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

by

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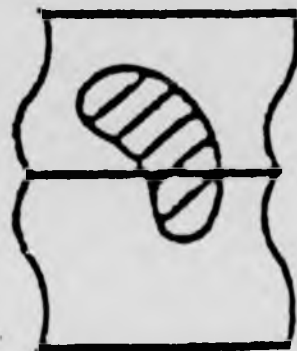
DEDICATION

To my husband, Don,
whose love and encouragement has kept me going
and made this all possible.

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ACKNOWLEDGEMENTS

Finally, I would like to say a big 'thank-you' to
Mrs. Carol Howes for her speed and accuracy in the typing
and presentation of this thesis.

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ABREVIATIONS

A	Absorbance
ATP	Adenosine 5'-triphosphate
bp	Base pairs
$btuB_E$	*btuB* gene from *Escherichia coli*
$btuB_S$	*btuB* gene from *Salmonella typhimurium*
$^\circ$C	Degrees celsius
CFU	Colony forming units
cm	Centimetre
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytosine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dUTP	2'-deoxyuracil 5'-triphosphate
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Diaminoethane tetraacetic acid
xg	Gravitational force
g	Gramme
h	Hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG	β-D-thiogalactopyranoside
Kb	Kilobase
M	Molar
μg	Microgram
mg	Milligram
mM	Millimolar

μF	Microfarads
M9	Mineral salts medium
M_r	Relative molecular mass
min	Minute
nM	Nanometre
NTG	N-methyl-N-nitro-N-nitrosoguanidine
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
PPO	2,5-diphenyloxazole
%	Percentage
rpm	Revolutions per minute
sp.	Species
subsp.	Sub-species
sec	Second
SDS	Sodium dodecyl sulphate
ss	Single strand
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris-hydroxymethylaminomethane
Tn	Transposon
Tween 20	Polyoxyethylenesorbitan monolaurate
UV	Ultraviolet radiation
v/v	Volume by volume
w/v	Weight by volume
X-Gal	5-Bromo-4-chloro-3-indoxyl β-D-galactoside

SUMMARY

Initial attempts to isolate the gene encoding Vitamin B_{12} receptor protein (btuB) from *Lactobacillus leichmannii*, resulted in the isolation of the 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 *Hind*III 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 re-arrangements. 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.

CHAPTER 1 - INTRODUCTION

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 cobalamins will play an important part".

<div align="right">

Linnell and Matthews, 1984.

</div>

In 1963 Lindstrand and Ståhlberg, using the technique of
chromatography and bioautography, showed that human sera or
plasma contained several cobalamins, 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 cobalamins 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 B$_{12}$.

Vitamin B$_{12}$ 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 B$_{12}$ 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 B$_{12}$, 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 B$_{12}$, 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).
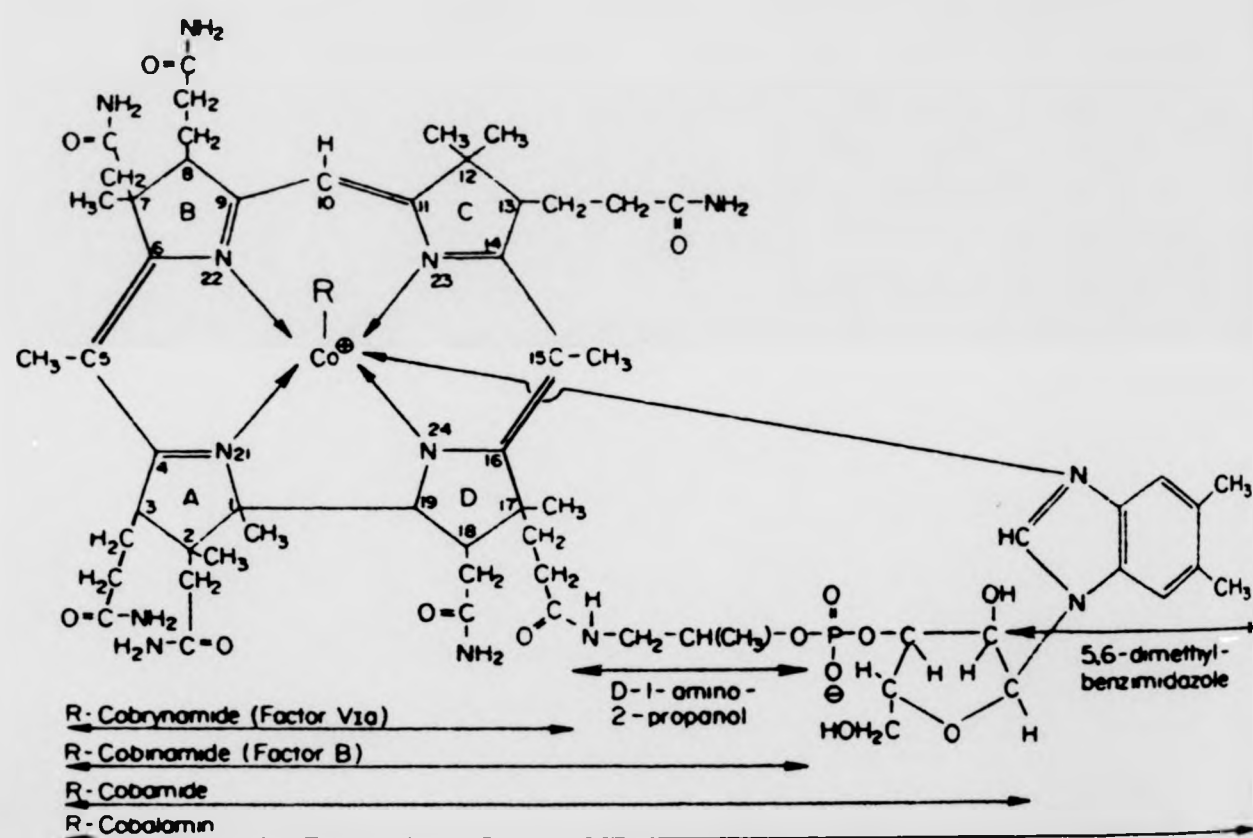
-R	PERMISSIVE NAME
-CN	cyanocobalamin
-OH	hydroxcobalamin
-H$_2$O	aquacobalamin
-NO$_2$	nitritocobalamin
-CH$_3$	methylocobalamin
5'-deoxyadenosyl	5'deoxyadenosylcobalamin (coenzyme B$_{12}$)

R-Cobrynomide (Factor Vio)
R-Cobinamide (Factor B)
R-Cobamide
R-Cobolamin

D-l-amino-2-propanol

HOH$_2$C

5,6-dimethyl-benzimidazole

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 B_{12} coenzymes to sustain activity and viability. Although more than ten B_{12}-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 B_{12}: 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.

Systematic name abbreviation	Common name	Occurrence and biological activity	Structure and formula
CN-Cbl	Cyanocobalamin, Vitamin B_{12}, Factor II	Artificial product obtained from other cobalamins by addition of KCN. Organisms convert it quickly to other forms, mainly aq-Cbl, CH_3-Cbl and Ado Cbl (Ellenbogen, 1974). Active in man, animals and microorganisms. Not active in enzyme systems.	$C_{63}H_{89}O_{14}N_{14}PCo$ M_r of 1355.5

Systematic name abbreviation	Common name	Occurrence and biological activity	Structure and formula
aq-Cbl	Aquacobalamin Vitamin B$_{12}$	Produced by microbes, occurs in man, animals, sewage sludge, soil and plant roots.	$C_{62}H_{91}O_{15}N_{13}PCo$ M_r of 1347.5
OH-Cbl	Hydroxcobalamin Vitamin B$_{12}$	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.	$C_{62}H_{90}O_{15}N_{13}PCo$ M_r of 1346.5

Systematic name abbreviation	Common name	Occurrence and biological activity	Structure and formula
Ado-Cbl	Vitamin B$_{12}$ coenzyme CoB$_{12}$	Present in man, animals and microbes. Obtained by chemical and enzymatic synthesis from CN-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	$C_{72}H_{101}O_{17}N_{18}PCo$ M_r of 1579.6

Systematic name abbreviation	Common name	Occurrence and biological activity	Structure and formula
Me-Cbl	Methylcobalamin Vitamin B_{12}	Present in man and animals (Lindstrand, 1964). In microbes, present in various amounts depending on species and growth conditions. Co-factor for enzymes involved in hydrogen transfer in bacteria, higher plants, animals and man (Stadtman, 1971).	$C_{63}H_{92}O_{14}N_{13}PCo$ M_r of 1344.5

Finally, a word about terminology, since some authors use the more familiar 'Vitamin B_{12}', 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 B_{12}.

Vitamin B_{12} 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 B_{12} 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 B_{12} was obtained in pure crystalline state in 1948 and its structure was elucidated in the following years. The production of Vitamin B_{12} on an industrial scale in the early fifties enabled its world-wide application in medicine to treat pernicious anaemia.

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 B_{12} 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 B_{12} deficiency. They are, in addition, particularly valuable in the diagnosis of Vitamin B_{12} neuropathy.

Table 1.2 **Nutritional requirement of _Lactobacillus leichmannii_ (ATCC 7830) for Vitamin B$_{12}$ (cyanocobalamin) of a deoxyribonucleoside.**

(taken from Beck, 1983).

Additions to assay medium	Turbidity[a]
None	0
Cyanocobalamin	127
Thymidine	105
Deoxycytidine	119
Deoxyadenosine	92
Deoxyuridine	100
Deoxyguanosine	112
Deoxyribose	0
Cytidine	0
Adenosine	0
Uridine	0
Guanosine	0

a   Turbidity was determined in a Klett colorimeter (Filter 66) after cultivation for 8 hours.   Final concentrations of additions were: cyanocobalamin 0.5 ng ml^{-1}, deoxyribonucleosides and ribonucleosides 2.5 μg ml^{-1} and deoxyribose 5.0 μg ml^{-1}.   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 B_{12} 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 B_{12} analogues in addition to those detected with an IF assay. Since then, it has been shown that the forms of B_{12} 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 $[^{57}Co]$ B_{12} from aqueous solution. IF was more effective than R-binder in removing $[^{57}Co]$ 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_{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_{12} (with B_{12} deficiency defined independently of a serum B_{12} assay). When laboratory assay results differ from clinical

A

B

C

D

Figure 1.2   **Plasma cobalamins separated by two-dimensional chromatography and bioautography**. (taken from Linnell & Matthews, 1984).
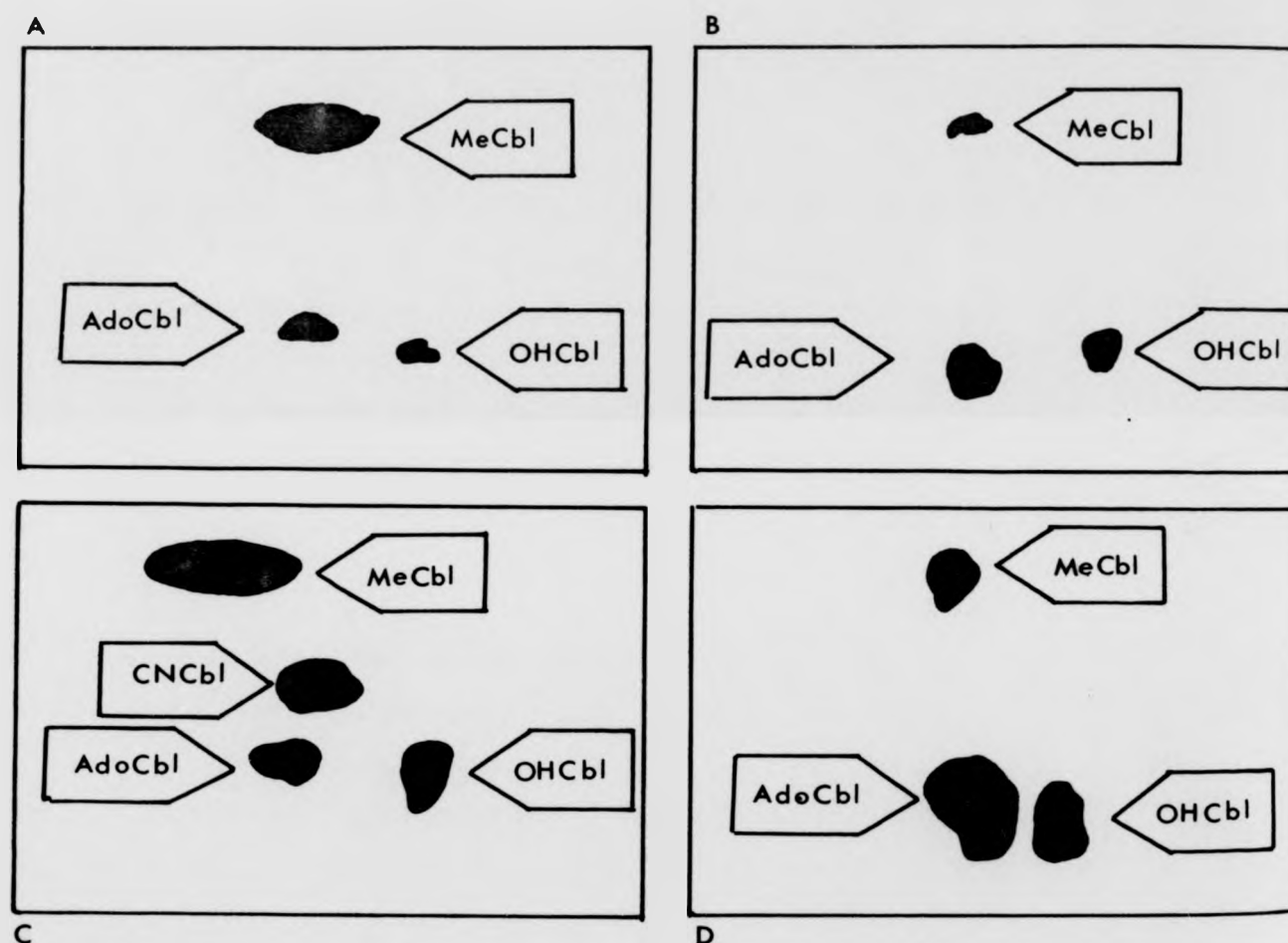
(a)   Normal subject (total Cbl 460 pg ml^{-1}).

(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_{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_{12} in these products and other foods. Microbiological assays using *L. leichmannii* or other microorganisms are the most widely used methods for measuring Vitamin B_{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_{12} in serum (Pratt & Woldring, 1982).

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

1.4 Non-enzymatic Vitamin B_{12} binding proteins.

The absorption and transport of Vitamin B_{12} in man and animals is exclusively mediated by proteins. That Vitamin B_{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 B_{12} 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 B_{12}-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 B_{12}-binding β-globulin is primarily a transport protein (Retief *et al*, 1967), whereas the B_{12} binding α-globulin functions mainly to conserve the vitamin. B_{12} binding α-globulin has a greater affinity for B_{12} and retains the vitamin more tenaciously than does the B_{12} 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 B_{12}; 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 B_{12} transport proteins (Hall, 1969). The other B_{12} transport protein of human plasma known at the time, then referred to as the B_{12} binding protein, but now known as TCI, did not take up much of the recently absorbed B_{12}. This led to the concept of two transport proteins carrying B_{12} 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}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

Figure 1.3   **Proposed pathway of uptake of cobalamin by**
E. coli.
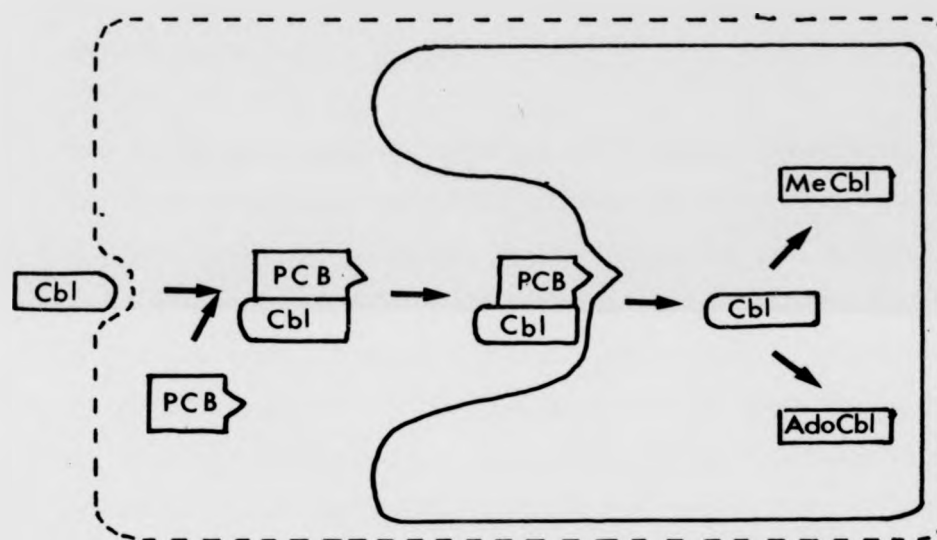
The outer membrane of the bacterial envelope is shown by the
dashed line; the inner membrane by the solid line; and the
periplasmic space by the space between the two membranes,
expanded at left.   PCB, periplasmic cobalamin binding
protein; AdoCbl, adenosylcobalamin; MeCbl, methylcobalamin.
See text 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 *btu*B.

