues, with a predicted Mr of 17,388. ORF 2b consisted of 633 bp (nucleotide position 283 to 920) and encoded a polypeptide of 211 amino acid residues, with a predicted Mr of 23,013. The second putative ORF, ORF 3 was preceded by a consensus Shine-Dalgarno (SD) sequence - AGGAGGA - (Shine-Dalgarno, 1974) 11 bp upstream from the
Plasmid DNA from the ten sub-clones (presented in Figure 5.1) were restricted with both EcoRV and BglII and fractionated on an agarose gel, to determine orientation of the 2.0 Kb DNA insert.

Key to tracks:

<table>
<thead>
<tr>
<th>Track</th>
<th>Clone</th>
<th>Orientation</th>
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<tbody>
<tr>
<td>A</td>
<td>clone 1</td>
<td>pBS(b)</td>
</tr>
<tr>
<td>B</td>
<td>clone 2</td>
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</tr>
<tr>
<td>C</td>
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<tr>
<td>J</td>
<td>clone 10</td>
<td>pBS(b)</td>
</tr>
</tbody>
</table>

(a) and (b) refer to orientation of the insert. See text for details.
Figure 5.2 Isolation of pBS3 and pBS7.

Plasmid DNA from the ten sub-clones (presented in Figure 5.1) were restricted with both EcoRV and BglII and fractionated on an agarose gel, to determine orientation of the 2.0 Kb DNA insert.

Key to tracks:-

A  clone 1 - pBS(b)
B  clone 2 - pBS(b)
C  clone 3 - pBS(a)
D  clone 4 - pBS(b)
E  clone 5 - pBS(a)
F  clone 6 - pBS(a)
G  clone 7 - pBS(b)
H  clone 8 - pBS(b)
I  clone 9 - pBS(a)
J  clone 10 - pBS(b)

(a) and (b) refer to orientation of the insert. See text for details.
Figure 5.3 Sequencing strategy for the *L. leichmannii* 2.0 Kb DNA insert from pSEC1.

Key:

<table>
<thead>
<tr>
<th>Key</th>
<th>Enzyme</th>
</tr>
</thead>
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<tr>
<td>A</td>
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<td>B</td>
<td>BgIII</td>
</tr>
<tr>
<td>E</td>
<td>EcoRV</td>
</tr>
<tr>
<td>P</td>
<td>PvuII</td>
</tr>
</tbody>
</table>

Arrows indicate the direction and extent of sequence data obtained from independent primers.

Open squares indicate the beginning of reverse complement synthetic oligonucleotides used as primers and closed squares indicate the positions of the 17-mer M13 sequencing primer (kind donation by H. Baybutt).
presumptive start codon (ATG). This ORF consisted of 492 bp (nucleotide position 1498 to 1989) and encoded a polypeptide of 164 amino acid residues, with a predicted $M_r$ of 17,792. In addition, a possible open reading frame (ORF 1) of 217 bp exists, which is located at nucleotide positions 4 to 234. However, the codon ATG at nucleotide 4 may not be the initiator codon, but a codon for an internal methionine. Further cloning and sequencing of a larger \textit{L. leichmannii} genomic DNA fragment would be necessary to ascertain this fact.

5.3.2 Upstream sequences of the putative ORFs.

The putative promoter regions for ORFs 2a, 2b and 3 are presented in Figures 5.5, 5.6 and 5.7 respectively.

5.3.2.1 ORF 2a.

Sequences beginning at nucleotide 352 (TTGCCA; -35) and nucleotide 381 (TATATT; -10) exhibited much similarity to reported promoter consensus sequences for both Gram-positive (Graves & Rabinowitz, 1986) and Gram-negative organisms (Rosenberg & Court, 1979; Hawley & McClure, 1983). Comparison of the putative \textit{L. leichmannii} promoter with either the Gram-negative or Gram-positive consensus promoters exhibited identity in 5 out of 6 nucleotides in both the -35 and -10 sequences. The spacing, however, between the -35 and -10 sequences was 23 nucleotides, which is greater than the distance normally found for the majority of bacterial promoters reported to-date (see Figure 5.8).
The sequence consists of 2,000 bp containing two open reading frames (ORFs). Putative -35 and -10 regions are as indicated, along with putative ribosome binding sites (SD).
Figure 5.5  The nucleotide sequence of the transcriptional control region of ORF 2a.

```
310 320 330 340 350 360
TGGAGATGCTGATCGTGGGCCGAAAGATTTTAGGGATGCCTGCTGCTCTGCTGGCAGCA

370 380 390 400 410 420
GCAGCTCAGGTACACCAACCTATATTGAAACGCCTGGTCACAAGGTGTTAGTGGATGCCC

430 440 450 460 470 480
GTGTCTAGSCAAGATAAGACACTGATGAAAGACATCGCGAGATCTAACCGATG
  MetLysSerIleGlyArgAspLeuThrAspV

490 500 510 520 530 540
TTGACGTGTATTTATCAGCGATGAAATACATAGCGATCATGCGTGCGTGGTAGGCGGTGG
  AspSerValPheIleThrHisGluHisSerAspHisValArgGlyValGlyValLeuA
```
**Figure 5.6** The nucleotide sequence of the transcriptional control region of ORF 2b.
Figure 5.7  The nucleotide sequence of the transcriptional control region of ORF 3.

```
1210 1220 1230 1240 1250 1260
GGTTACTGTCTGGATCGACATGGCCATTGGAAGCTTTGACGATGAGCTACGTTACG

1270 1280 1290 1300 1310 1320
TCGGACACCATGATTTCTGGATTTACGCGACATACGTCACGGTTAAGGACCTCAG

1330 1340 1350 1360 1370 1380
GCAAAGATCAACCCCTGCTCATACGCCCTGGGAAACTTACAACCTGATCCCTAAAGC

1390 1400 1410 1420 1430 1440
CGATTGCGCTCAGGAAAAATACCGTATTGGTTTCATAGGGTGCAATACG

1450 1460 1470 1480 1490 1500
CAATATCTTTGTAACACAGGTTGAGTGGTAACTGCGGTTACGAGGGAAGACGC

1510 1520 1530 1540 1550 1560
AACGGAAGTCATAACTTTCGGAGGACGCCACCCGAAACGCGACCACCGTGCGGCAA

1570 1580 1590 1600 1610 1620
GCGAGAGGATGCTTACCACTGACCTGACATTGCTATGCTGGTGGCACTTGGTGG

1630 1640 1650 1660 1670 1680
CGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT

AsnGlySerHisAsnProGluValGlutyrThrThrGlulAlaGluArgSerAlaGluLys

```

```
ProLysMetValGlulAlaAspCysAsnCysCysCysCysGlyCysLeuAspTrpTrpTrp
```

```
ArgValGlyGlyGlyThrAlafyrTrpAlaIleAsnHisThrThrSerLeuGlyValThr
```
### Figure 5.8  Compilation of promoter sequences from Gram-positive organisms.

(adaptation from Graves & Rabinowitz, 1986).

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Sequences are aligned by the -35 and Pribnow regions. The 3' nucleotide listed is the major site of initiation, which often occurred at more than one nucleotide. The consensus sequence derived from the compiled list is given below. The E. coli consensus sequence (McClure, 1985) is also provided for comparative purposes.