1.5.1 BtuB in *E. coli*.

The *btu*B 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 *ton*B 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 *btu*B encoded B_{12} receptor is *ton*B dependent, the entry of the E colicins and phage BF23, which use the same receptor is not (Bassford *et al*, 1976; Bradbeer *et al*, 1976; Davies & Reeves, 1975). These activities seem to be only transitory *in vivo* and restricted to a particular sub-class of BtuB proteins; most probably those which are newly synthesized and not evenly distributed within the outer membrane. However, cells retain full activity for Vitamin B_{12} uptake (Kadner & McElhaney, 1980).

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

Previous studies have suggested that Vitamin B_{12} is involved in the repression of the *btu*B gene (Kadner, 1978). Growth in the presence of 100 nM B_{12} reduces the B_{12} uptake by as much as 90%. BtuB is usually found at a low level (200 - 300 copies per cell) when Vitamin B_{12} is omitted from the culture media (Di Masi *et al*, 1973).

There is also good evidence to suggest that B_{12}, in the presence of the genes encoding methyltransferase, *met*H and *met*F, represses the gene encoding homocysteine transmethylase (*met*E) (Mulligan *et al*, 1982). The methionine (*met*) regulon of *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 *btu*B 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 *btu*R, required for repression of *btu*B. It appears to encode a repressor of *btu*B transcription, but has no apparent role in the biosynthesis of methionine or in the

transport of Vitamin B_{12}. The gene is not located near any of the genes of methionine or Vitamin B_{12} transport. Cellular levels of the BtuR repressor and the location of the operator site on the cloned *btu*B region are presently under investigation.

Earlier this year Lundrigan and Kadner published results to suggest that BtuR does not in fact regulate *btu*B 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 *btu*B) 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 *btu*B 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 B_{12} uptake suggested a common region on the *btu*B 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 *Pst*I deletion of their cloned *btu*B gene and yet it was still capable of imparting sensitivity to phage BF23 despite being defective in Vitamin B_{12}-binding activity.

Moir and colleagues (1987) found that altering the C-terminus of the *btu*B 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 *btu*C and *btu*D, two genes in the *btu*CED operon.

1.5.2 BtuCED operon in *E. coli*.

The *btu*CED 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 *btu*E might be the periplasmic CN-Cbl binding protein. However, Rioux and Kadner (1989a) have examined the role of the *btu*E gene and despite its genetic location in the transport operon, the *btu*E 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 *btu*C gene, has been found to influence Vitamin B_{12} uptake or utilisation. The *btu*C function is required for the growth response to Vitamin B_{12} when the outer membrane transport process (*btu*B and *ton*B function) is defective. However, even in a wild-type strain, *btu*C is required for proper transport of Vitamin B_{12} (DeVeaux & Kadner, 1985).

In contrast to the genes *btu*B and *ton*B, *btu*C 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 *et al*, in 1971, who showed that such strains, which were also *met*E, 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 *ton*B gene product are involved in the release of cobalamin from the outer membrane receptor and that the *btu*C gene product is necessary for cobalamin transport across the inner membrane.

Mutations in *btu*D confer a less extreme deficiency in Vitamin B_{12} utilisation than do *btu*C mutations, although transport was strongly impaired (DeVeaux & Kadner, 1985).

The *btu*CED region may encode a transport system for passage of Vitamin 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 *btu*C, *btu*D and *btu*E 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 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 *btu*E showed that the absence of the *btu*E 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 *btu*E mutants is likely to be encoded outside *btu*CED.

They also found an error in the published sequence of *btu*C. 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 *met*H gene) for which a cobalamin-independent alternative (encoded by the *met*E 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 *btu*CED.**

(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 *Sma*I site located at the 3' end of *him*A. 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 B_{12} 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. colicins (Guterman *et al*, 1975). Resistance to these lethal agents results from mutations in *bfe* (susceptibility to phage BF23 and the E. colicins), which is located at the position on the *S. typhimurium* chromosome map analogous to that of the *E. coli btu*B gene. In the latest version of the *S. typhimurium* genetic map (Sanderson & Roth, 1988), the *bfe* locus was renamed *btu*B.

Rioux and Kadner (1989b), investigated the involvement of an outer membrane transport component for Vitamin B_{12} uptake in *S. typhimurium* analogous to the *btu*B product in *E. coli*. An M_r 60,000 protein in the *S. typhimurium* outer membrane was repressed by growth with Vitamin B_{12} and was eliminated in a *btu*B_S mutant. The *btu*B_S product thus appears to play the same role in Vitamin B_{12} transport by *S. typhimurium* as does the *E. coli btu*B_E 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 *ton*B 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 (*btu*B) of the Vitamin B_{12} receptor. Vitamin B_{12} was absorbed, but was not transported into cells carrying the *btu*B 451 mutation. This mutation could be suppressed by two mutations in *ton*B, 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 *fhu*A gene encodes an outer membrane protein which is an essential constitutent 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 *fhu*C 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 *Btu*D 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 *ton*B and possibly the *exb*B 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 1.3   **Industrial applications of** *Lactobacillus* **strains**.

(taken from Chassy, 1985).

Product	Organism
Yoghurt	*L. bulgaricus*
Fermented milks	*L. acidophilus*
	L. casei
	L. bulgaricus
Cheeses	*L. bulgaricus*
	L. helveticus
Soy sauce	*L. delbrueckii*
Crackers	*L. plantarum*
Pickles	*L. plantarum*
Cured ham	*L. casei*
	L. plantarum
Sausages, meats	*L. plantarum*
	L. reuteri
Distillery mashes	*L. casei*
	L. fermentum
	L. plantarum
	L. delbrueckii

Product	Organism
Feed additives	*L. acidophilus*
	L. bulgaricus
	L. lactis
Silage starters	*L. plantarum*
Lactic acid	*L. delbrueckii*

Table 1.4 **Advantages of** *Lactobacillus* **strains for industrial processes**.

(taken from Chassy, 1985).

Methods already exist for large-scale cultivation

Non-pathogenic

No toxins or toxic products formed

Aerotolerant

No aeration required

Moderately thermophilic (51-54°C)

Can withstand low pH

Natural products discourage contamination and spoilage

Ferment diverse carbohydrate feedstocks (whey, silage, plant juices and hydrolysed starch)

Rapid and abundant growth

Cultures are stable and viable

Non spore-forming

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*

(Boizet *et al*, 1988; Shimizu-Kadota & Kudo, 1984; Chassy, 1987; Cosby *et al*, 1988).

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.

Organism	Reference
Streptomyces coelicolor	Hopwood & Wright, 1978
Bacillus subtilis	Chang & Cohen, 1979
Bacillus megaterium	Vorobjeva *et al*, 1980
Streptococcus lactis	Kondo & McKay, 1982
Clostridium acetobutylicum	Reid *et al*, 1983

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 *B. subtilis* i.e. PEG induced DNA uptake by protoplasts.

The following year, Morelli and colleagues reported a method for the transformation of *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 *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 pAMβ1 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.

pAMβ1 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), *Staphyloccus 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 pAMβ1 to *L. plantarum* by a filter mating method. The conjugal transfer frequency of pAMβ1 from Streptococci to Lactobacilli has been reported to be generally low. They examined various factors affecting the transfer frequency of

this plasmid from *S. faecalis* to *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 *S. faecalis* into
commercially used strains of lactic streptococci and from
these strains into two strains of *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 *L.*
helveticus and from *L. helveticus* back to *S. faecalis*.

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

(i)   a potentially broader host range than pAMβ1

(ii)   it carries two antibiotic resistant markers allowing
        the scoring of the unselected marker as evidence for
        plasmid transfer (Er^r and Cm^r).

(iii)   it has been the source of DNA used for the
        construction of potential cloning vectors (Behnke *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 1.6 <u>Conjugative transfer</u>

Organism transferred from	Vector/ Plasmid	Organism transferred to	Comments	Reference
S. avium	pAMβ1	*L. casei*	-	Gibson et al, 1979
Streptococcus sp.	pAMβ1 and pIP501	*L. plantarum*	-	West & Warner 1985
S. sanguis	pVA838 & pSA3	*L. plantarum*	Mobilised by pVA797 via cointegrate formation	Shrago & Dobrososz, 1988

Organism transferred from	Vector/ Plasmid	Organism transferred to	Comments	Reference
S. lactis	pAMβ1	L reuteri, L. acidophilus, L. salivarius	-	Vescovo et al, 1983
S. lactis	pAMβ1	L. reuteri, L. murinus, L. fermentum	-	Tannock, 1987
S. faecalis	pIP501	L. helveticus	via commercially used strains of lactic Streptococci	Thompson & Collins, 1988

Organism transferred from	Vector/ Plasmid	Organism transferred to	Comments	Reference
S. faecalis	pIP501, pVA797	L. plantarum	-	Shrago & Dobrososz, 1988
Lactococcus lactis	pIP501	L. helveticus, L. delbruckii	-	Langella & Chopin, 1989

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.

Plasmids are widely distributed among members of the genus *Lactobacillus* (Chassy *et al*, 1976; Chassy *et al*, 1978; Hofer, 1977; Ishiwa and Iwata, 1980; Klaenhammer, 1984; Klaenhammer & Sutherland, 1980; Morelli *et al*, 1983; Smiley & Fryder, 1978; Vescovo *et al*, 1981, 1982; Nes, 1984).

Lactose metabolism in *L. casei* (Chassy *et al*, 1978; Hofer, 1977) has been shown to be dependent on the presence of plasmids. The phospho-β-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^r) 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 1.7 _Lactobacillus_ DNA cloned in recent years.

Organism	Gene	Cloned	Nucleotide Sequenced	Expressed	Reference
L. casei	β-D-phospho-galactoside galactohydrolase gene	shot-gun clone bank	-	in _E. coli_	Lee et al, 1982
L. casei	dihydrofolate reductase gene	using pBR322	-	-	Davies & Gronenborn 1982
L. casei	dihydrofolate reductase gene	-	√	-	Andrews et al, 1985

Table 1.7 continued.

Organism	Gene/Description	Cloning	Sequencing	Expression	Reference
L. delbruecki	gene for malolactic fermentation of wine	using pBR322 in yeast & *E. coli*	-	in *E. coli* and *S. cerevisiae*	Williams et al, 1983, 1984
Lactobacillus 30a	Prohistidine decarboxylase	√	dideoxy sequencing	-	Vanderslice et al, 1986
L. bulgaricus	plasmid encoded β-galactosidase gene from *L. casei*	in *E. coli*	-	-	Flickinger et al, 1986
L. bulgaricus	β-galactosidase gene	√	√	√	Schmidt et al, 1989

Table 1.7 continued.

Organism	Gene/Description	Cloning	Sequencing	Expression	Reference
L. bulgaricus	gene encoding serine tRNA with the anticodon CGA	√	-	expressed & active in *E. coli*	Hottinger et al, 1987
L. casei	Thymidylate synthase gene	√	dideoxy sequencing	high expression & active in *E. coli*	Pinter et al, 1988
L. casei	Factor IIIlac gene	using pUC8 into *E. coli*	dideoxy sequencing	-	Alpert & Chassy, 1988

Table 1.7 continued.

L. casei	β-D-phospho-galactoside galactohydrolase gene	shotgun cloned	dideoxy sequencing	√	Porter & Chassy, 1988
L. bulgaricus	gene encoding (UCG) tRNAser	√	-	-	Hottinger et al, 1989
L. delbrueckii	gene encoding a minor (UCG) tRNAser	√	√	-	Zwahlen & Hottinger, 1989
L. casei	D-2-hydroxyiso-caproate dehydrogenase gene	using pBR322	dideoxy sequencing	expression in E. coli	Lerch et al, 1989

Table 1.7 continued.

L. helveticus subsp. jugurti	cryptic plasmid pLJ1	using pBR329	√	√	Takiguchi et al, 1989
L. bulgaricus	DNA fragment containing a 5S RNA gene adjacent to a cluster of five tRNA genes	√	-	-	Pittet & Hottinger 1989a
L. bulgaricus	Hexameric tRNA gene cluster associated with rRNA genes	-	dideoxy sequencing	-	Pittet & Hottinger, 1989b

'√' 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 Cm^r (from pC194) and Er^r (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.

Organism	Vector	Transformation/ Transfection	Reference
S. lactis	–	transformation	Harlander, 1986
L. casei	PL-1 (40 kb)	transfection	Chassy & Flickinger, 1987
	PLZ15 (28 kb)	transformation	Chassy & Flickinger, 1987
	pSA3 (Er^r) pLP825 (Cm^r) pNZ12 (Cm_r)	transformation	Chassy & Flickinger, 1987
E. carotovora	pBR329	transformation	Ito *et al*, 1988
L. plantarum	pTV1	transformation	Aukrust & Nes, 1988
L. plantarum	Tn917	(transposition)	Aukrust & Nes, 1988

E. coli	pUC18	transformation	Calvin & Hanawalt, 1988
S. cremoris	pMU1328 pMU1329	transformation	Powell *et al*, 1988
S. lactis LMO230 (now *Lactococcus* *lactis* subsp. *lactis*	pMU1328 pMU1329 pLS1 pAMβ1	transformation	Powell *et al*, 1988
E. coli	pUC12	transformation	Wilson & Gough, 1988
E. coli	α3RF M13mp19RF	transfection	Taketo, 1988
E. coli	pSA3 pBR322	transformation	Taketo, 1988
E. coli	QB phage RNA	transfection of RNA	Taketo, 1989
B. thuringiensis	pC194 pUB110	transformation	Bone & Ellar, 1989

Leuconostoc paramesenteroides	pAMβ1	transformation	David *et al*, 1989
Bordetella	pRK404 (broad host range cloning vector)	transformation	Zealey *et al*, 1988
Clostridium perfringens	pAMβ1 pHR106 (7.9 kb shuttle vector)	transformation	Allen & Blaschek, 1988
Clostridium acetobutylicum	pMTL500E (shuttle vector - Er^r gene and replication machinery of pAMβ1	transformation	Oultram *et al*, 1988
Lactococcus lactis subsp. *lactis*	pSA3 pGB301	transformation	McIntyre & Harlander, 1989a
S. pyogenes	pGK12 (Er^{r}Cm^r)	transformation	Suvorov *et al*, 1988

B. thuringiensis	pC194	transformation	Mahillon *et al,*
	pE194		1989
Brevibacterium	pUL340	transformation	Haynes & Britz,
lactofermentum			1989
Corynebacterium	pUL340	transformation	Haynes & Britz,
glutamicum			1989
B. thuringiensis	pX193	transformation	Schurter
B. cereus			*et al,* 1989
E. coli			
K. pneumoniae	pKT231	transformation	Wirth
Pseudomonas	(broad host-		*et al,* 1989
putida	range for gram		
Erwinia carotovora	negative bacteria,		
	expressing		
	Km^r & Sm^r		
L. acidophilus	pAMβ1		
	pC194		
	pGB354[a]	transformation	Luchansky
	pGKV1[b]		*et al,* 1988
	pSA3		
	pTRK13[c]		
	pTV1[d]		
	pVA797[e]		

Bacillus,			
Enterococcus,			
Lactococcus,	pGK12	transformation	Luchansky
Leuconostoc,			*et al,* 1988
Listeria,			
Pediococcus,			
Propionibacterium and			
Staphylococcus			
E. coli TG1	M13 DNA	transfection	Heery & Dunican 1989
Lactococcus lactis subsp. *lactis*	pGB301	transformation	McIntyre & Harlander, 1989b

a   6.2 kb, Cm^r (Behnke & Gilmore, 1981)

b   4.6 kb, $Cm^r Er^r$ (van der Vossen *et al*, 1985)

c   12.5 kb, Cm^r (Luchansky *et al*, 1988)

d   12.4 kb, $Cm^r Er^r$ (Youngman *et al*, 1983)

e   30.7 kb, $Cm^r Tra^+$ (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, Rodriquez-Quinones *et al*, (1982) reported NTG mutagenesis in *L. plantarum*. They showed that efficiency was good with optimal concentrations 500 μg 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

Strain	Genotype	Source	Reference
Lactobacillus leichmannii 8964	-	NCIB	White, 1958
Lactobacillus leichmannii 4797	-	ATCC	Bergey et al, 1925
Lactobacillus casei 7473	-	NCIB	
Lactobacillus plantarum NCDO 1752	-	P. Warner	West & Warner, 1985
Escherichia coli DH1	F$^-$, *rec*A1, *end*A1 *gyr*A96 *thi*-1, *hsd*R17 $(r_k M_k)$, *sup*E44. λ^-	D. Cardy	Hanahan, 1983
Escherichia coli HB101	F$^-$, *hsd*S20 (r_B, M_B), *rec*AB, *ara*-14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20 *xyl*-5, *mtl*-1, *sup*E44, λ^-	D. Cardy	Boyer & Roulland-Dussoiz, 1969

Strain	Genotype	Source	Reference
Escherichia coli CSH26ΔF6	F$^-$, *ara*, Δ(*lac-pro*), *thi*, *rpsL*, Δ(*recA-srl*) F6, *sup*^{o}, λ$^-$	D. Cardy	Jones & Holland, 1984
Escherichia coli TG1	(*pro-lac*),$^\nabla$*supE*, *thi*, F$^-$, *traO*, *proAB*$^+$, *lacI*^{q}, *lacZ*$^{\nabla 15}$	D. Cardy	Carter *et al*, 1985
Escherichia coli L1-431	*argF*40, *argR*11, *rpsL*40, *btuB*	J-P. Bohin	Aufrère *et al*, 1986
Escherichia coli NFB362	MC400, *argE*3, *rpoB*, *recA*6, *srl*-300 :: Tn10	J-P. Bohin	Aufrère *et al*, 1986

Table 2.2 **Plasmids**

Plasmid	Characteristics	Phenotype	Source	Reference
pBR327	Multicopy cloning vector	$Ap^r Tc^r$	D. Hodgson	Soberon *et al*, 1980
pBR325	Multicopy cloning vector	$Ap^r Tc^r Cm^r$	D. Cardy	Bolivar, 1978
pBR329	Multicopy cloning vector	$Ap^r Tc^r Cm^r$	D. Hodgson	Covarrubias & Bolivar, 1982
pAT153	High copy variant of pBR322	$Ap^r Tc^r$	C. Oakley	Twigg & Sherratt, 1980
pACYC184	Stringent cloning vector	$Tc^r Cm^r$	C. Oakley	Chang & Cohen, 1978
pNF48	High copy number vector harbouring *btu*B gene	$Ap^r Sm^r$	J. P. Bohin	Aufrère *et al*, 1986
pSDC1	Derivative of pBR327	Ap^r	-	This work

Plasmid	Characteristics	Phenotype	Source	Reference
pSDC2	Derivative of pBR327	Ap^r	–	This work
pSDC3	Derivative of pBR327	Ap^r	–	This work
pAMβ1	Broad host range plasmid	Er^r	M. Gasson	Clewell et al, 1974
pC194	High copy number plasmid	Cm^r	R. Saunders	Iordanescu, 1975
pSA3	E. coli-Streptococcus Shuttle vector	Tc^r $Cm^r Er^r$	M. Gasson	Dao & Ferretti, 1985
pSEC1	Derivative of pBR325	$Ap^r Cm^r$	–	This work
pSEC2	Derivative of pBR325	$Ap^r Cm^r$	–	This work
pBS	phagemid vector	Ap^r	I. Garner	Stratagene
pBS3	Derivative of pBS	Ap^r	–	This work

Plasmid	Characteristics	Phenotype	Source	Reference
pBS7	Derivative of pBS	Ap^r	-	This work
pCK1	High copy number plasmid	$Cm^r Km^r$	M. Gasson	Gasson & Anderson, 1985

Table 2.3 **Bacteriophage**.

Phage	Characteristics	Source	References
M13tg130	-	Amersham Int.	Kieny *et al*, 1983
M13tg131	-	Amersham Int.	Kieny *et al*, 1983
VCS-M13	helper phage	I. Garner	Stratagene
BF23	T_5-like phage	R. E. Glass	Heller, 1984

2.3.4 Antibiotics.

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

Antibiotic	Stock concentration $(mg\ ml^{-1})$	Final concentration $(\mu g\ ml^{-1})$
Ampicillin (Ap)	100	100
Chloramphenicol (Cm)	35	15
Kanamycin (Km)	50	50
Streptomycin (Sm)	20	20
Tetracycline (Tc)	20	20
Erythromycin (Er)	20	10

Antibiotic stock solutions were prepared as described in Maniatis *et al.*, 1982.

2.3.5 Chemicals used in this study.

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

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 mls 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, $4^{o}C$), 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 $37^{o}C$ 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 $37^{o}C$ 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, $20^{o}C$. 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)   10 g Bacto tryptone
 (per litre)   5 g Bacto yeast extract
                                    5 g NaCl (pH 7.2)

Phenol (neutralised, for DNA   Neutralised with 1 M
 extraction)   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)	60 g $Na_2 HPO_4$ anhydrous
(per litre)	30 g $KH_2 PO_4$ anhydrous
	10 g NH_4Cl
	5 g NaCl (pH 7.4)
M9 minimal medium	100 ml (x 10) M9 salts
(per litre)	10 ml 10 mM $CaCl_2$
	0.1 M $MgSO_4$
K medium	M9 minimal medium
(per litre)	50 ml 20% (w/v) Bacto
	casamino acids
	0.1 ml 0.1% (w/v) thiamine
	10 ml 20% (w/v) glucose
Western Transfer	14 g glycine
buffer (WTB)	2.42 g Tris
(per litre)	200 ml Methanol
Tris-buffered	20 mM Tris pH 7.6
saline (TBS)	0.15 M NaCl

Hershey salts	3.0 g KCl
(per litre)	5.4 g NaCl
	1.1 g NH_4Cl
	15 mg $CaCl_2$ $2H_2O$
	0.2 g $MgCl_2$ $6H_2O$
	0.2 mg $FeCl_3$ $6H_2O$
	87 mg KH_2 PO_4 anhydrous
	12.1 g Tris-HCl (pH 7.4)

Hershey medium	Hershey salts
(per litre)	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 $MgCl_2$, 1 mM $ZnCl_2$

    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 H_2O, 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 *Hind*III 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^+ (*Hind*III 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 A_{600} 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 $MgCl_2$.

Cell washing was repeated in 20 ml of 0.1 M $CaCl_2$ (Grade 1 $CaCl_2$ Sigma) and final resuspension left the cells in 2 ml of 0.1 M $CaCl_2$. 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 $42^{\circ}C$ 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 $37^{\circ}C$, 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.

X μl	DNA in TE (250 ng)
1 μl	1 mM dATP
1 μl	1 mM dTTP
1 μl	1 mM dCTP
1 μl	^{32}P-dGTP (10 μCi μl^{-1})
4 μl	10 x Nick translation buffer
0.8 μl	DNA polymerase I
1 μl	DNAse I (1 μl of 1 mg ml^{-1} stock in 50 μl H_2O, then 1 μl of above in 50 ml H_2O)
Y μl	H_2O

X and Y were adjusted to give 20 μl final volume.

The above constituents were incubated at 15°C for 3 hours.
Reaction was terminated by the addition of 5 μl 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 1-1½ 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.

% acrylamide	Bromophenol blue	Xylene cyanol
10	12 mer	55 mer
12	10 mer	46 mer
16	9 mer	35 mer
20	8 mer	28 mer

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 mls of elution buffer (10 mM Mg acetate, 300 mM NH_4 acetate, 0.1% (w/v) SDS and 1 mM EDTA) at 37^oC, 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 NH_4HCO_3 and oligo' eluted with 50 mM NH_4HCO_3/1 M NaCl. A_{260} 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 $MgCl_2$, 50 mM DTT), 2 μl of T_4 polynucleotide kinase and 2 μl (20 μCi) of [γ-^{32}P] ATP. The mixture was incubated for 45 min at 37^oC.

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 x SSC
     1 x Denhardts (Denhardt, 1966)
     200 μg ml^{-1} sheared, heat-treated Herring sperm DNA.
     Room temperature, 1 hour.

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

     3 x SSC
     1 x Denhardts
     100 μg ml^{-1} sheared, heat-treated Herring sperm DNA.
     45°C, 1 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 *Hind*III. 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 *Hind*III 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 [α-^{32}P]-labelled pNF48 1.5 kb *Eco*RI insert (see Figure 2.1) or [γ-^{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 x 10^8 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 μg 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 μl 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**.

pNF48
4100 bp
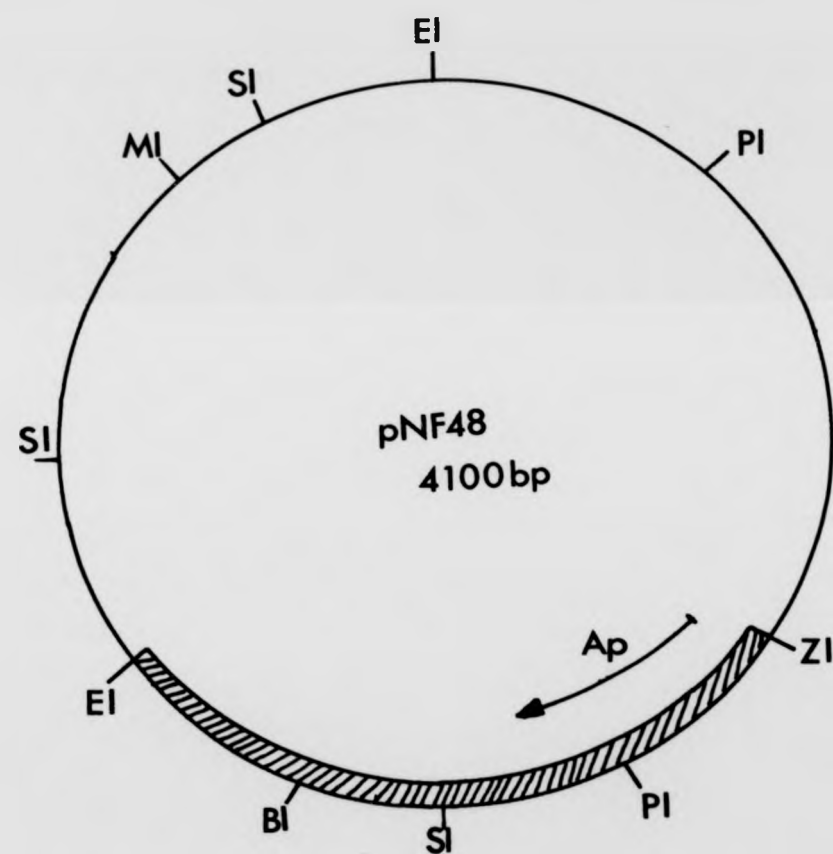
B1	*Bam*HI
E1	*Eco*RI
S1	*Sal*I
M1	*Mlu*I
P1	*Pst*I
Z1	*Sma*I
Ap	ampicillin resistance gene
⫽	pUC8

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 $MgCl_2$, 60 mM NH_4Cl, 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 A_{260} 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 $MgCl_2$ adjusted to 12 mM. Each assay contained 2 A_{260} units of S30, 2 μg plasmid DNA (added last), 8 μl synthesis mix (see below) and 2 μl [^{35}S]-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

folinic 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 $5\frac{1}{2}$ 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 NH_4 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 colE1 origin, ampicillin resistance, T3 and T7 promoters flanking the pUC19 polylinker and a *lac*Z 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 x $10^{11}/10^{12}$ 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 x 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

Figure 2.2 **Phagemid vector pBS (+/-).**
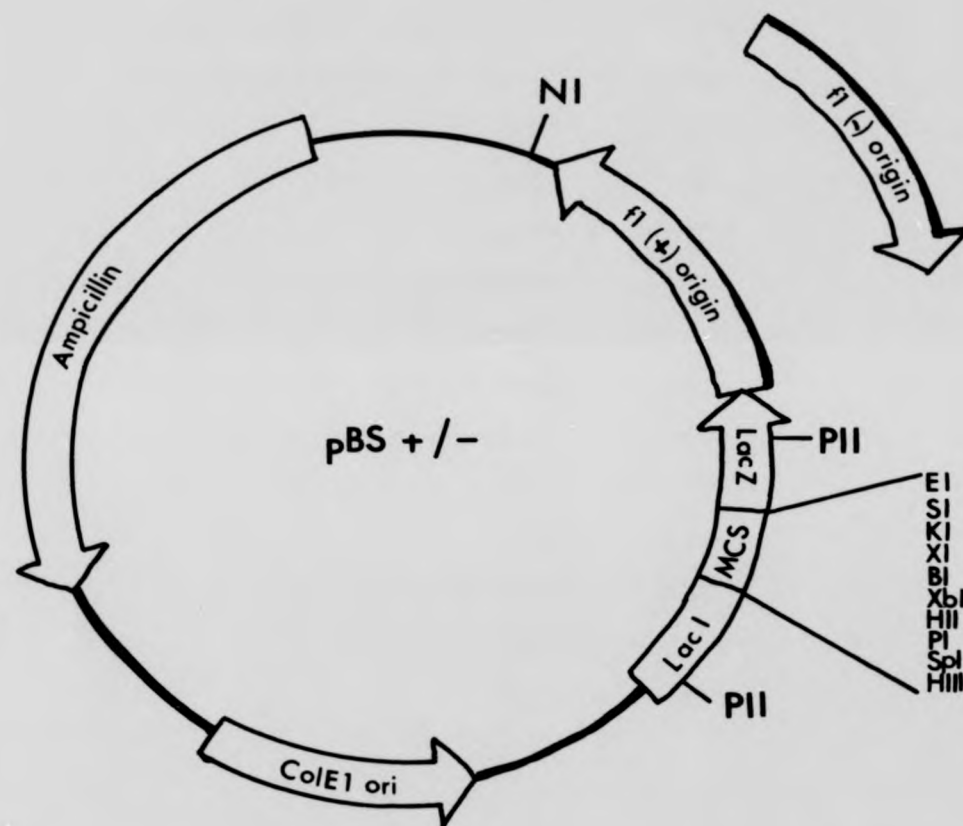
The pBS (+/-) phagemids are 3.2 kb colony forming hybrids, derived from the phage f1 and Stratagene's pBS plasmid.

Key:

ori	origin of replication
Ap	ampicillin resistance gene
MCS	multiple cloning site
E1	*Eco*RI
S1	*Sac*I
K1	*Kpn*I
X1	*Sma*I/*Xma*I
B1	*Bam*HI
Xb1	*Xba*I
H11	*Hinc*II
P1	*Pst*I
Sp1	*Sph*I
H111	*Hind*III
P11	*Pvu*II
N1	*Nde*I

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 mls 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 H_2O 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

Stock solution	Composition
Acrylamide A	25% (w/v) acrylamide
	1.25% (w/v) bisacrylamide
Acrylamide B	60% (w/v) acrylamide
	0.3 % (w/v) bisacrylamide
Resolving gel buffer	3M Tris-HCl (pH 8.8)
Ammonium persulphate (Amps)	10% (w/v)
Temed	used as supplied
Electrophoresis buffer	0.05 M Tris
	0.26 M Glycine
Sample buffer	10 μl 75% (v/v) glycerol
(per 100 μl sample)	10 μl 0.1% (w/v) bromophenol
	blue

Table 2.4.2 **Composition of non-denaturing exponential gradient gels**.

Stock Solution	4%	15%
Acrylamide A	4.6 ml	-
Acrylamide B	-	7.2 ml
Resolving buffer	3.75 ml	3.75 ml
double distilled water	20.4 ml	7.2 ml
30% (v/v) glycerol	-	10.625 ml
Temed	17.5 μl	7.5 μl
Amps	150 μl	75 μl

Table 2.5.1   Stock solutions for SDS-PAGE.

Stock Solutions	Composition
Resolving gel buffer	0.75 M Tris-HCl pH 8.8 0.2 % (w/v) SDS
Stacking gel buffer	0.25 M Tris-HCl pH 6.8 0.2 % (w/v) SDS
Acrylamide stock	44% (w/v) acrylamide 0.8% (w/v) bisacrylamide
Ammonium persulphate (Amps)	1% (w/v)
TEMED	As supplied by manufacturer
Electrophoresis buffer	0.025 M Tris 0.129 M Glycine 0.1 % (w/v) SDS
Sample buffer (x2)	0.025 M Tris-HCl pH 6.8 0.8 % (w/v) SDS 10 % (v/v) glycerol 5 % (v/v) β-mercaptoethanol Bromophenol blue

Table 2.5.2   Composition of SDS-PAGE resolving gels.

Stock Solution	15%	11%	8.5%
Resolving gel buffer	27.0 ml	27.0 ml	27.0 ml
Acrylamide stock	18.4 ml	13.6 ml	10.6 ml
double distilled water	7.2 ml	12.0 ml	15.0 ml
Amps	1.9 ml	1.9 ml	1.9 ml
Temed	0.1 ml	0.13 ml	0.13 ml

Table 2.5.3 Composition of SDS-PAGE stacking gels.