Bases that appear in any given position in more than 41% of the examples are in small letters, those more than 50% in capital letters, and those more than 75% are in tall capital letters.
Sequences are from:

1. *S. aureus* β-lactamase (McLaughlin et al., 1981)
2. *B. subtilis* phage φ29G3b (Murray & Rabinowitz, 1982)
5. *B. subtilis* veg (Moran et al., 1982)
8. *B. subtilis* trp operon (Shimotu & Henner, 1984)
9. *S. aureus* erm C (Gryczan et al., 1980)
10. *S. aureus* erm A (Murphy, 1985a)
11. *B. amyloliquefaciens* α-amylase (Lehtovaara et al., 1984)
12. *Corynebacterium diphtheriae* toxin (Kaczorek et al., 1985)
13. *B. licheniformis* spo OH (Ramakrishna et al., 1984)
14. *B. subtilis* rrnB p1 (Stewart & Bott, 1983)
15. *B. subtilis* rrnB p2 (Stewart & Bott, 1983)
16. *B. subtilis* rrn0 p1 (Ogasawara et al., 1983)
17. *B. subtilis* rrn0 p2 (Ogasawara et al., 1983)
18. *B. subtilis* rrnA p1 (Ogasawara et al., 1983)
20. *C. pasteurianum* Fd (Graves & Rabinowitz, 1986)
21-27. *B. subtilis* replication origin region and are TR1 through TR7 respectively (Ogasawara et al., 1985).
28. *S. aureus* AAD9 (Murphy, 1985b)
30. *L. leichmannii* ORF 2a (this work).
31. *L. leichmannii* ORF 2b (this work).
32. *L. leichmannii* ORF 3 (this work).
Covering the region 6-10 bp upstream of the presumptive initiator codon (ATG) is the sequence AGGAG. This is a shorter version of the consensus for the Shine-Dalgarno sequence of Gram-positive cells (Murray & Rabinowitz, 1982), as well as for *E. coli*, as mentioned previously, and presumably serves the same function in *Lactobacillus*.

5.3.2.2 ORF 2b.

Sequences beginning at nucleotide 209 (TTATGA; -35) and nucleotide 231 (TTAAGT; -10) exhibited much weaker similarity with the reported consensus sequences, only exhibiting identity in 3 out of 6 nucleotides in both the -35 and -10 sequences. The spacing between the sequences was more in line with the distance found for the majority of bacterial promoters reported to-date (i.e. 17-18 bp). Figure 5.8 mentioned previously shows a survey carried out by Graves and Rabinowitz (1986) on the promoter sites of Gram-positive cells. Their tabulations are based only on those systems for which the control regions have actually been identified by chemical means. However, if the promoter regions mentioned in this study are compared to other putative *Lactobacillus* transcriptional control regions, it can be seen that the spacing between the -35 and -10 sequences is quite varied - as presented in Figure 5.9.
### Figure 5.9 Compilation of promoter sequences from *Lactobacillus* genes.

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<td>3.</td>
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<td>ACGGTCAAA TTTAAC GGGACTG GC</td>
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<td>6.</td>
<td>TCAGTGTCC TTGCA GCCACAGCTCAGTACGTAACGGCAACC TATATT GAAACG</td>
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<td>7.</td>
<td>CTCGACCG ATTAAT AAAACCC</td>
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<td>CCTGAGCTT GCCATA GTTTCTGCA</td>
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<td>9.</td>
<td>CAATAATTT TAAAAC GAATCATT</td>
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Gram +ve TTGACA T TATAAT AATT

Sequences are aligned by the -35 and Pribnow regions. The consensus sequence derived from the compiled list of Graves and Rabinowitz (1986) is given below. Sequences are from:-

6. *L. leichmannii* ORF 2a (this work).
7. *L. leichmannii* ORF 2b (this work).
8. *L. leichmannii* ORF 3 (this work).
Covering the region 9-13 bp upstream of the presumptive initiator codon (ATG) is the sequence AGGAGG, which is slightly nearer to the consensus Shine-Dalgarno sequence, already mentioned.

5.3.2.3 ORF 3.

Sequences beginning at nucleotide 1413 (TTCATA; -35) and nucleotide 1439 (TACAAT; -10) exhibited good similarity with the reported consensus sequence of Gram-positive organisms, but again the spacing between the two sequences was slightly more than that in other bacterial promoters. Covering the region 11-18 bp upstream of the presumptive start codon ATG is the consensus SD sequence AGGAGGA, as mentioned previously.

5.3.2.4 Extended promoter region in Gram-positive organisms.

In addition to the putative Pribnow box around position -10 from the proposed transcription origin, it is suspected that other upstream bases may play a role in an extended promoter site (Graves & Rabinowitz, 1986). The presence of TG at -16 and -15 is noteworthy, with the T residue being the seventh most highly conserved base in the promoter region (76%) and the G less highly conserved (62%). 83% of the promoter regions compared by Graves and Rabinowitz contained at least one TG pair in the -18 to -14 region and in several cases,
The TGTG doublet appeared. These nucleotides are only weakly conserved (less than 44%) in *E. coli* promoters. This feature however does not appear to be conserved in the promoter regions proposed in this study and appears only in one case in Figure 5.9; that of the dihydrofolate reductase gene of methotrexate-resistant *L. casei* (Andrews et al., 1985).

Graves and Rabinowitz also found that downstream of the -10 region, A residues are conserved at -7, -6 and -4 and T residues at -5 and -3, whereas no analogous conservation exists in *E. coli*. There is seen to be some conservation in this area in all three reading frames discussed in this section. Finally, A residues are again shown to be conserved in a cluster at positions 41-45 with a greater than 50% conservation rate. This can be seen in the promoter regions shown in Figure 5.8 but not unfortunately in the ORFs studied or indeed to such an extent in any of the promoter regions presented in Figure 5.9, other than that of histidine carboxylase in *Lactobacillus 30a* (Vanderslice et al., 1986).

The two additional conserved areas on either side of the typical Pribnow box broaden the promoter element to span the region from residues -18 to -4. In this extended region, out of the 29 promoter regions studied by Graves and Rabinowitz, 12 nucleotides were conserved at a rate greater than 50%. This extended promoter consensus could be important for recognition by, or binding of, RNA polymerase.
Although all three of the putative promoter regions described contained few of the features of the extended consensus proposed for Gram-positive promoter sequences (Graves & Rabinowitz, 1986), they do share this with other Lactobacillus putative promoter regions (see Figure 5.9).

5.3.3 Downstream sequences of the putative open reading frames.

A sequence resembling a Rho-independent transcription terminator (Platt, 1986) was found 3 nucleotides following the presumed stop codon (TGA) for ORF 2. The RNA transcribed from this region could possibly form a large stem-loop structure with 27 bp in the stem and 34 bp in the loop (Figure 5.10a). The calculated free energy for this structure is -16.6 Kcal, which is within the range typically observed for Rho-independent terminators (Platt, 1986). A smaller stem-loop structure could also be found (see Figure 5.10b) 307 nucleotides following the stop codon (TGA) of the putative ORF 2, which had a higher free energy of formation than the former (-20.6 Kcal). Another significant small stem-loop structure was found 116 nucleotides following the stop codon (TAA) of the putative ORF 1, which also had a high free energy of formation, -21.0 Kcal (Figure 5.10c).
**Figure 5.10a**  Structure of a putative Rho-independent terminator for ORF 2.

```
TGAGGGGCATT
```
Figure 5.10b  
Structure of a second putative Rho-independent terminator of ORF 2.
Figure 5.10c  Structure of a putative Rho-independent terminator for ORF 1.
5.3.4 Analysis of the coding regions of the
L. leichmannii 2.0 Kb DNA insert.

5.3.4.1 Nucleotide sequence analysis.

L. leichmannii 4797 DNA has a G + C content of 50.5% (Miller et al., 1970). The open reading frames under discussion; ORF 2a, 2b and 3 have G + C content values of 50.3%, 49.8% and 47.8% respectively.