Stock Solution	4.5%		7.5%	
Stacking gel buffer	10	ml	10	ml
Acrylamide stock	3	ml	3.3	ml
double distilled water	7	ml	6.7	ml
Amps	0.5	ml	0.5	ml
Temed	0.04	ml	0.04	ml

of ddw and slowly added to a solution containing 42 ml 0.36%
NaOH and 2.5 ml NH_3. 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 $[^{35}S]$-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 $60^{\circ}C$ 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$_{12}$ 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$_{12}$ (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^{-1} of walls.   This was followed by incubation, with
stirring, at 37°C.   The Vitamin B$_{12}$ 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.

A$_{280}$ and A$_{361}$ 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 colormetrically.

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) $CuSO_45H_2O$ dissolved in 1% (w/v) sodium potassium tartrate were added to 50 mls of 5% (w/v) Na_2CO_3 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-Ciocolteu reagent (BDH) rapidly added and mixed well. Samples were left to stand at room temperature for 30 min and A_{750} 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 μg 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°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

Chapter 3. **The isolation and cloning of** *L. leichmannii* **genomic DNA exhibiting homology to the** *E. coli* **gene** (*btu*B) **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 (*btu*B) 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 btu*B
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 *btu*B gene. The
mutational lesion was determined by nucleotide sequencing
and a single amino acid substitution near the amino terminus
of *btu*B 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 *btu*B gene (Figure 3.2). The
deduced nucleotide sequence of the mixed oligonucleotide

Figure 3.1   Nucleotide sequence of the structural gene

for the Vitamin B$_{12}$ receptor (btuB) in

Escherichia coli.

Figure 3.2   Codon usage of the *Escherichia coli btuB* **gene.**

TTT	Phe	11 (1.8)	TCT	Ser	8 (1.3)
TTC	Phe	7 (1.1)	TCC	Ser	7 (1.1)
TTA	Leu	5 (0.8)	TCA	Ser	7 (1.1)
TTG	Leu	8 (1.3)	TCG	Ser	4 (0.7)
CTT	Leu	6 (1.0)	CCT	Pro	2 (0.3)
CTC	Leu	7 (1.1)	CCC	Pro	3 (0.5)
CTA	Leu	0 (0.0)	CCA	Pro	3 (0.5)
CTG	Leu	19 (3.1)	CCG	Pro	10 (1.6)
ATT	Ile	14 (2.3)	ACT	Thr	10 (1.6)
ATC	Ile	9 (1.5)	ACC	Thr	25 (4.1)
ATA	Ile	2 (0.3)	ACA	Thr	7 (1.1)
ATG	Met	3 (0.5)	ACG	Thr	14 (2.3)
GTT	Val	11 (1.8)	GCT	Ala	7 (1.1)
GTC	Val	15 (2.4)	GCC	Ala	10 (1.6)
GTA	Val	2 (0.3)	GCA	Ala	7 (1.1)
GTG	Val	13 (2.1)	GCG	Ala	15 (2.4)
TAT	Tyr	38 (6.2)	TGT	Cys	1 (0.2)
TAC	Tyr	11 (1.8)	TGC	Cys	0 (0.0)
TAA	End	0 (0.0)	TGA	End	0 (0.0)
TAG	End	0 (0.0)	TGG	Trp	14 (2.3)
CAT	His	8 (1.3)	CGT	Arg	14 (2.3)
CAC	His	3 (0.5)	CGC	Arg	14 (2.3)
CAA	Gln	14 (2.3)	CGA	Arg	0 (0.0)
CAG	Gln	20 (3.3)	CGG	Arg	3 (0.5)
AAT	Asn	14 (2.3)	AGT	Ser	9 (1.5)
AAC	Asn	14 (2.3)	AGC	Ser	13 (2.1)
AAA	Lys	16 (2.6)	AGA	Arg	1 (0.2)
AAG	Lys	5 (0.8)	AGG	Arg	0 (0.0)
GAT	Asp	36 (5.9)	GGT	Gly	22 (3.6)
GAC	Asp	13 (2.1)	GGC	Gly	21 (3.4)
GAA	Glu	12 (2.0)	GGA	Gly	10 (1.6)
GAG	Glu	6 (1.0)	GGG	Gly	11 (1.8)

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 *omp*C, *omp*F and *pho*E, the 'diagon' sub-routine of the 'Staden' program was employed to determine the level of homology between the *btu*B 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-^{32}P]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/TCCGGATACTCTC/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.
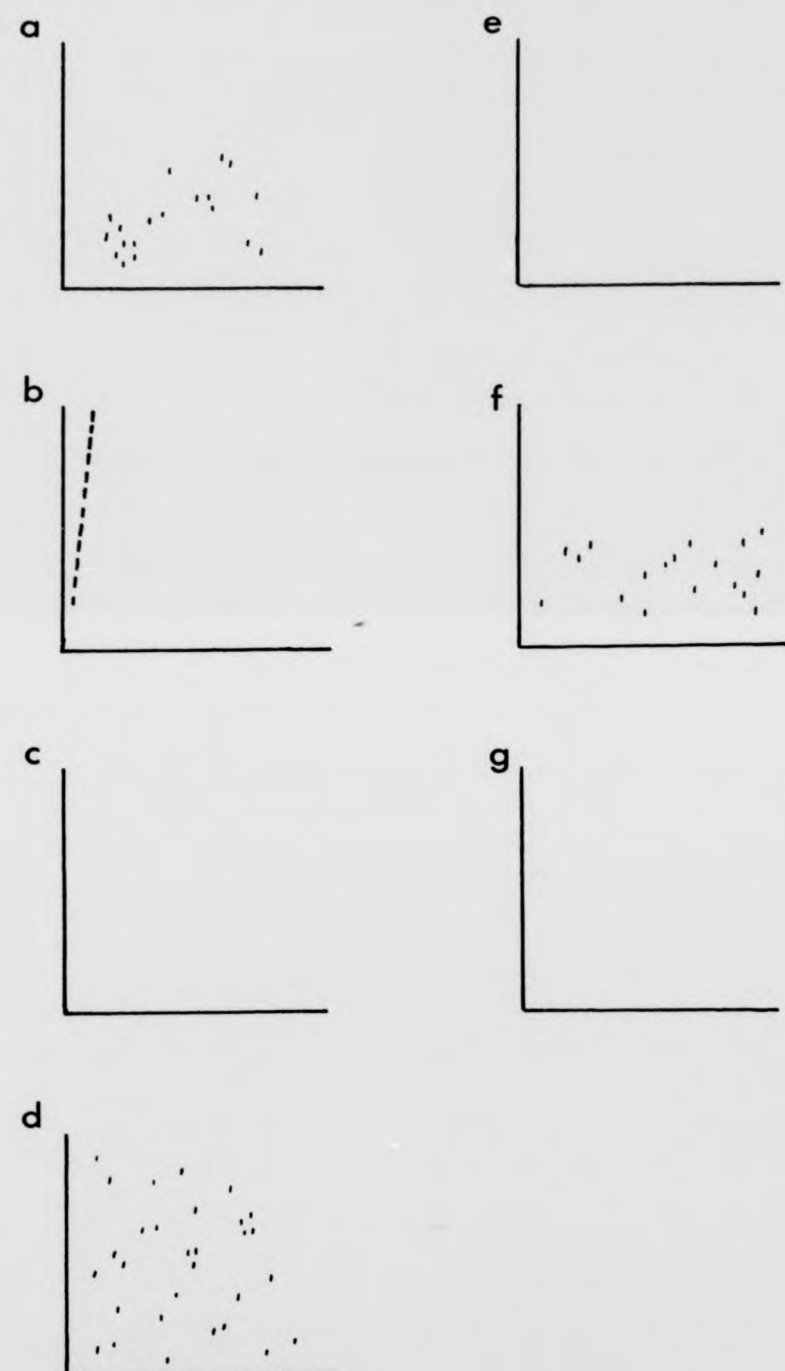
Figure 3.4   Comparison of the proposed synthetic oligonucleotide with other outer membrane proteins.

Key:-

(a)   *btu*B ORF vs *omp*F ORF

(b)   *btu*B ORF vs mixed oligonucleotide probe

(c)   *omp*F ORF vs mixed oligonucleotide probe

(d)   *btu*B ORF vs *omp*C ORF

(e)   *omp*C ORF vs mixed oligonucleotide probe

(f)   *btu*B ORF vs *pho*E ORF

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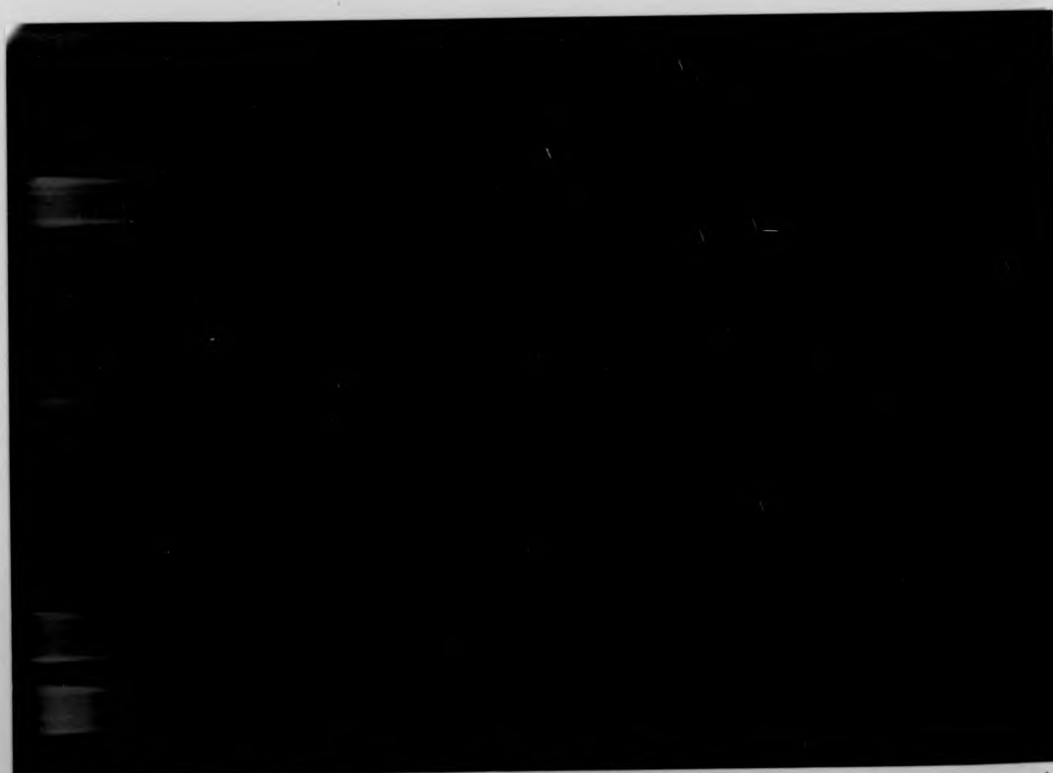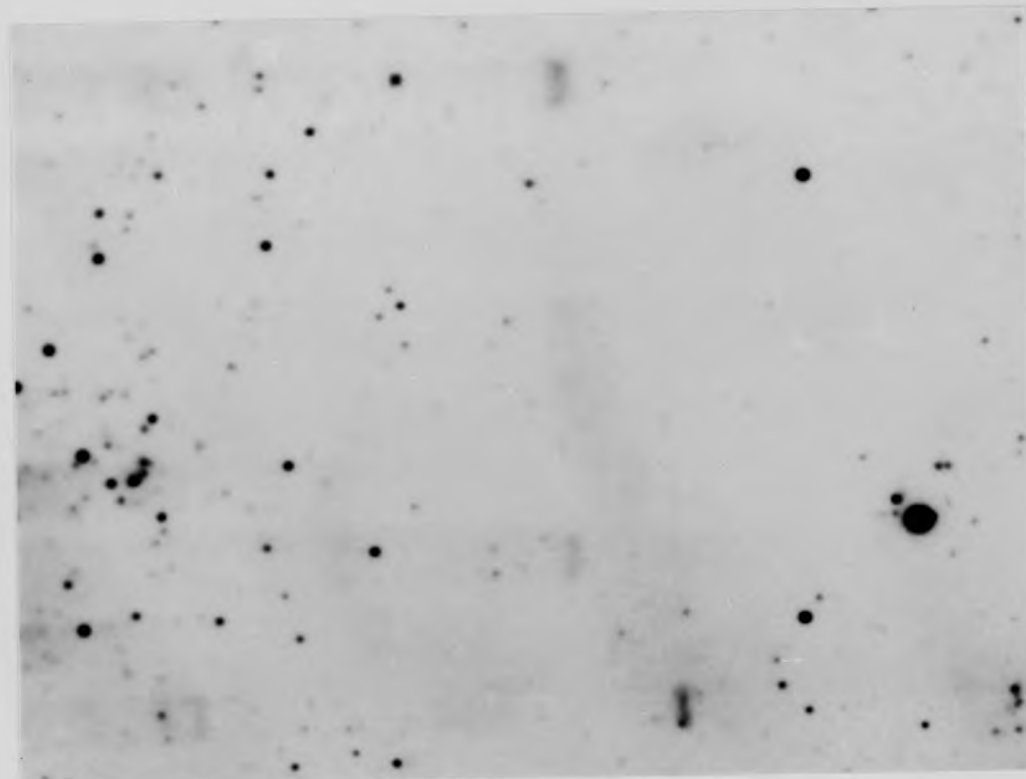
Figure 3.5   Examination of *L. leichmannii* **genomic DNA for**
homology with the synthetic oligonucleotide.

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 [γ^{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 *Eco*RI

B   *L. leichmannii* 4797 genomic DNA restricted with *Sal*I

C   *L. leichmannii* 4797 genomic DNA restricted with *Hind*III

D   *L. leichmannii* 4797 genomic DNA restricted with *Eco*RI

E   *L. casei* 7473 genomic DNA restricted with *Sal*I

F   *L. casei* 7473 genomic DNA restricted with *Hind*III

G   *L. casei* 7473 genomic DNA restricted with *Eco*RI

H   *L. leichmannii* 8964 genomic DNA restricted with *Sal*I

I   *L. leichmannii* 8964 genomic DNA restricted with *Hind*III

J   *L. leichmannii* 8964 genomic DNA restricted with *Eco*RI

Tracks K to T are autoradiographs of corresponding tracks A
to J probed with [γ^{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 *Hin*dIII 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 *Hin*dIII site of pBR327; the resulting library being screened by hybridisation.

Due to possible sequence homology between the synthetic oligonucleotide probe and the resident *btu*B 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 $[\gamma-^{32}P]dATP$ 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 *Hin*dIII, fractionated by agarose gel electrophoresis, Southern blotted and probed with the labelled oligonucleotide probe. Both clones harboured a single 2.0 Kb *Hin*dIII DNA fragment

Figure 3.6   Cloning strategy adopted for the isolation of
              L. leichmannii DNA exhibiting homology to the
              synthetic oligonucleotide.

clone 1

clone 2

A B
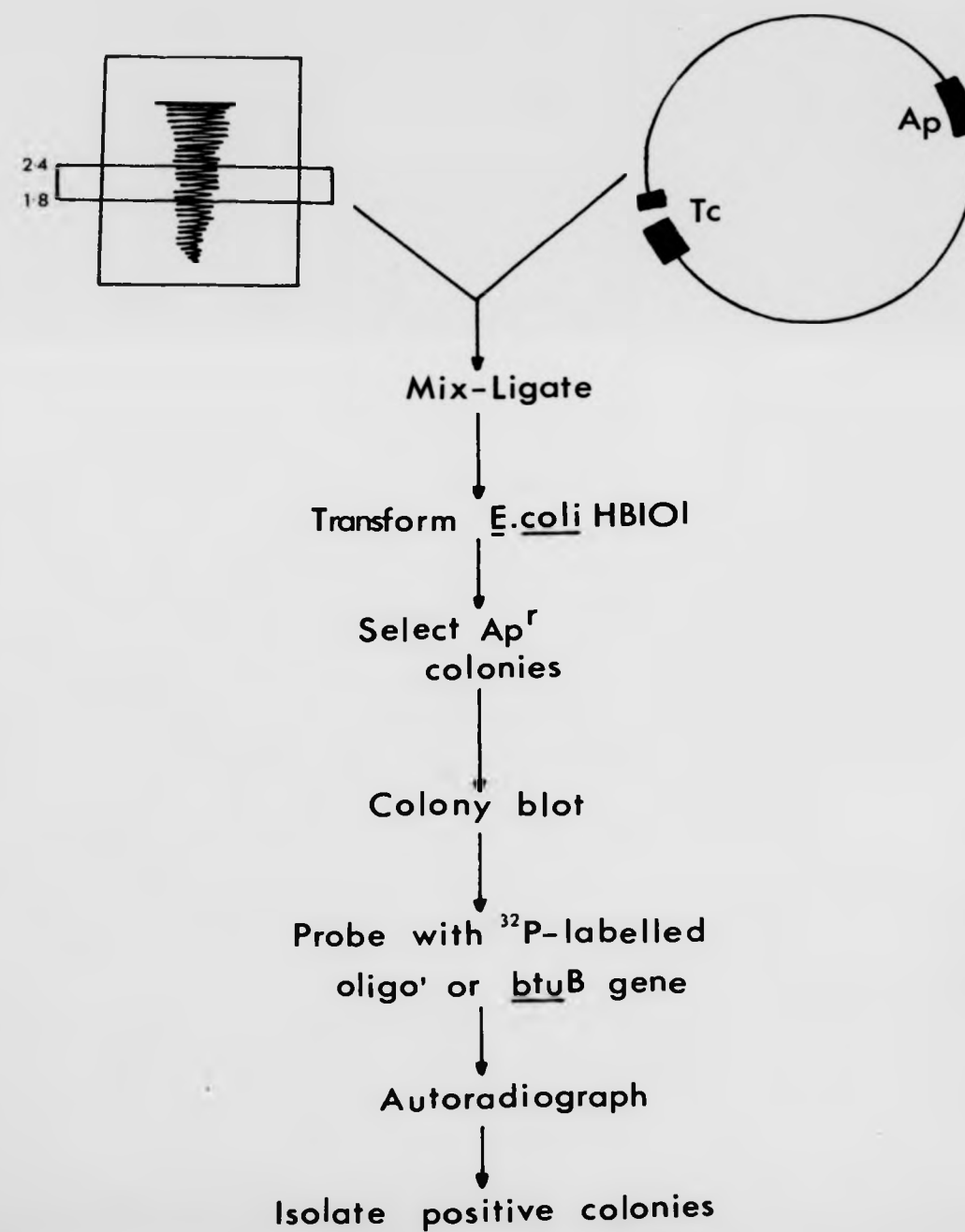
Figure 3.7 **Screening of a selective** *L. leichmannii* **gene bank for sequences homologous to the synthetic oligonucleotide.**

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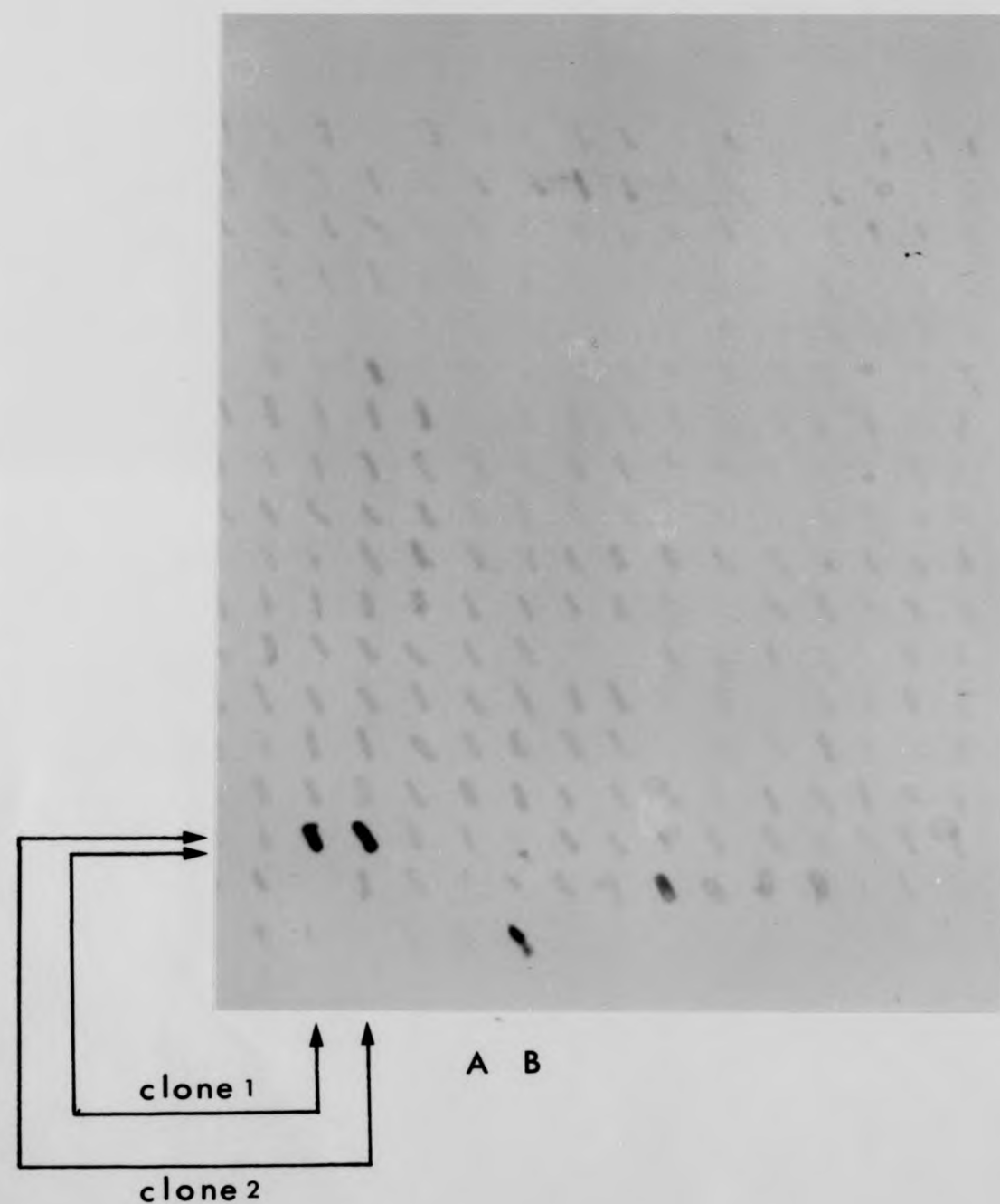
which exhibited strong homology to the oligonucleotide
probe. These clones were redesignated pSDC1 and pSDC2.

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

Screening of the *Lactobacillus* limited genomic library was
also carried out with a [α-^{32}P]dATP labelled 1.5 Kb *Eco*RI
fragment isolated from plasmid pNF48 (Figure 2.1) harbouring
the *E. coli btu*B 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 anomolies 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 *sph*I).

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
L. 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

A B C D E F G H I J K L M N O P Q R S T
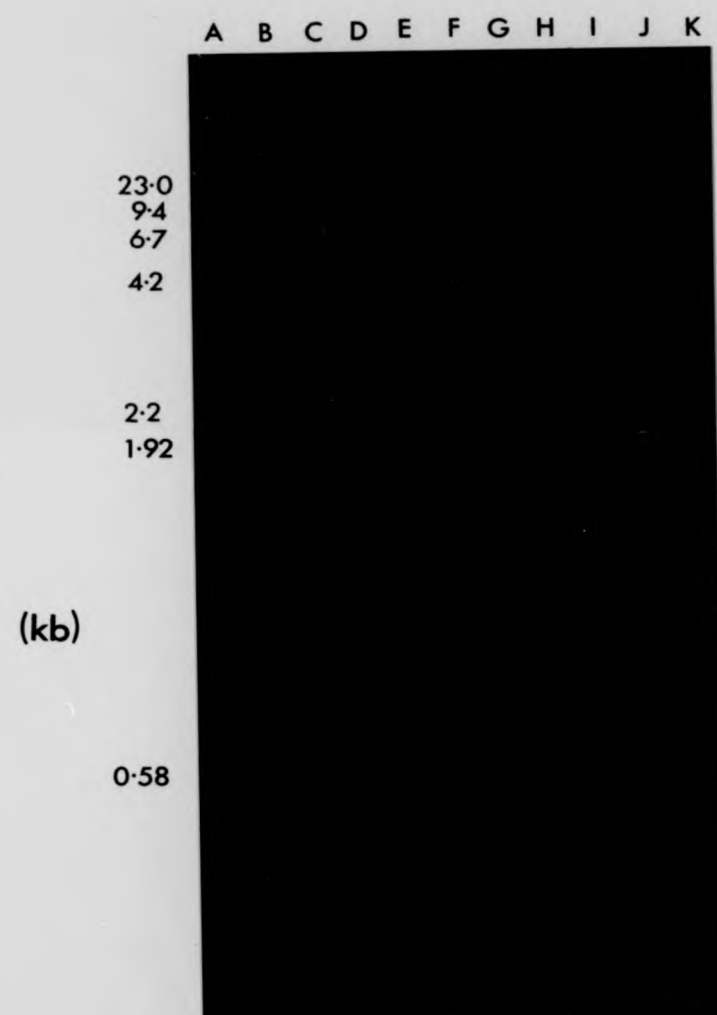
Figure 3.9   Restriction endonuclease mapping of the
            L. leichmannii **clone pSDC1.**

Keys to tracks:-

A   λ *Hind*III DNA markers

B   pBR327 DNA

C   pSDC1   DNA

D   pSDC2   DNA

E   pBR327 DNA restricted with *Hind*III

F   pBR327 DNA restricted with *Hinc*II

G   pBR327 DNA restricted with *Ava*I

H   pBR327 DNA restricted with *Bam*HI

I   pBR327 DNA restricted with *Pst*I

J   pSDC1   DNA restricted with *Hind*III

K   pSDC1   DNA restricted with *Hinc*II

L   pSDC1   DNA restricted with *Ava*I

M   pSDC1   DNA restricted with *Bam*HI

N   pSDC1   DNA restricted with *Pst*I

O   pBR327 DNA restricted with *Cla*I

P   pBR327 DNA restricted with *Sph*I

Q   pBR327 DNA restricted with *Eco*RI

R   pSDC1   DNA restricted with *Cla*I

S   pSDC1   DNA restricted with *Sph*I

T   pSDC1   DNA restricted with *Eco*RI

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 B_{12} 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 btu*B⁻ 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 *btu*B lesion due to an intact and functional *btu*B gene, located within the 2.0 Kb *Hind*III 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 *btu*B. This hypothesis

Figure 3.10 Restriction endonuclease mapping of pSDC1.

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

Key to tracks:-

A	λ *Hind*III DNA markers
B	pSDC1 DNA restricted with *Pst*I and *Pvu*II
C	pSDC1 DNA restricted with *Pst*I and *Eco*RV
D	pSDC1 DNA restricted with *Pst*I and *Sal*I
E	pSDC1 DNA restricted with *Pst*I and *Ava*I
F	pSDC1 DNA restricted with *Pst*I and *Hinc*II
G	pSDC1 DNA restricted with *Pst*I and *Hind*III
H	pSDC1 DNA restricted with *Pst*I only
I	pSDC1 DNA restricted with *Pvu*II and *Eco*RI
J	pSDC1 DNA restricted with *Eco*RV and *Sal*I

A B C D E F G H I J K

230
9·4
6·7

4·2

2·2
1·9
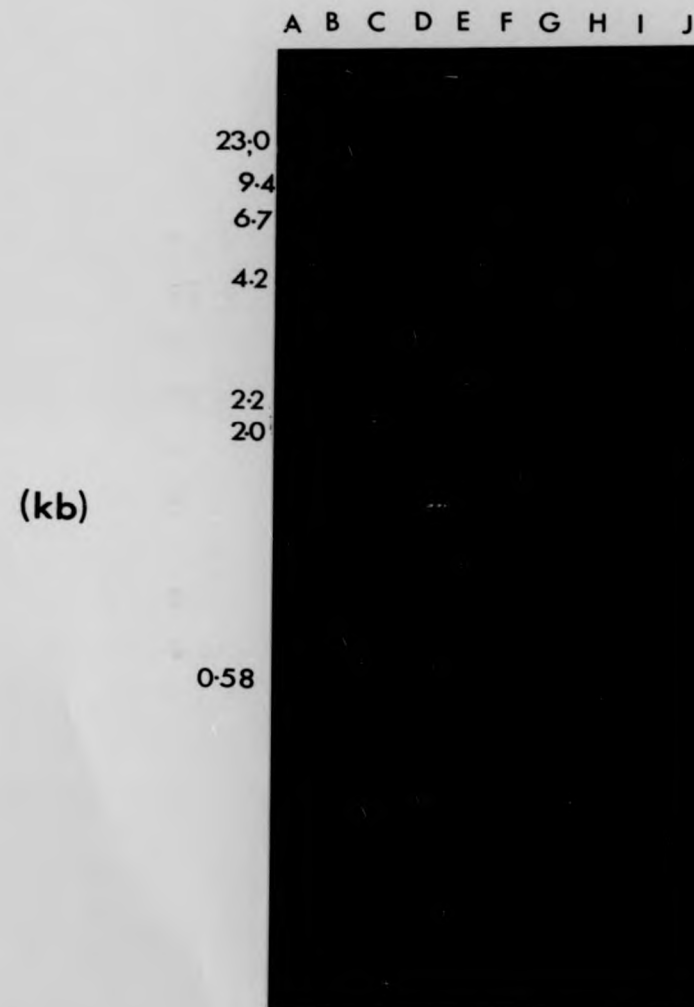
Figure 3.11   Restriction endonuclease mapping of pSDC1.
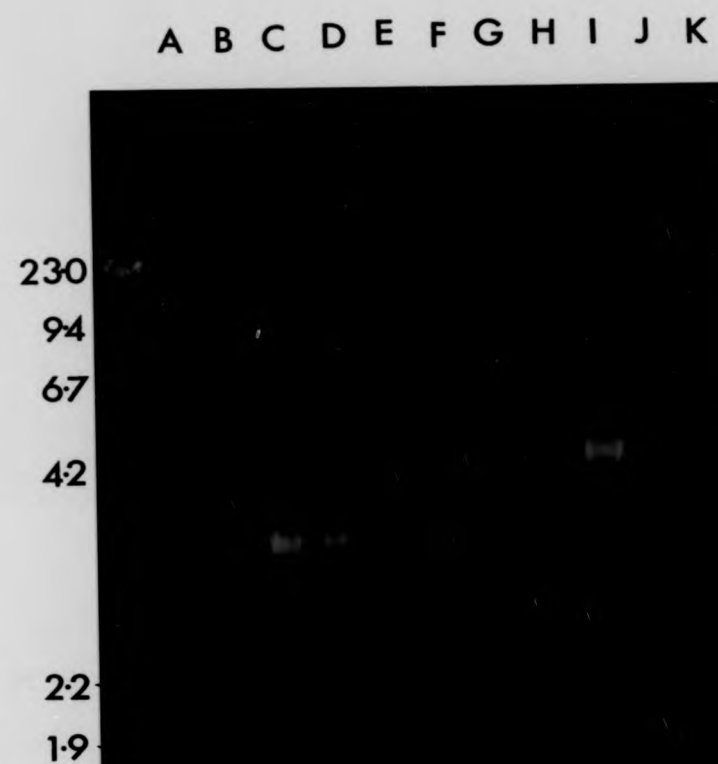
Key to tracks:-

A   pSDC1 DNA restricted with *Eco*RV and *Bgl*II

B   pSDC1 DNA restricted with *Eco*RI and *Eco*RV

C   pSDC1 DNA restricted with *Eco*RI and *Hinc*II

D   pSDC1 DNA restricted with *Eco*RI and *Sac*I

E   pSDC1 DNA restricted with *Eco*RI and *Sal*I

F   pSDC1 DNA restricted with *Eco*RI and *Xho*I

G   pSDC1 DNA restricted with *Hinc*II and *Eco*RI

H   pSDC1 DNA restricted with *Hinc*II and *Pvu*II

I   pSDC1 DNA restricted with *Pst*I and *Cla*I

J   pSDC1 DNA restricted with *Pst*I and *Eco*RI

K   pSDC1 DNA restricted with *Pst*I and *Hind*III

A B C D E F G H I J K L M

Figure 3.12   Restriction endonuclease mapping of pSDC1.

Key to tracks:-

A   λ *Hin*dIII DNA markers

B   pSDC1 DNA restricted with *Eco*RI and *Pvu*II

C   pSDC1 DNA restricted with *Eco*RI and *Eco*RV

D   pSDC1 DNA restricted with *Eco*RI and *Hin*dIII

E   pSDC1 DNA restricted with *Eco*RI

F   pSDC1 DNA restricted with *Hinc*II and *Pst*I

G   pSDC1 DNA restricted with *Hinc*II and *Pvu*II

H   pSDC1 DNA restricted with *Hinc*II and *Eco*RI

I   pSDC1 DNA restricted with *Hinc*II

J   pSDC1 DNA restricted with *Pst*I and *Hin*dIII

K   pSDC1 DNA restricted with *Pst*I and *Hinc*II

L   pSDC1 DNA restricted with *Pst*I and *Eco*RI

M   pSDC1 DNA restricted with *Pst*I

Figure 3.13 Restriction endonuclease map of pSDC1 with the published map of pBR327 for comparison.