Codon usage of the three open reading frames were compared with data from other Lactobacillus genes and is outlined in Table 5.1. Lerch et al. (1989) also carried out a comparative analysis, stating that out of the sequences compared (comprising 1805 codons), only one example of AUA (isoleucine) codon usage was observed (Porter & Chassy, 1988). They did not however include ORF 2 from the insertion element ISL1, isolated from L. casei bacteriophage (Shimizu-Kadota et al., 1985). This open reading frame was larger than ORF 1 and contained 3 AUA codons. Perhaps Lerch et al. (1989) did not include ORF 2 in their calculations because a putative ribosome binding site was found only for ORF 1 and not for ORF 2. Lerch and his colleagues also stated that the codons AGA/G (Arginine) and UAG (Terminator) have not been found to be used by Lactobacillus genes so far. Yet again, ORF 2 of the insertion element ISL1 (Shimizu-Kadota et al., 1985) seems to differ. It uses the terminator UAG and contains two AGA codons and four AGG
### Table 5.1 Codon usage comparison of some *Lactobacillus* genes.

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1. β-galactosidase gene from L. bulgaricus (Schmidt et al, 1989).


3. histidine decarboxylase gene from Lactobacillus 30a (Vanderslice et al, 1986).

4. Orf 1 (this work).

5. Orf 2a (this work).

6. Orf 2b (this work).

7. Orf 3 (this work).


9. ORF 1 from insertion element ISL1 from L. casei bacteriophage (Shimizu-Kadota et al, 1985).

10. ORF 2 from insertion element ISL1 from L. casei bacteriophage (Shimizu-Kadota et al, 1985).
codons. A few months following this publication, Schmidt et al. (1989) presented the nucleotide sequence of the β-galactoside gene from *L. bulgaricus* and it could be seen that it used five AGA codons and one AGG codon.

Taking the three putative reading frames in the 2.0 Kb HindIII *Lactobacillus* genomic DNA insert, ORF 1, ORF 2a/b and ORF 3, it can be seen that ORF 1 differs from the others. It uses the UAA (ochre) termination codon like the majority of the *Lactobacillus* genes, whereas the others (ORF 2a/b and ORF 3) use the UGA codon. ORF 1 does not use AGA or AGG to code for arginine, whereas the others do. In fact, ORF 3 uses two AGA codons and 3 AGG codons. ORF 3 also uses the codon AUA five times and ORF 2a, once.

No significant trend in codon usage for the *Lactobacillus* genus can be seen by comparing the codon usage of the putative open reading frames with the other *Lactobacillus* genes in Table 5.1, other than perhaps the more frequent use of the codon UUU for Phenylalanine, the codon GAA for glutamine and the non-use of codon UAG for termination (with the exception of ORF 2 from insertion element ISL1).

Considering the type of amino acids encoded by the ORFs under discussion, they all have a majority of hydrophobic amino acids as shown in Table 5.2.
Table 5.2

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<th>ORF 2a</th>
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<td>Basic (Arg + Lys)</td>
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<td>Aromatic (Phe + Trp + Tyr)</td>
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<tr>
<td>Hydrophobic (Aromatic + Ile + Leu + Met + Val)</td>
<td>49</td>
<td>70</td>
<td>54</td>
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</table>

This data is backed up by the hydropathy profiles, presented in Figure 5.11. These plots were performed, using a subroutine of the 'Staden-plus' programme. This routine plots the hydrophobicity of each section of the sequence using the hydrophobicity values of Kyte and Doolittle (1982). A window of size 'span' is slid along the sequence and a sum calculated for each position.

A comparison of nucleotide sequences between the ORFs contained within the *L. leichmannii* 2.0 Kb genomic HindIII fragment and the *btu* genes (*btuB* and *btuCED*) revealed no significant homology, even though the G + C content of *E. coli* is approximately 50% (similar to 50.5% of *L. leichmannii*) and the preferred codon usage of the *E. coli btuB* gene (see Figure 3.2) does not appear to have any major significant differences from that of *L. leichmannii*. 
Figure 5.11  Hydropathic profiles of the deduced polypeptides encoded by the three putative ORFs of *L. leichmanni* 2.0 Kb HindIII genomic DNA fragment.

The hydropathic profiles shown are based upon the scale of Kyte and Doolittle (1982). A span of eleven consecutive residues was used.

**ORF1**

![Hydropathic profile for ORF1](image1)

**ORF2**

![Hydropathic profile for ORF2](image2)

**ORF3**

![Hydropathic profile for ORF3](image3)
It is likely that the two nucleotide sequences could be dissimilar and in fact the gene encoding the *L. leichmannii* Vitamin B$_{12}$ receptor may not even be 'btuB-like', since recent findings have shown that *S. typhimurium* possesses not only a high affinity btuB dependent system, but also another cobalamin uptake system not present in *E. coli* (Rioux & Kadner, 1989b). It is unlikely that this system is tonB dependent; it did not confer susceptibility to phage BF23 or to the E colicins, and the DNA insert (encoding a polypeptide with an M$_r$ of 84,000) did not hybridise to the btuB gene. However, a degree of homology between the polypeptides encoded by these genes would be expected at the site where binding of the Vitamin occurs.

5.3.4.2 Nucleotide sequence derived - amino acid analysis.

The nucleotide sequence derived BtuB, BtuC, BtuD and BtuE sequences of *E. coli* were compared to the nucleotide sequence derived (as yet, unknown) polypeptides of *L. leichmannii*, using the IBM AT Microgenie protein alignment sub-routine.

A region of homology was found between *E. coli* BtuB and the polypeptides encoded by the cloned *L. leichmannii* genomic DNA, illustrated in Figure 5.12. The homology between amino acid residues 61 and 68 of *L. leichmannii* ORF 1 and residues 507 and 514 of the *E. coli* BtuB polypeptide is thought to be
### Figure 5.12 Comparison of amino acid sequences.

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<th>E. coli BtuB</th>
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<tr>
<td>61 Arg Val Asn Ala Ile Thr Arg Thr</td>
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</table>
significant as Moir et al. (1987) stated that, removal of
the C-terminus of the E. coli btuB gene prevents receptor
function (being tested through both complementation and
negative complementation). They also presented data to
argue strongly that at least 216 C-terminal residues were
not required for transfer to the outer membrane, but may be
necessary for correct localisation in the membrane. It is
therefore the C-terminal end of the protein which appears to
be involved in B_{12} binding and so the region of homology to
E. coli BtuB, found between residues 61 to 68 of ORF 1 from
L. leichmannii may also be involved in B_{12} binding. It is
unfortunate that the start of ORF 1 is unknown. The ATG
codon taken to be the start site of translation predicts a
polypeptide with an M_r of only 8,602 consisting of 77 amino
acid residues. This data suggests that the entire gene may
not be present. To ascertain whether there is more to ORF 1
then represented in Figure 5.4, would require the cloning of
a larger fragment of L. leichmannii genomic DNA and further
nucleotide sequence analysis. Whether ORF 1 in its possible
extended form encodes a Vitamin B_{12} receptor protein in
L. leichmannii remains to be elucidated.