Key :-

EI	*Eco*RI
HIII	*Hin*dIII
BI	*Bam*HI
EV	*Eco*RV
PII	*Pvu*II
PI	*Pst*I
SI	*Sal*I
HII	*Hin*cII
AI	*Ava*I
XI	*Xma*I
CI	*Cla*I
SpI	*Sph*I
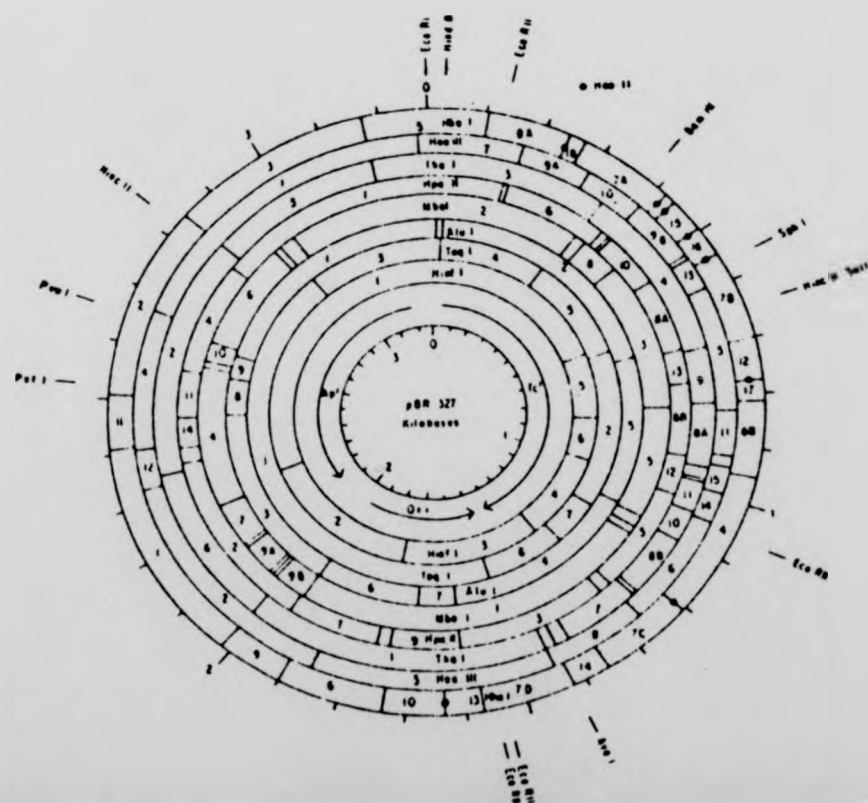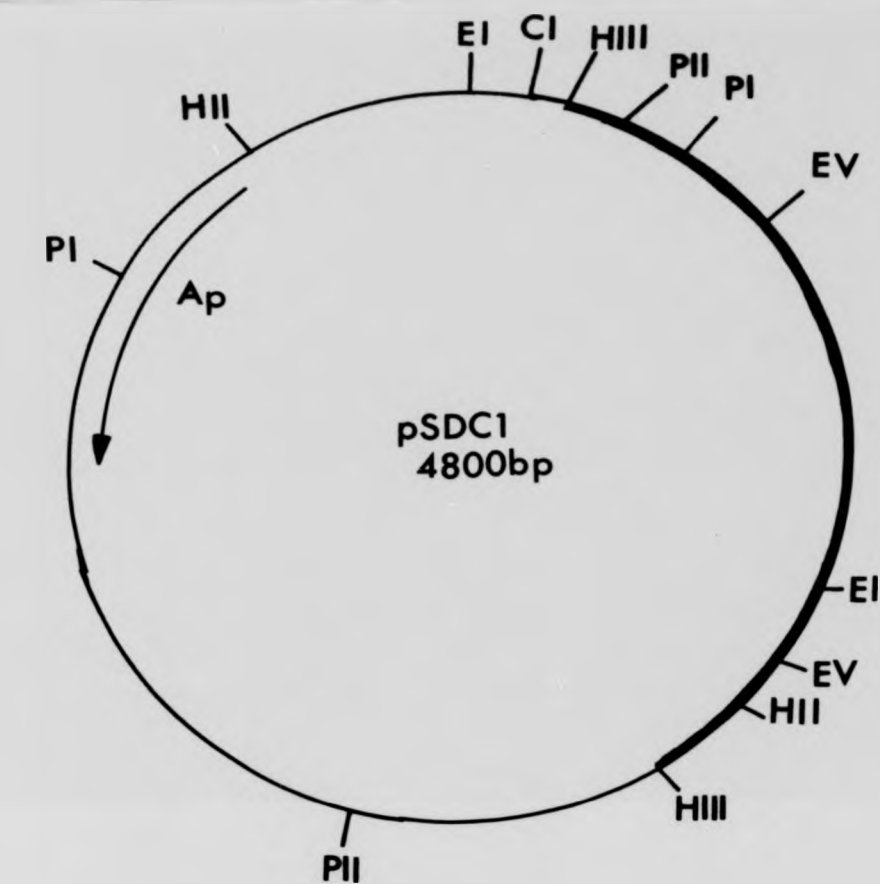
held true for *E. coli* L1-431 transformed with pSDC1, as did
E. coli L1-431 transformed with pNF48.

The 2.0 Kb *Hin*dIII insert of pSDC1 was subsequently recloned
in the opposite orientation in pBR327, by restriction of
pSDC1 with *Hin*dIII, 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 *btu*B
lesion in L1-431 with pSDC3 revealed that the cloned *btu*B$_L$
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 Hin*dIII fragment, it was necessary to attempt
to express the gene located on plasmid pSDC1 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}S-methionine and samples analysed by SDS-PAGE.

The plasmids pSDC1, pNF48, pSDC2 and pBR327 were transformed into the *E. coli* maxicell strain CSH26ΔF6 (Stoker *et al.*, 1984). The resulting transformants were allowed to synthesise proteins in the presence of ^{35}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.

3.2.5.3 *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   CSH26ΔF6 control

E   pBR327 encoded polypeptides
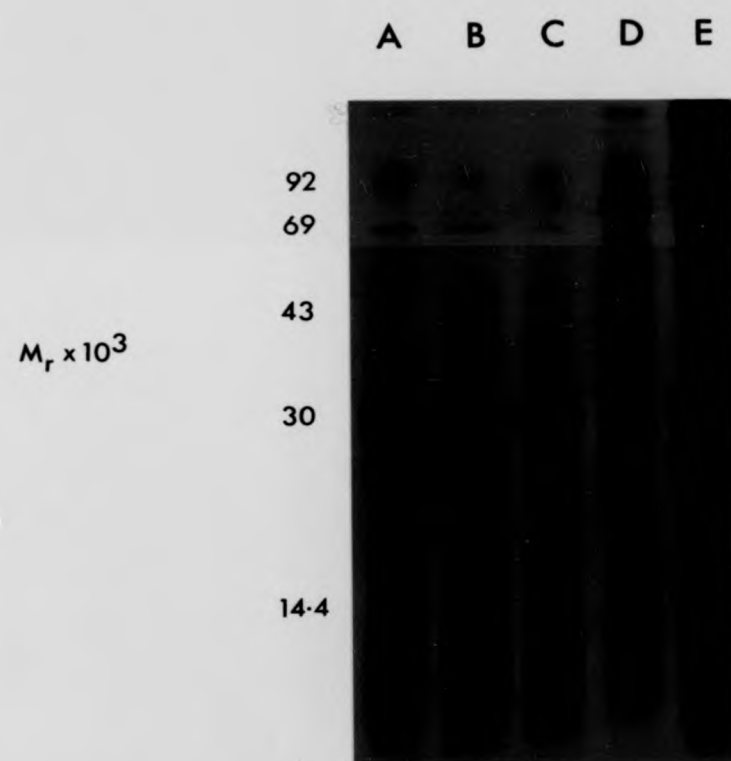
been cloned together with its own promoter, is frequently difficult to establish using *in vivo* systems. However, *in vitro* systems circumvent these problems. Two further advantages of the *in vitro* system, first described by De Vries and Zubay (1967) are that, incorporation of radioactive label into 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 ^{35}S-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* *btu*B gene. This polypeptide was thus proposed to be the product of the *L. leichmannii* *btu*B 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 *Hind*III initially which resulted in the loss of the

Figure 3.15 *In vitro* **translation products of pSDC1 and pSDC2**.

The fluorograph shows the various ^{35}S-methionine labelled plasmid encoded products. The M_r 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
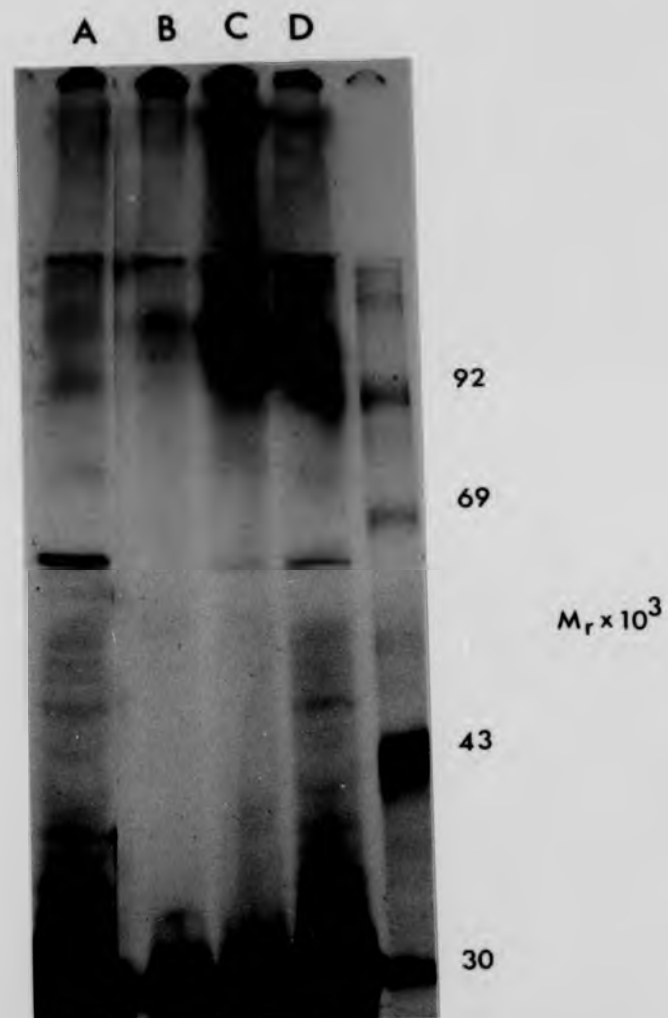
M_r-66,400 protein, as produced by pNF48 (positive control) and pSDC1. This implies that the 2.0 Kb HindIII insert did not in fact possess its own promoter, but possibly the 66,400 polypeptide was the result of read-through from vector sequences. This data correlates well with the complementation data. 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 M_r 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 M_r-66,400 protein, although there was expression of an M_r-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-^{32}P]dATP labelled 1.5 Kb EcoR1 fragment from pNF48. Clones were designated pSDC41 and pSDC42, in accordance with pSDC1 and pSDC3 respectively.

Figure 3.16 *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 β-lactamase of the vector pBR327.

Key to tracks:

A   pSDC1 encoded products

B   pSDC1/*Pst*I encoded products

C   pSDC1/*Pvu*II encoded products
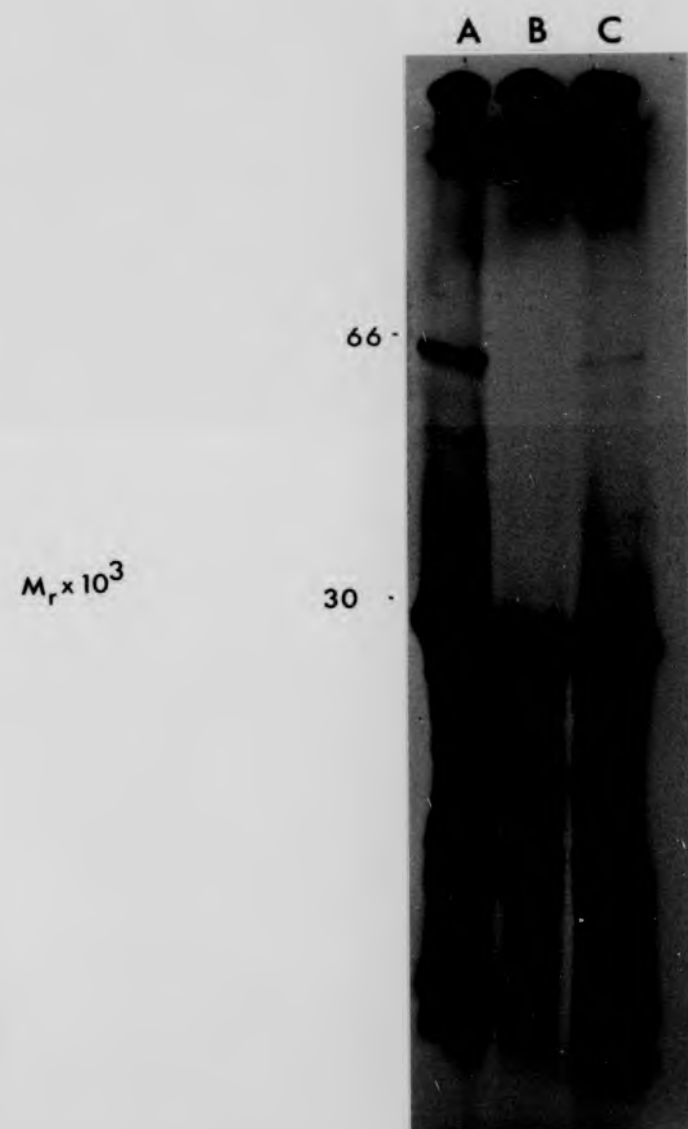
Figure 3.17   Plasmid analysis of putative sub-clones of pSDC1.

Nineteen putative clones were subjected to plasmid analysis, restricted with both *Pvu*II and *Hind*III (separately) and fractionated on an agarose gel.

Key:

    a tracks - DNA digested with *Pvu*II

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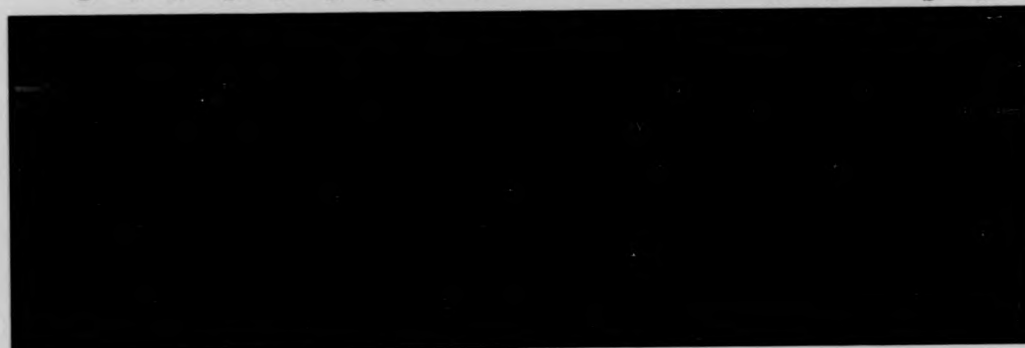
After further restriction analysis (data not shown) of pSDC41, in order to correlate with the data produced from analysis of pSDC1, the final restriction map is presented in Figure 3.18. It was this construct, pSDC41, which was used in subsequent studies.

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 btu*B gene.

3.2.6   Nucleotide sequence of the *L. leichmannii btu*B gene ($btuB_L$).

The restriction map of the $btuB_L$ 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 pBR329 for comparison.

Key:-

PII	PvuII
HIII	HindIII
EV	EcoRV
PI	PstI
EI	EcoRI
BI	BamHI
AI	AvaI
SpI	SphI
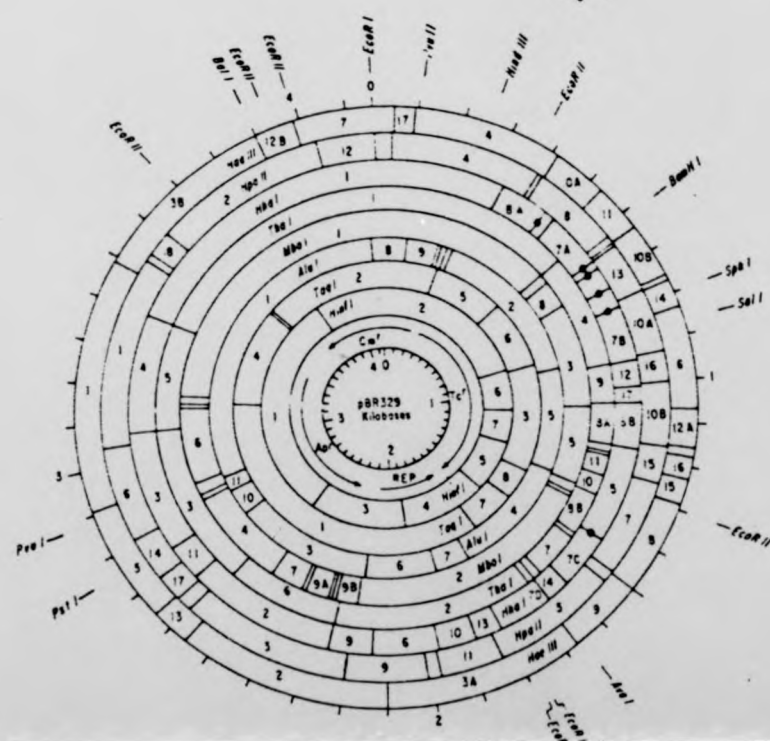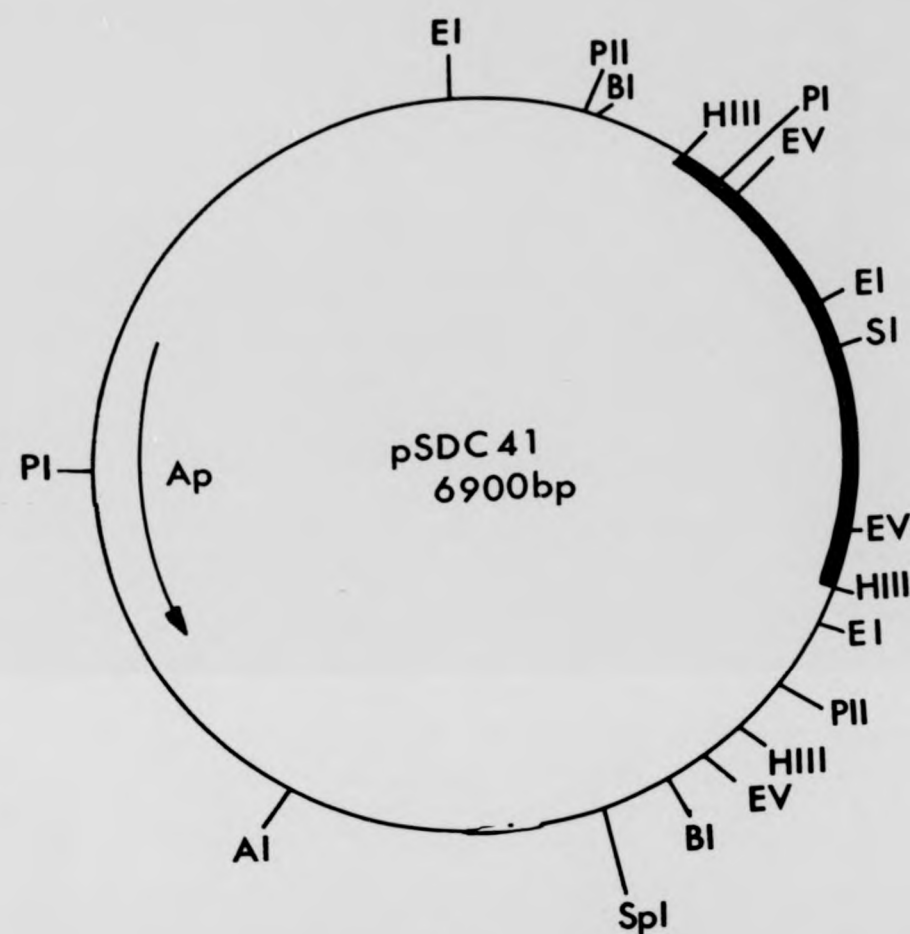
Figure 3.19   Sequencing strategy for the *L. leichmannii btuR*
gene region.

Key:-

HIII	*Hind*III
HII	*Hinc*II
PI	*Pst*I
EV	*Eco*RV
SI	*Sal*I
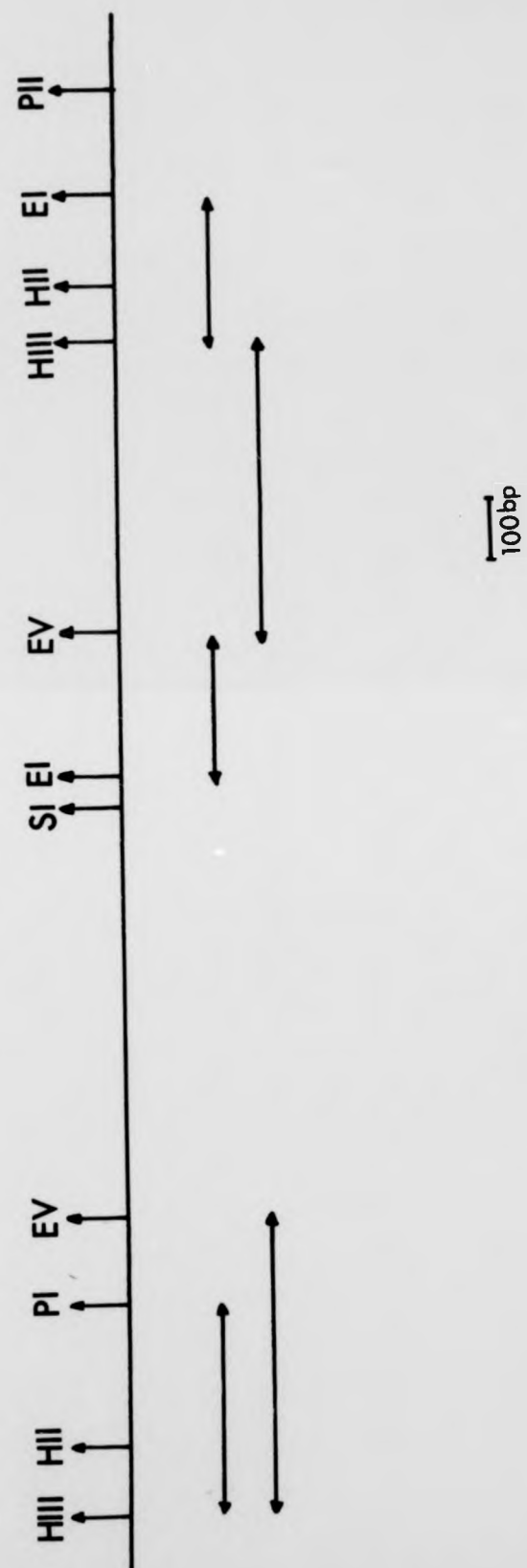
dideoxynucleotide chain termination procedure (Sanger *et al.*, 1977).

Nucleotide sequence data was generated initially from the 5' and 3' ends of the 2.45 Kb *Pvu*II DNA insert. When this sequence data was compared for homology with the nucleotide sequence of the *E. coli btu*B 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 *btu*B 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 (*btu*B) had been cloned from *L. leichmannii* by heterologous hybridisation, utilising an internal *btu*B gene fragment from *E. coli* and a synthetic oligonucleotide, derived from the *E. coli btu*B gene, as gene probes.

A heterologous hybridisation approach was used to isolate the putative *btu*B 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 *Hin*dIII DNA fragment (thought to have been cloned from *L. leichmannii*) was shown to complement the *btu*B lesion in *E. coli* L1-431, only as part of the recombinant pSDC1, 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 M_r-66,400 polypeptide produced was the functional product of the *L. leichmannii btu*B gene. *In vitro* transcription and translation studies, together with complementation studies also revealed the direction of transcription of the proposed *btu*B$_L$ gene to be in the orientation presented in Figure 3.19.

The fact that the cloned *btu*B 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 *rec*A$^-$ 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 *rec*A$^-$.

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 *btu*B 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 *btu*B, since it was found to be at the
analagous map position as the *E. coli* homolog (Sanderson &
Roth, 1988)], which encodes for a Vitamin B_{12} receptor
protein of similar size to *E. coli*; the Vitamin B_{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 *btu*B gene
cloned was indeed isolated from *E. coli* and not *L.
leichmannii*.

With no (or very little) information available on genes
encoding Vitamin B_{12} receptor proteins in other organisms,
particularly Gram-positive organisms, it was necessary to
attempt to re-clone the gene encoding the Vitamin B_{12}
binding protein in *L. leichmannii*.

CHAPTER 4

CHAPTER 4 - CLONING OF THE STRUCTURAL GENE FOR THE VITAMIN B_{12} RECEPTOR PROTEIN FROM *LACTOBACILLUS LEICHMANNII*

4.1 Introduction.

Initial cloning studies were based on the presumption that the Vitamin B_{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_{12} requiring Lactobacilli characteristically take up and preserve large amounts of B_{12} *in vivo*, from the surrounding media. The cellular site of B_{12} accumulation was shown to be located in the cell wall, where a B_{12}-binding principle exists (Sasaki & Kitahara, 1963a; 1963b; 1964b). Initial attempts at isolation and purification of the B_{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_{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 *Hind*III 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 *Hind*III 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 $[\gamma-^{32}P]$ 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 *Hind*III 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 *Hind*III 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

Figure 4.2   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 _Hin_dIII
2	pSEC1 DNA restricted with _Hin_dIII
3	pSEC1 DNA restricted with _Eco_RI
4	pSEC1 DNA restricted with _Eco_RV
5	pSEC1 DNA restricted with _Pvu_II
6	pSEC1 DNA restricted with _Pst_I
7	pSEC1 DNA restricted with _Hinc_II
8	pSEC1 DNA restricted with _Bam_HI
9	pSEC1 DNA restricted with _Cla_I
10	pSEC1 DNA restricted with _Bgl_II
11	λ DNA restricted with _Hin_dIII
12	pSEC1 DNA restricted with _Nco_I
13	pSEC1 DNA restricted with _Kpn_I
14	pSEC1 DNA restricted with _Ava_I
15	pSEC1 DNA restricted with _Sal_I
16	pSEC1 DNA restricted with _Sma_I
17	pSEC1 DNA restricted with _Pvu_I
18	pSEC1 DNA restricted with _Mlu_I
19	pSEC1 DNA restricted with _Xho_I
20	pSEC1 DNA restricted with _Sau_3a
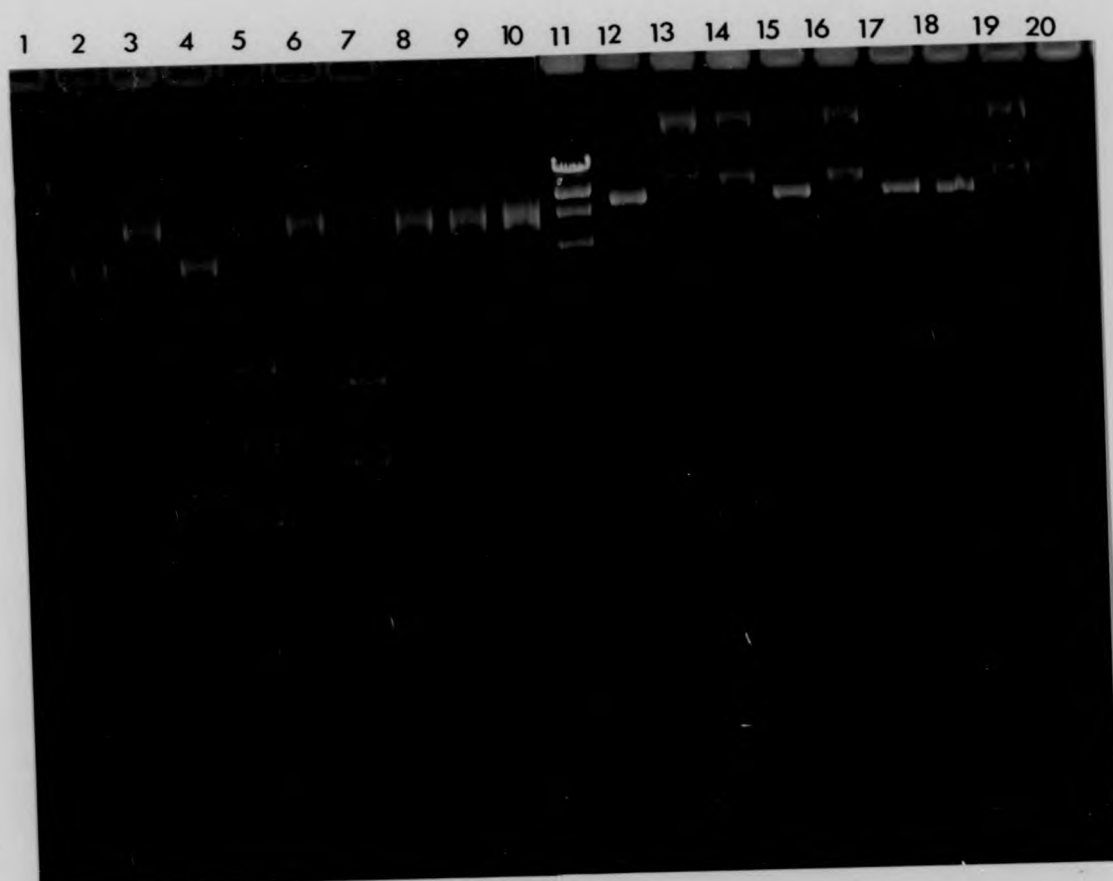
Figure 4.3   Restriction endonuclease mapping of the
L. leichmannii recombinant pSEC1.