According to Sasaki and Kitahara (1964c), there are
pertinent features to be noted regarding the amino acid
composition of the Vitamin B_{12} complex. They found that the
complex was indeed a B_{12}-peptide consisting of no sugar or
base. It lacked cysteine and histidine, which was
characteristic of being obtained from bacterial walls and
possessed phenylalanine and tyrosine, two aromatic amino
acids thought to be involved in conjunction of the walls with the Vitamin and had up till then not been reported in the cell walls of lactic acid bacteria. From the data generated from ORF 1 in these studies, it appears that this was the only possible coding region within the 2.0 Kb L. leichmannii genomic fragment, which bears all of these features. However, analysis of sequences upstream of the L. leichmannii genome is required to either refute or confirm this hypothesis.
Chapter 6  **Expression analysis of the cloned *L. leichmannii* genomic DNA 2.0 Kb HindIII fragment.**

6.1 Introduction.

The Vitamin B$_{12}$-binding property of *L. leichmannii* has been studied. Kashket et al. (1962) found *L. leichmannii* to be capable of rapidly binding [${}^{60}$Co] Vitamin B$_{12}$ in amounts greatly in excess of maximal growth requirements. The quantity of Vitamin B$_{12}$ stored was sufficient to support 9000-fold increase in cell number when the cells were subsequently inoculated into Vitamin B$_{12}$-free media. It was initially thought that the major binding site appeared to be a ribosomal glycoprotein (Kashket et al., 1962). However, in later years, it was shown that three quarters of the B$_{12}$ bound to the cell was found in the cell wall and the remaining quarter found in the particulate (ribosome) fraction (Sasaki, 1972). Apparently, the binding mechanism in *L. leichmannii* is a simple adsorptive process requiring the expenditure of little energy. This is suggested by the low activation energy of the binding phenomenon ($\mu = 1900$ cal.) and the ineffectiveness of various metabolic inhibitors in altering the rate or amount of [${}^{60}$Co] Vitamin B$_{12}$ binding by intact cells.

In order to determine whether the cloned *L. leichmannii* DNA fragment (outlined in Chapter 4) harbours the gene encoding the Vitamin B$_{12}$ receptor protein from *L. leichmannii*, it was not only necessary to subject the fragment to expression
analysis, but also to have a means of identifying the gene product.

Sasaki (1972) described a procedure of purifying the $B_{12}$-complex ($B_{12}$ bound to a polypeptide) from the cell walls of *Lactobacillus*. Therefore, the $B_{12}$-complex was purified from the cell walls of *L. leichmannii* and polyclonal antibodies raised against it. These antibodies were then be used as a 'probe' for the identification of the gene product.

### 6.2 Results and Discussion.

**6.2.1 Complementation analysis.**

The *E. coli btuB* strain, L1-431, was transformed with the recombinant pSEC1 and pNF48 (positive control) and tested for complementation, as described in Section 3.2.5.1. However, there appeared to be no BtuB$^+$ transformants when pSEC1 was used to transform the *E. coli btuB* mutant L1-431 (i.e. no plaques formed upon infection of transformants phage with BF23). The 2.0 Kb HindIII insert of pSEC1 was subsequently recloned in the opposite orientation in pBR325 by restriction of pSEC1 with HindIII, followed by religation and transformation into *E. coli* DH1. The resultant plasmid was designated pSEC2 and the orientation of the insert confirmed by restriction analysis. Subsequent transformation and complementation analysis of the btuB lesion in L1-431 (as described above) with pSEC2 gave the same result as for pSEC1.
The absence of plaques does not necessarily mean that the btuB lesion in *E. coli* L1-431 was not complemented by the inserts contained within pSEC1 and pSEC2, since the binding protein in *L. leichmannii* may not be suitable as a receptor for phage BF23. This could be due to several reasons, for example, a BF23 binding domain may not be present on this smaller protein and if one does exist, the orientation of the receptor in the cell of *Lactobacillus* may be such that binding is not permitted. Another reason may be that phage BF23 cannot replicate in *Lactobacillus*, although it may infect the cell.

Other lesions in *E. coli* have been shown to be complemented by *Lactobacillus* genes; for example, the *E. coli* leuB mutation was complemented by a *L. bulgaricus* gene encoding a serine tRNA (Hottinger et al., 1987) and it is thought that the restoration of the mutant leuB allele occurred by missense suppression. Also, the *Streptococcus pneumoniae* polA gene can functionally substitute for the *E. coli* polA gene (López et al., 1987).

6.2.2 **Purification of the Vitamin B₁₂-binding protein from the cell walls of *L. leichmannii***

A Vitamin B₁₂ binding protein was isolated from the cell walls of *L. leichmannii* by treatment with 0.2 M HCl and purified, as described in the Methods section 2.21. Owing
to the stabilising effect of Vitamin B$_{12}$, the binding protein was initially isolated as a B$_{12}$-complex.

Figure 6.1 is a diagrammatic representation of the structure of the B$_{12}$ binding protein complex in the cell walls of *Lactobacillus* sp. Sasaki and Kitahara (1964b) believed that B$_{12}$ binds to the site where pepsin acts since, if B$_{12}$ is bound to the wall, pepsin cannot release the B$_{12}$ complex and therefore destroy the binding activity. That is, B$_{12}$ protects the B$_{12}$-binding protein complex from the action of pepsin. However, they found that 0.2 M HCl destroyed the B$_{12}$-binding activity of the wall since the Vitamin bound to the wall was also released by treatment with 0.2 M HCl, showing that 0.2 M HCl acts at some other point closer to the basal structure of the wall than the site upon which pepsin acts. Their view was confirmed by evidence that material released from walls by treatment with 0.2 M HCl could still bind B$_{12}$, whilst that with pepsin could not. Lipase and ribonuclease have no significant effect on the B$_{12}$-binding activity of the wall (Sasaki, 1972).

CM-cellulose column chromatography, using gradient elution with phosphate buffer was found by Kitahara and Sasaki (1963) to give the most satisfactory purification. (Retention on CM-cellulose is characteristic of proteins with a net positive charge). As shown in Figure 6.2, the complex was eluted between fractions between 25 and 32. The B$_{12}$ complex was purified by rechromatography and was subsequently concentrated by using a small column of CM-
Figure 6.1  Representation of the structure of the $B_{12}$-binding complex in the wall.

A dot represents one molecule of $B_{12}$. Arrows indicate the sites where 0.2 M HCl (→) and pepsin (←) acts, respectively.
Figure 6.2  CM-cellulose column chromatography of the B₁₂-complex of the cell wall from *L. leichmannii*.

A B₁₂ complex solution in 0.01 M phosphate buffer (pH 7.0) containing 0.1 M NaCl (PBN). 200 ml with optical densities: 280 nm = 1.44 and 361 nm = 0.62 was placed on a CM-cellulose column (1.5 by 20 cm) equilibrated with PBN. Elution was performed as described in Methods and Materials.

Symbols: optical density at 361 nm (- - -), at 280 nm (---) and concentration of phosphate buffer (----).
cellulose, followed by dialysis. This was electrophoresed through a polyacrylamide gel and yielded a diffuse band of low molecular mass, shown in Figure 6.3.

Resemblance of the absorption spectrum of the complex to that of B$_{12}$, over the range of 330 nm to 560 nm (Figure 6.4) clearly indicates that the complex contained a cyanocobalamin molecule. Slight shifts of peaks around 361 nm and 550 nm to shorter wavelengths was observed for the B$_{12}$ complex. The increased peak at 278 nm of the B$_{12}$ complex indicates involvement of polypeptide(s) in this complex.

Sephadex G-75 equilibrated with PBN was used with a column of 1.5 x 84 cm. The B$_{12}$ complex in the equilibration buffer (3 ml) was layered on the top and eluted. The elution profile was monitored with a Uvicord monitor at 280 nm. The void and total volumes were determined by using blue dextran 2000 and B$_{12}$ respectively. Pepsin, soybean trypsin inhibitor and cytochrome c were used as reference standards in molecular weight determination of the B$_{12}$ complex, as described by Andrews (1964). The B$_{12}$ complex eluted at a similar position to that of trypsin inhibitor (see Figure 6.5), which suggests that the molecular weight of the B$_{12}$ complex was in the region of 21,500, which corresponds well with the value obtained by Sasaki (1972).
Figure 6.3 Polycrylamide gel electrophoresis of the purified B$_{12}$ complex of the cell walls from *L. leichmannii*.

Key to tracks:

**A** Purified B$_{12}$-complex after CM-cellulose chromatography.

**B** B$_{12}$-complex solution before CM-cellulose chromatography.
Figure 6.4  Absorption spectrum of the purified B₁₂ complex of the cell wall.

Key:

(-----) B₁₂ complex

(----) B₁₂

Absorption spectra were recorded using a Beckman DU-70 spectrophotometer.
Figure 6.5  Gel-filtration on sephadex G-75 of the purified $B_{12}$-complex from the cell wall of
*L. leichmannii*.

![Diagram]

**Key:**

- $A_{280}$
- $A_{361}$
- BD  Blue Dextran
- P  pepsin
- TI  Trypsin inhibitor
- CC  Cytochrome c
- $B_{12}$  free $B_{12}$
The free binding protein and not the $\text{B}_{12}$ complex was required for the production of antibodies. Preparation of the free binding protein was achieved by dissociation of the Vitamin $\text{B}_{12}$-binding protein complex. Gräsbeck had found dissociation to occur at high pH (12.9). When the pH of the $\text{B}_{12}$ complex solution was brought to pH 12.9 with Gräsbeck's buffer, consisting of NaOH, glycine and NaCl (Gräsbeck et al., 1968), by elution through a 2 x 30 cm Biogel P-10 polyacrylamide column, the detached polypeptide and Vitamin $\text{B}_{12}$ eluted separately from the column. The column had previously been calibrated so that the elution volumes for large and small volumes were known. To ensure rapid neutralisation, the eluate was collected in tubes containing 0.5 M NaH$_2$PO$_4$.

6.2.3 Antibody production.

Purified protein was administered to a New Zealand white rabbit, initially mixed with Freund's complete adjuvant, followed by two further administrations of protein mixed with Freund's incomplete adjuvant, and antisera obtained as detailed in Methods section 2.24. Antisera to the $E. \text{coli}$ Vitamin $\text{B}_{12}$ receptor protein (BtuB) was also made available (kind donation by J. Armstrong), which allowed some comparative analysis.
A preparation of outer membranes of a BtuB+ strain, NFB362 (harbouring the plasmid pNF48) was made and electrophoresed through an SDS-polyacrylamide gel, together with cell wall extracts from *L. leichmannii*. A duplicate gel was run and both were Western blotted onto nitrocellulose (Section 2.25). Antisera raised against *E. coli* BtuB was used to probe one blot and antisera raised against *L. leichmannii* Vitamin B\textsubscript{12} binding protein used to probe the other. Peroxidase conjugated goat anti-rabbit IgG was used as the secondary antibody and chloronaphthol (Sigma) as the colour reagent reaction. It appeared from Figure 6.6 that the antisera raised against the *L. leichmannii* B\textsubscript{12} binding protein was able to detect the *E. coli* Vitamin B\textsubscript{12} receptor (BtuB), shown quite clearly in (b). However, antisera raised against *E. coli* BtuB could only just detect the *Lactobacillus* B\textsubscript{12}-binding protein. This difference in specificity could be because the antisera raised against *L. leichmannii* B\textsubscript{12} binding protein was still in a ‘crude’ form, whereas that raised against *E. coli* BtuB had been immunoabsorbed against a total *E. coli* extract to remove anti-*E. coli* antibodies and therefore non-specific detection of bacterial proteins was markedly reduced.

6.2.4 Expression of the cloned 2.0 Kb genomic DNA HindIII fragment from *L. leichmannii* in an *E. coli* system.

There are many examples of *Lactobacillus* genes being efficiently expressed in *E. coli*; for example, \(\beta\)-D-
Figure 6.6  Western Blot Analysis.

a) Probed with antisera raised against *E. coli* BtuB

b) Probed with antisera raised against *L. leichmannii* B12 binding protein.

Key to tracks:

A  *L. leichmannii* cell wall extract (40 µg protein).
B  *L. leichmannii* cell wall extract (20 µg protein).
C  *E. coli* NFB362 outer membrane (20 µg protein).
phosphogalactoside galactohydrolase gene from *E. coli* has been expressed as efficiently as that from *E. coli* itself (Lee et al., 1982); high expression (catalytically active) of the thymidylate synthase gene has been achieved from *L. casei* (Pinter et al., 1988); the β-galactosidase gene from *L. bulgaricus* has been efficiently expressed (Schmidt et al., 1989) and the D-2-hydroxyisocaproate dehydrogenase gene (D-HiCDH) from *L. casei* (Lerch et al., 1989) also.

An *in vitro* cell free coupled transcription-translation system obtained in kit form (Amersham) was used to identify the protein products of the cloned 2.0 Kb HindIII fragment from *L. leichmannii* genomic DNA. To reiterate from Chapter 3, this method, first described by De Vries and Zubay (1967) allows the expression of genes contained on a bacterial plasmid provided that the relevant control signals are present for initiation of transcription and translation.

Out of the three putative open reading frames encoded by the 2.0 Kb fragment, only ORF 2 (a or b) and ORF 3 possessed the relevant control signals necessary for *in vitro* expression in the Zubays reactions. Both ORF 2a and ORF 3 had predicted Mr's of 17,388 and 17,792 respectively, whereas ORF 2b had a predicted Mr of 23,013; none of which correspond to the value estimated by Sasaki (1972) or that obtained in these studies, which was in the region of 21,500. However, ORF 1 which is possibly incomplete, has no relevant control signals (Pribnow box, Shine-Dalgarno sequence) present upstream of the presumptive initiation codon, within the
2.0 Kb HindIII genomic fragment cloned from *L. leichmannii*. However, transcription of ORF 1 could theoretically be possible if there was 'read through' from the tetracycline resistance gene promoter in pBR325. Normally this is inactivated when DNA is cloned into the HindIII site. However, since the HindIII site of pBR325 lies within the promoter region rather than within a structural gene, insertional inactivation depends on whether the cloned DNA carries a promoter-like sequence able to initiate transcription of the tetracycline resistance gene.

To determine whether any of the protein products, expressed by any of these putative coding regions, is indeed the Vitamin B₁₂-binding protein from *L. leichmannii*, pSEC1 and pSEC2 were subjected to expression analysis and the resulting proteins electrophoresed in duplicate on 15% SDS-denaturing polyacrylamide gels, followed by Western blotting onto nitrocellulose. One blot was probed with antisera raised against *E. coli* BtuB and the other probed with antisera to the B₁₂-binding protein from *L. leichmannii*. Again, the secondary antibody used was peroxidase conjugated goat anti-rabbit IgG and chloronaphthol as the colour reagent reaction. Figure 6.7 shows the Western blot analysis.

It appears that, although the banding patterns of the two probings appear to be different, unfortunately there does not seem to be any difference between the tracks on each blot. The detection of a Mr-66,400 polypeptide on track A
**Figure 6.7** Western blot analysis of Zubays produced polypeptides.

a) Western blot probed with antisera raised against *E. coli* BtuB.

b) Western blot probed with antisera raised against *L. leichmannii* B₁₂-binding protein.

Key to tracks:

- A  plasmid pNF48 (harbouring *E. coli* btuB gene)
- B  plasmid pSEC2
- C  plasmid pSEC1
- D  plasmid pBR325
when probed with antisera to *E. coli* BtuB was expected (positive control). Without this control, there is no way of determining whether a negative result is due to the fact that, none of the putative open-reading frames (ORF 2a, ORF 2b or ORF 3) encode for the B$_{12}$-binding protein from *L. leichmannii* or, that ORF 1 should encode the B$_{12}$-binding protein but does not, because it is not present in its entirety on the 2.0 Kb HindIII genomic fragment, cloned from *L. leichmannii* and therefore does not possess the relevant control signals for transcription and translation.

Expression studies were also carried out, using *E. coli* maxicells (as described in Methods section 2.17.1). However, it appeared that not enough protein was transferred and the detection system used was not sufficiently sensitive.

6.2.5 Expression of the cloned 2.0 Kb genomic DNA HindIII fragment from *L. leichmannii* in a Gram-positive system.

Expression studies were attempted using the method described by Thomson *et al.* (1984), outlined in Section 2.17.3. This method, although functional in *Streptomyces lividans*, did not appear to result in any *de novo* proteins being synthesised from the recombinant *L. leichmannii* DNA.
6.3 Summary and Overview.

The $\text{B}_{12}$-binding protein from *L. leichmannii* was isolated and purified, in order to raise antibodies against it to use as a 'probe', for the identification of any gene products encoded by the putative open-reading frames, located on the cloned 2.0 Kb HindIII genomic DNA fragment.

Cross-reactivity was found between the *E. coli* BtuB protein and the antisera raised against the *L. leichmannii* binding protein. Likewise, cross-reactivity was found between the *L. leichmannii* $\text{B}_{12}$ binding protein and the antisera raised against the *E. coli* BtuB protein. This data suggests that a degree of homology exists between the two proteins.

The use of the antisera raised in these studies as a 'probe', for the detection of the $\text{B}_{12}$ binding protein, which may be encoded by any of the putative open-reading frames located on the cloned 2.0 Kb *L. leichmannii* DNA fragment, failed. No plasmid encoded protein were found to be expressed by the Gram-positive system used and those expressed in the Gram-negative system (Zubays) were not detected by the 'probe' used. The failure to detect a $\text{B}_{12}$ binding protein by this method does not suggest that the $\text{B}_{12}$ binding protein was not encoded by any of the putative ORFs, but that the detection system used was not sensitive enough. The *E. coli* $\text{B}_{12}$ receptor protein (BtuB) is expressed from a weak promoter and it may be that the amount of protein
transferred to the nitrocellulose was not sufficient, to be detected.

In order to determine whether any of the putative ORFs do encode the B$_{12}$-binding protein from *L. leichmannii*, it would now be necessary to obtain N-terminal amino acid sequence information from the B$_{12}$ binding protein purified in these studies. A direct comparison could then be made between the amino acid sequence of the B$_{12}$ binding protein from *L. leichmannii* and the nucleotide sequence derived-amino acid sequences of the putative ORFs located on the cloned 2.0 Kb *L. leichmannii* genomic DNA fragment.
Chapter 7  Transformation of *Lactobacillus leichmannii*.

7.1 Introduction.

The introduction of DNA into bacteria by transformation is an essential step in the construction of recombinant strains. Members of at least fifteen genera of bacteria are naturally competent (i.e. they can take up DNA and thus acquire new genetic traits); current knowledge of the natural DNA uptake process has been reviewed (Stewart & Carlson, 1985; Saunders et al., 1984). Without natural DNA uptake, an alternative means of transferring DNA into target cells, in order to perform genetic manipulation, must be found. In some cases, this task is simple; for example, the induction of 'pseudocompetence' in *E. coli* by pretreatment of cells with Ca$^{2+}$ or other divalent metal ions (Hanahan, 1983). In other cases, extreme measures must be taken. One approach, used first for the transformation of certain Bacilli (Chang & Cohen, 1979) and Lactobacilli (Boizet et al., 1988; Morelli et al., 1987), is to remove the bacterial cell wall, which is thought to present a barrier to DNA, with lytic enzymes. Transforming DNA can then be introduced into the resulting 'protoplasts' by the action of a fusant, such as polyethylene glycol (PEG). Walls are then induced to reform on protoplasts by growing them on regeneration media. Such methods tend to take time to develop and perform and they are usually restricted in scope to the specific strain for which they were developed.
Recently the situation has changed radically. The technique of 'electroporation' - the formation of holes or pores in the cell membrane by high voltage electricity - has found widespread application. Unlike other methods of gene transfer (with the exception of microinjection or protoplast fusion), electroporation is a physical, rather than a biochemical technique and this probably accounts for its wide applicability. Table 1.8 (Chapter 1, page 56) reviews the bacteria including many Lactobacillus sp. that have been successfully electroporated, to date.

7.2 Experimental considerations.

For practical reasons, capacitor discharge circuits are most commonly employed to reproducibly deliver pulses of an intensity and duration suitable for electroporation. The pulse generator shown in Figure 7.1 generates an electric pulse characterised by an exponential decay waveform.

The parameters of central importance for bacterial electroporation are:

(i) the initial electric field strength \(E_0\), and
(ii) the time constant \(T\).

The electric field strength is the applied voltage divided by the distance between parallel electrodes in the sample chamber and can be adjusted, either by setting the voltage output of the power supply, or by using cuvettes with a
Figure 7.1 Components of the Gene Pulser electroporation apparatus - as shown in part A.
(from Miller, 1988).

A capacitor \( C_1 \) is charged to the desired voltage when switch \( S_1 \) places it in series with the power supply. Discharge of the capacitor across the pulse controller and sample results in an electric field that decays exponentially as shown in part B. The rate of decay is inversely proportional to the time constant \( T \). \( T \) (in seconds) is defined as the product of the total resistance \( (R_T, \text{in ohms}) \) and capacitance \( (C_1, \text{in farads}) \). \( T \) is also a measure of pulse duration and is equal to the time required for \( E_0 \) \( (\text{the initial electric field}) \) to decline to \( E_0/e \).
particular gap distance. The time constant is a measure of the duration of the discharge curve. As shown in Figure 7.1, \( T \) is equal to the time required for the initial electric field \( (E_0) \) to decay to approximately 37% of its original value.

Using data from a variety of bacterial systems, it is possible to draw some general conclusions concerning the electrical parameters important for electroporation. With short pulses, increases in field strength lead to increased transformation efficiency. Similarly, at low field strength, increased pulse lengths are usually beneficial. In general, electric field strength and time constant have compensatory effects. Higher voltages require shorter time constants and longer pulses reduce the voltage requirement (Miller et al., 1988; Dower et al., 1988).

Experiments with \( E. \) coli have shown that the frequency of transformation depends on the DNA concentration; the yield of transformants is a product of this frequency and the number of cells present (Dower et al., 1988). Thus, high efficiency (tfs/µg) transformation of \( E. \) coli is partly due to the use of very concentrated cell preparations \( (4 \times 10^{10} \text{ ml}^{-1}) \) and small volumes \( (40 \mu l) \), thereby maintaining a high DNA concentration. These conditions are likely to be useful for \( Lactobacillus \) sp.
Little information is available on the effects of plasmid size and topology on electroporation efficiency and a clear cut relationship between plasmid size and transformation by electroporation has not been observed. Large plasmids, exceeding 25 Kb, have been efficiently introduced into \textit{Lactobacillus} and \textit{Streptococcus} species (Chassy & Flickinger, 1987; Labigne-Roussel et al., 1987). DNA molecules introduced into cells by electroporation appear to be intact and free of rearrangements.

The cell surfaces of Gram-positive and Gram-negative bacteria are complex structures that must be traversed by transforming DNA. Gram-positive organisms surround their cytoplasmic membrane with a thick, rigid, highly cross-linked peptidoglycan cell wall, and this appears to present a partial barrier to incoming DNA. Although \textit{S. lactis} and \textit{L. casei} have been electroporated at efficiencies ranging from $10^4$ to $10^5$ tfs/µg, transformation of the former species is increased several-hundred fold by mild treatment of the cells with lysozyme (Powell et al., 1988). This may be a general technique useful for \textit{Lactobacillus} sp.

It has been seen that cells harvested in the early to mid-exponential growth phase are generally optimal for electroporation (Miller et al., 1988; Powell et al., 1988; Taketo, 1988; Labigne-Roussel, 1987), although variable responses have been reported.
Finally, components of the electroporation medium have a pronounced effect on the efficiency of transformation. Ionic strength, osmolarity, pH and other parameters may require adjustment for optimal effects on survival and efficiency. Therefore, an investigation of parameters influencing the frequency of transformation of *Lactobacillus leichmannii* was carried out. Parameters evaluated were; voltage, DNA concentration, type of buffer and buffer strength.

### 7.3 Experimental details.

Cells for electroporation were propagated in MRS media (Oxoid) and harvested at an $A_{600} = 0.7$ (approximately $4 \times 10^7$ CFU ml$^{-1}$). Electroporation of *L. leichmannii* was carried out using a ‘Gene Pulser’, supplied by Bio-Rad Laboratories U.S.A., set at 25 $\mu$F. Electrode gaps of either 0.4 cm or 0.2 cm were used, providing field strengths of up to 6.25 KVCm$^{-1}$ and 12.5 KVCm$^{-1}$ respectively. When the 0.2 cm cuvettes were used the output of the pulse generator was directed through a Pulse Controller unit (Bio-Rad) containing a high power, 20 $\Omega$ resistor in series with the sample, and a selection of resistors of 100 to 1000 $\Omega$ in parallel with the sample. The effective resistance placed in parallel with the electrodes is much lower than that of the sample and determines the time constant of the pulse. The pulse controller was set at 200 $\Omega$ for these studies.
7.3.1 0.4 cm electrode gap.

800 µl of cells were transferred to a cold 1.5 ml polypropylene tube, 0.5-1 µg DNA solution (in a low ionic strength medium; TE) was added, the suspension mixed vigorously and left on ice for 10 mins. The cell/DNA mixture was placed between the chilled electrodes, the cuvette placed in the safety chamber and the appropriate pulse applied. Following the pulse, the cells were left on ice for a further ten minutes. Cells were appropriately diluted and plated onto selective MRS agar to screen for transformants.

7.3.2 0.2 cm electrode gap.

Only 40 µl of cells were used and there was no chilling on ice before and after electroporation. Following the pulse, 960 µl of MRS media was added to the cells and selective agar used to screen for transformants.

In both cases, after dilution of the cells, the samples were incubated for 1 hour at 37°C to allow for expression of antibiotic resistance genes. Transformation efficiency was calculated as CFU/µg of plasmid DNA added. Dilutions were plated on non-selective agar to assess cell survival. Transformation frequency was calculated as transformants/survivors.
7.4 Results and Discussion.

7.4.1 Transformation by electroporation with plasmid DNA.

The vectors pSA3 (Dao & Ferretti, 1985), pC194 (Horinouchi & Weisblum, 1982) and pCK1 (Gasson & Anderson, 1985), but not pAMβl (LeBlanc & Lee, 1984) transformed *L. leichmannii* to chloramphenicol resistance by electroporation. Plasmid DNA was isolated from transformants (as described in Section 2.6.1) and analysed by agarose gel electrophoresis (Figure 7.2). Restriction enzyme analysis confirmed the pSA3, pC194 and pCK1 restriction patterns of the 'new' plasmids appearing in the transformed *L. leichmannii* (data not shown).

7.4.2 Effect of voltage on survival and transformation.

Variation of voltage had an effect on both survival and transformation efficiency and frequency. As can be seen in Table 7.1, at 2500 Vcm⁻¹, 83% of the starting CFU count was recovered and the transformation frequency and efficiency were low. Increasing the voltage to 6250 Vcm⁻¹ raised the efficiency and frequency, while slightly lowering the survival to 68%. Again, increasing the voltage to 7500 Vcm⁻¹ raised the efficiency and frequency and the survival rate dropped to 50%. Higher voltages resulted in higher survival CFU scores but no transformants were observed. Chassy and Flickinger (1987) found this same phenomenon with voltages
Figure 7.1  Agarose gel electrophores of plasmids isolated from *L. leichmannii* 4797.

Plasmid DNA was isolated from *L. leichmannii* transformed with the vectors pSA3, pC194 and pCK1 separately and electrophoresed through a 1% agarose gel.

Key to tracks:

- A pCK1 transforming DNA
- B pCK1 transformant DNA
- C pC194 transforming DNA
- D pC194 transformant DNA
- E pSA3 transforming DNA
- F pSA3 transformant DNA
- G λ HindIII DNA markers
Table 7.1  Transformation of *L. leichmannii* by electroporation.

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<td>CFUobs</td>
<td>CFUobs/μg DNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CFUtot</td>
<td>CFUrec</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAMβ1</td>
<td>2.50</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>5.00</td>
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<td></td>
<td>6.25</td>
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<td>7.50</td>
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<td>10.00</td>
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<tr>
<td></td>
<td>12.50</td>
<td>-</td>
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</tr>
</tbody>
</table>

**CFU_{tot}** = total colony forming units used in the electroporation experiment, as determined by dilution and plating on MRS agar plates.

**CFU_{rec}** = colony forming units recovered after electroporation, dilution and plating.

**CFU_{obs}** = colony forming units observed as antibiotic resistant on selective plates. Equals total number of cells transformed by electroporation.
of 6750 Vcm\(^{-1}\) and above with \(L.\) \textit{casei}. They suggested this may be due to electroporation-induced 'dechaining' of \(L.\) \textit{casei} cells, rather than a net increase in the number of viable cells.

7.4.3 Effect of DNA concentration.

To determine the effect of DNA concentration, different concentrations of pC194 were mixed with \(L.\) \textit{leichmannii} 4797 and electroporated. As shown in Figure 7.3, the total number of transformants increased progressively from 0.1 to 5 µg. Transformants were not recovered if pC194 was not added to cells or if the cell–DNA mixture was plated prior to delivery of the electric pulse. The transformation efficiency, however, was greatest using 2 µg total DNA, even though the total number of transformants continued to increase with increasing concentrations of pC194.

7.4.4 Effect of buffer type.

Electroporation was carried out with three different electroporation buffers. 'HEB' = 7 mM N-2-hydroxy-ethylpiperazine-N’-2-ethanesulfonic acid (HEPES), pH 7.4; 272 mM sucrose, 1 mM MgCl\(_2\). 'PEB': 7 mM Potassium phosphate, pH 7.4, 1 mM MgCl\(_2\), 272 mM sucrose. 'EB': 10% glucose, after washes in distilled water. The lower-resistance buffer (PEB) generated time constants (decay time from peak voltage to approximately 37% of peak voltage)
Figure 7.3  The effect of pC194 DNA concentration on the number of transformants of L. leichmannii 4797.

![Graph showing the effect of pC194 DNA concentration on the number of transformants of L. leichmannii 4797.]
ranging from 3-5.3 msec and the higher-resistance buffer (HEB) produced time constants ranging from 5.3-14.4 msec.

7.4.5 Effect of buffer strength.

The effect of varying the buffer strength on transformation frequency is shown in Figure 7.4. Both HEB and PEB yielded more transformants at buffer strengths greater than 1X. The best buffer concentrations for HEB (1.25X) and PEB (1.75X) resulted in approximately the same frequency of transformation.

7.5 Summary and Overview.

Electroporation has become increasingly popular as a tool for transforming bacterial cells. Although it is much simpler and faster than previous transformation methods using such chemicals as CaCl₂ (Hanahan, 1983) and polyethylene glycol (Kondo & McKay, 1984), reported electroporation efficiencies have been quite variable for bacteria, ranging from 0 to \(10^9\) transformants per µg of DNA.

This study has dealt with several parameters; final concentrations of cells have been suspended in a variety of buffers and buffer strengths, pulsed at field strengths ranging from 2.5 to 12.5 KVcm⁻¹ and with a range of DNA concentrations. The capacitance (25 µF) and resistance
Figure 7.4  Transformation frequency as a function of buffer strength.

Buffer strengths (x-fold) were obtained by proportionally increasing or decreasing all buffer components in HEB or PEB. Transformation frequencies obtained with *L. leichmannii* 4797 using HEB (Δ) and PEB (O).
were kept constant, since these particular values have been used in several successful bacterial electroporations to date.

The factors most likely to affect 'electrotransform-ability' of a bacterium are the membrane composition and the wall thickness, density and structure. Gram-positive bacteria have denser and thicker cell walls than Gram-negative bacteria, and, in general, Gram-negative strains electroporate more efficiently. Limited digestion of the cell wall with lysozyme has been shown to increase transformation efficiencies several-hundred fold (Powell et al., 1988) and this technique may be required to increase the transformation frequencies of L. leichmannii. Another factor, which may help in increasing the frequency is growth of the cells in the presence of glycine (Haynes & Britz, 1989).

However, to summarise briefly, cells of L. leichmannii were transformed to chloramphenicol resistance by the vectors pSA3, pC194 and pCK1 at efficiencies of 0.05 - 2.8 x 10^2 transformants per μg DNA. Approximately 50% of the CFU survived pulses of 7.5 KVcm\(^{-1}\) at 25 μF, conditions found to be optimal for the transformation process. Plasmid DNAs isolated from pSA3, pC194 and pCK1 transformants were indistinguishable from authentic preparations, by gel electrophoresis.
CHAPTER 8
Chapter 8  General Conclusions and Outlook.

8.1  Summary of the Main Results.

The initial aims of this project included the isolation and cloning of a gene encoding the Vitamin \( B_{12} \) binding protein from *Lactobacillus leichmannii*, followed by expression analysis of the cloned gene and the determination of its nucleotide sequence.

*L. leichmannii* genomic DNA sequences bearing homology to the gene encoding the Vitamin \( B_{12} \) receptor protein in *E. coli* (*btuB*) were thought to have been cloned into a high copy number vector, pBR327, using a heterologous hybridisation approach. Expression analysis, using both *in vivo* and *in vitro* systems appeared to confirm this and the data indicated that the protein encoded by the cloned fragment had the same molecular mass as the *BtuB* protein of *E. coli*. However, problems were encountered throughout, with the clumping of cells in liquid culture and the spurious results obtained with restriction endonuclease mapping, which suggested that the recombinants obtained, pSDC1 and pSDC2 contained DNA which had possibly undergone re-arrangements and/or deletions. It appeared that a cross-over event had occurred between the *E. coli* HB101 host strain and the cloned *L. leichmannii* DNA, resulting in pSDC1 and pSDC2 containing the *E. coli btuB* gene and not any homologous sequences from *L. leichmannii*. This theory was confirmed by
the data generated from the initiation of nucleotide sequencing of the 2.0 Kb DNA insert.

Different high and low copy number vectors were used, in an attempt to clone DNA sequences homologous to the synthetic mixed oligonucleotide probe (derived from the *E. coli* *btuB* gene), since it was subsequently determined that the B$_{12}$ binding protein in *L. leichmannii* was only a third of the size of that in *E. coli*. A 2.0 Kb *HindIII* *L. leichmannii* genomic DNA fragment was subsequently cloned into pBR325, where it was found to be stably maintained and had not undergone any re-arrangements during the cloning procedure. Nucleotide sequence data revealed three putative open-reading frames, one of which was variable in length. The first putative open-reading frame (ORF 1) was probably not complete as the presumptive initiator codon (ATG) may encode an internal methionine and the true initiator codon could be located further upstream on the *L. leichmannii* genome. This fact remains to be elucidated and would require further cloning and sequencing work to be carried out. The data generated from the nucleotide sequence of ORF 1 suggests that a degree of homology exists between the C-terminus of the *E. coli* *btuB* gene and the C-terminus of ORF 1 at the amino acid level, which looks very promising since, Moir et al. (1987) presented evidence that the C-terminus was responsible for the binding of the Vitamin.

The functions of ORF 2 and ORF 3 remain to be elucidated.
The cloned *L. leichmannii* 2.0 Kb genomic DNA insert was subjected to expression analysis. It was shown not to complement the *btuB* lesion in *E. coli* L1-431, although this may be due to the inability of phage BF23 to undergo replication in *L. leichmannii*. A way of testing for this would be to radiolabel the phage to determine whether the phage can attach to the cell, i.e. if the B₁₂ binding protein in *L. leichmannii* can act as a receptor for phage BF23, as does BtuB in *E. coli*.

The B₁₂-binding protein was purified from the cell walls of *L. leichmannii* and polyclonal antibodies raised to it. In theory, this antisera could then be used as a ‘probe’ to detect the polypeptides encoded by the cloned *L. leichmannii* 2.0 Kb genomic DNA fragment by means of an *in vitro* expression system. However, on analysis, it appeared that the peroxidase-conjugated system used did not have the sensitivity desired. It is known that the BtuB protein in *E. coli* is expressed by a weak promoter and therefore the protein transferred to the nitrocellulose filter was not present in sufficient amounts to be detected by the Western blot analysis system used.

To achieve the final aims of this project, it was necessary to develop a transformation system for *L. leichmannii* that was rapid and easy to perform. Electroporation — the technique of introducing transient holes or pores in the membranes of cells, to allow DNA molecules to enter — is fast becoming a generalised method for the transformation of
bacteria, although some bacteria still remain refractory to it.

Three plasmid vectors; pSA3 (Dao & Ferreti, 1985) which has previously been shown to replicate in Lactobacilli, pC194 (Horinouchi & Weisblum, 1982) and pCK1 (Gasson & Anderson, 1985) were found to transform *L. leichmannii* 4797 to chloramphenicol resistance by electroporation although the frequency of transformation observed was low. The method now requires optimisation. A range of electrical field strengths had the most marked effect on the efficiency of transformation, which was to be expected. This result paves the way for future molecular genetics with *L. leichmannii*.

8.2 Future work.

One of the main aims not achieved within the duration of this project was the site-specific mutagenesis of the gene encoding the B₁₂ binding protein in *L. leichmannii*. This would have enabled the specificity of a particular strain to be altered so that several strains of *L. leichmannii* would be specific to each of the different cobalamin analogues. However, it can be seen from these studies that much of the ground work has been carried out. Cloning of a larger *L. leichmannii* genomic DNA insert, containing the whole of ORF 1 would be necessary initially. Nucleotide sequence derived-amino acid sequence of ORF 1 could be compared to the N-terminal amino acid sequence data and site-specific mutagenesis of the gene achieved by the substitution of
synthetic oligonucleotides. Results could be evaluated by growth of the *L. leichmannii* strain harbouring the modified gene (introduced by electroporation) in the presence of different cobalamins. Since *L. leichmannii* requires B₁₂ to grow, evaluation would require only simple absorbance measurements.

These studies have reached a very interesting point and further work along the lines suggested could result in a new and exciting challenge to the Vitamin B₁₂ deficiency assay system in current use.
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Lactobacillus leichmannii as a probe for the quantitation of Vitamin B-12.

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