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 _Hin_dIII

2   pSEC1 DNA restricted with _Hin_dIII and _Mlu_I

3   pSEC1 DNA restricted with _Hin_dIII and _Ava_I

4   pSEC1 DNA restricted with _Hin_dIII and _Hinc_II

5   pSEC1 DNA restricted with _Hin_dIII and _Bgl_II

6   pSEC1 DNA restricted with _Hin_dIII and _Pvu_II

7   pSEC1 DNA restricted with _Eco_RV and _Ava_I

8   pSEC1 DNA restricted with _Eco_RV and _Hinc_II

9   pSEC1 DNA restricted with _Eco_RV and _Bgl_II

10   pSEC1 DNA restricted with _Eco_RV and _Pvu_II

11   pSEC1 DNA restricted with _Bgl_II and _Hinc_II

12   pSEC1 DNA restricted with _Bgl_II and _Pvu_II

13   pSEC1 DNA restricted with _Mlu_I and _Ava_I

14   pSEC1 DNA restricted with _Mlu_I and _Bgl_II
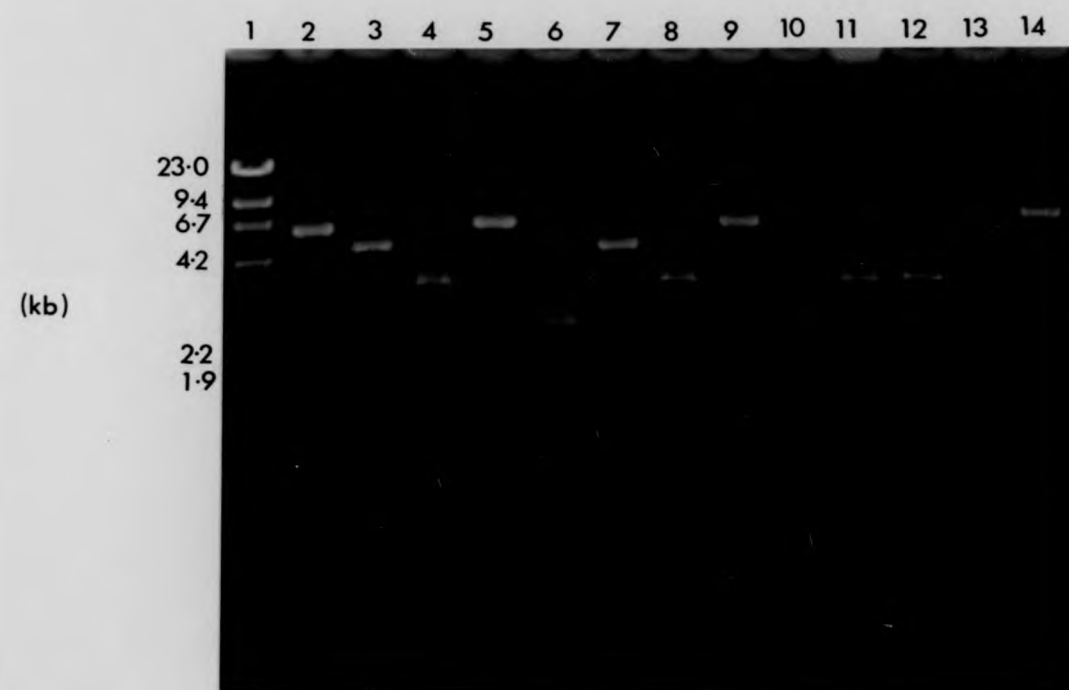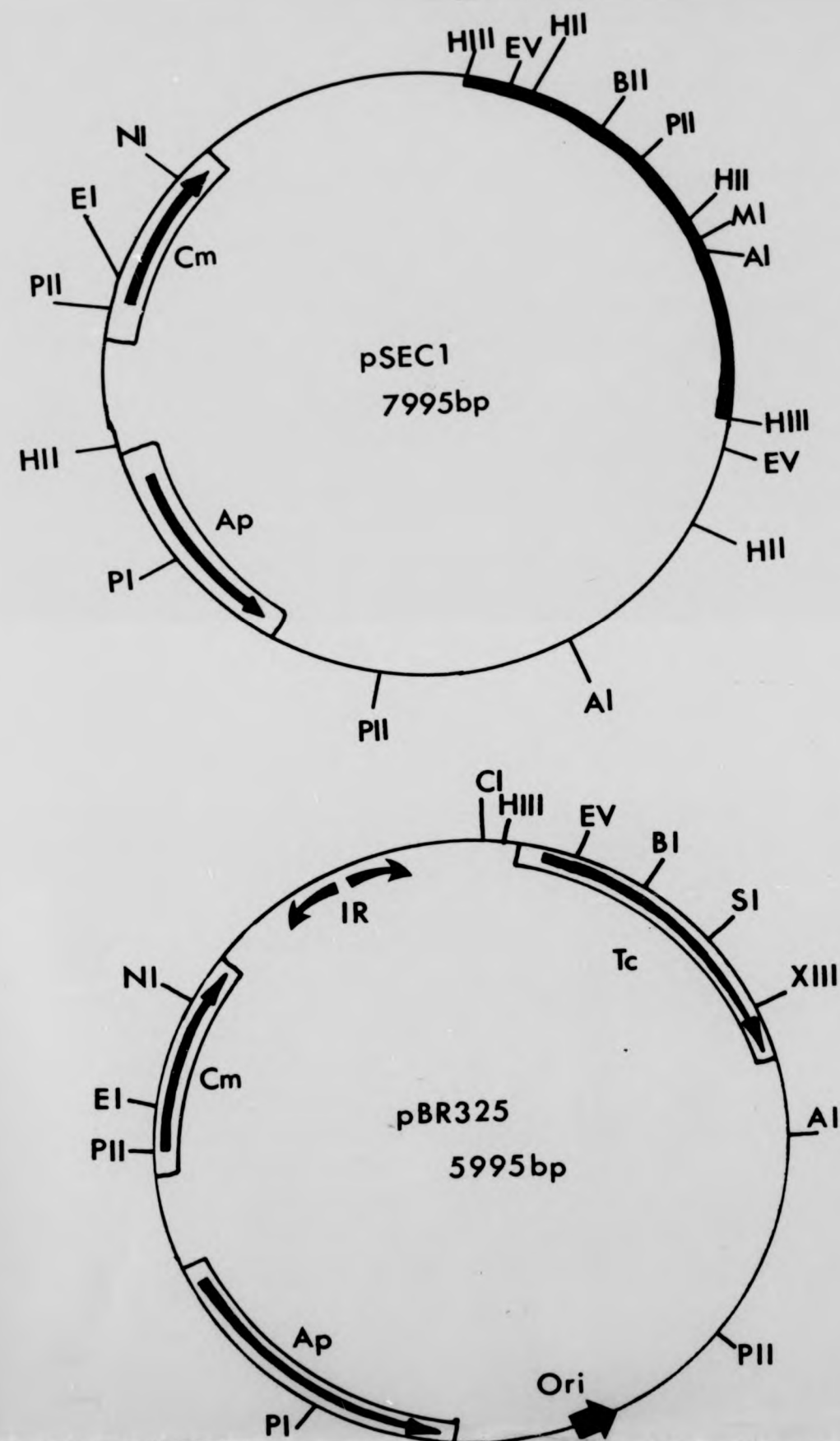
Figure 4.4   Restriction endonuclease map of pSEC1 (with published map of pBR325 for comparison).

The 2.0 Kb *Hind*III fragment within pSEC1 has mapped with respect to a number of restriction endonucleases.

Key:-

EI	*Eco*RI
NI	*Nco*I
PII	*Pvu*II
CI	*Cla*I
HIII	*Hind*III
EV	*Eco*RV
BI	*Bam*HI
SI	*Sal*I
AI	*Ava*I
PI	*Pst*I
BII	*Bgl*II
XI	*Xho*I
MI	*Mlu*I
HII	*Hinc*II
Ori	Origin of replication
IR	Inverted repeat
Tc	tetracycline resistance gene
Ap	ampicillin resistance gene
Cm	chloramphenicol resistance gene

None of the following sites were present:-

*Pst*I	*Eco*RI	*Kpn*I	*Nco*I	*Xho*I	*Sma*I
*Bam*HI	*Cla*I	*Sal*I	*Acc*I	*Pvu*I	

high stringency with $[\alpha-^{32}P]$-labelled pSEC1 2.0 Kb *Hin*dIII 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 *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.

4.3 Summary and Overview.

This chapter details the successful isolation and cloning of a 2.0 Kb *Hin*dIII genomic DNA fragment of *L. leichmannii* exhibiting homology to the synthetic oligonucleotide probe (described in Chapter 3), into a *rec*A⁻ strain of *E. coli* DH1.

There are many examples of genes cloned into *E. coli* from Gram-positive organisms including *Lactobacillus* genes. Examples of the latter have already been listed in Table 1.7. Examples of genes from other Gram-positive organisms cloned into *E. coli* include; two chloramphenicol acetyltransferase genes from *Clostridium butyricum* (Dubbert *et al.*, 1988); α-amylase gene from *Clostridium acetobutylicum* (Verhasselt *et al.*, 1989); the gene for phospho-β-galactosidase from *Staphylococcus aureus* (Breidt & Stewart, 1987); the tagatose 1,6-bisphosphate aldolase gene from *Steptococcus lactis* (Yu *et al.*, 1988) and the arginine

A B C D E F G H I J K L M
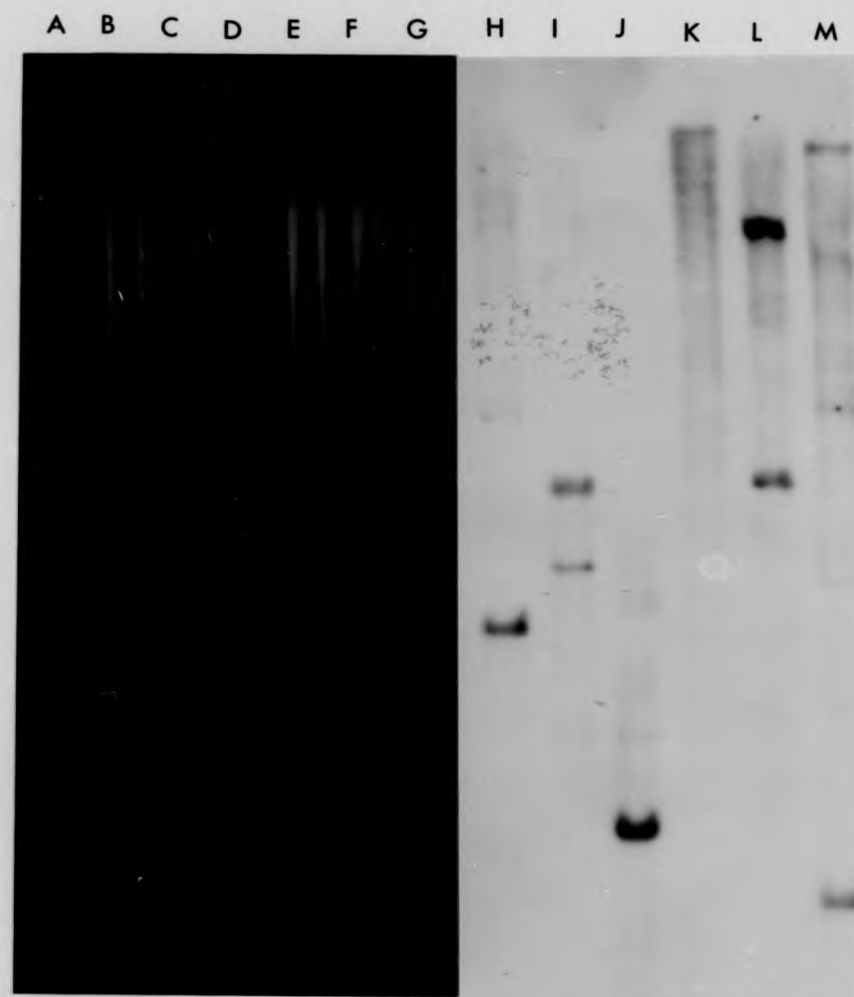
Verification of the origin and continuity of the *L. leichmannii* recombinant pSEC1.

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 *Hin*dIII insert at high stringency (assuming an appropriate 5% bp mismatch).

Key to tracks:-

A   λ DNA restricted with *Hin*dIII

B   *L. leichmannii* genomic DNA restricted with *Hin*dIII

C   *L. leichmannii* genomic DNA restricted with *Pvu*II

D   *L. leichmannii* genomic DNA restricted with *Hinc*II

E   *L. leichmannii* genomic DNA restricted with *Eco*RV

F   *L. leichmannii* genomic DNA restricted with *Bgl*II

G   *L. leichmannii* genomic DNA restricted with *Ava*I

Tracks H to M are autoradiographs of corresponding tracks B to G probed with pSEC1 2.0 Kb *Hin*dIII 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

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 M_r 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 B_{12} receptor protein from other organisms.

5.3 Results and Discussion.

5.3.1 Nucleotide sequence of the 2.0 Kb *Lactobacillus* DNA insert of pSEC1.

The 2.0 Kb *L. leichmannii* genomic DNA insert of pSEC1 was prepared for sequencing by the digestion of pSEC1 with *Hin*dIII, fractionated on an agarose gel and sub-cloned in both possible orientations into the *Hin*dIII site of a phagemid vector derived from pUC19 - pBS (+/-). The vector possessed ampicillin resistance, *lac*Z 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 *Hin*dIII, 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 *Eco*RV and *Bgl*II, 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 *Hind*III 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 *Hind*III and fractionated on
an agarose gel.

←2·0kb

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 *btu*B 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 M_r 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 M_r 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

Figure 5.2   Isolation of pBS3 and pBS7.

Plasmid DNA from the ten sub-clones (presented in Figure
5.1) were restricted with both *Eco*RV and *Bgl*II 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.

A B C D E F G H I J

1·5

(kb)

0·6

Figure 5.2   Isolation of pBS3 and pBS7.

Plasmid DNA from the ten sub-clones (presented in Figure 5.1) were restricted with both *Eco*RV and *Bgl*II 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.
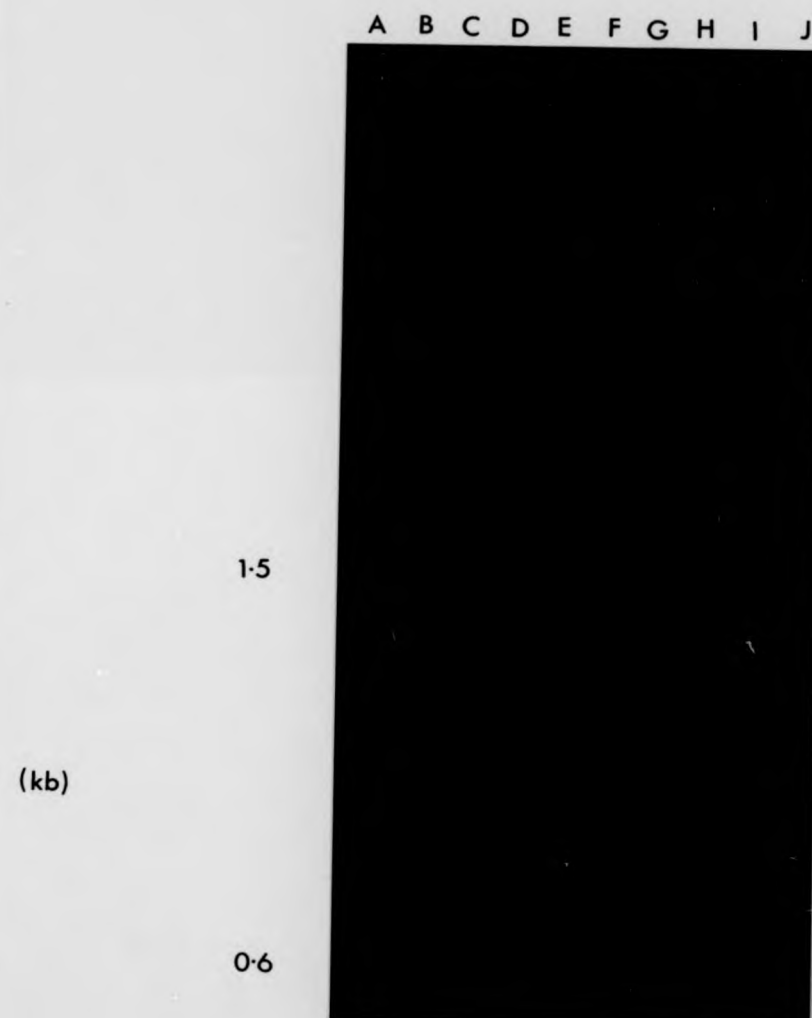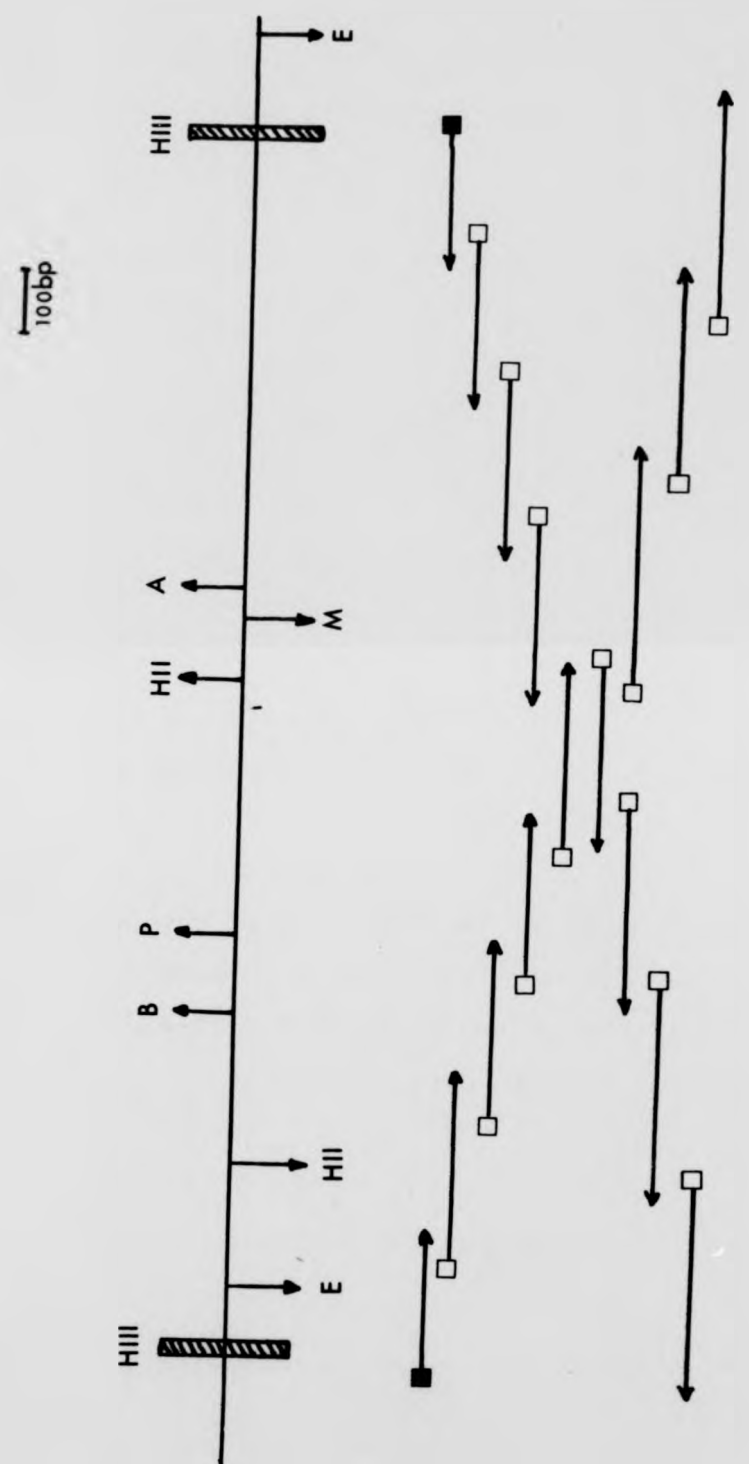
Figure 5.3 **Sequencing strategy for the _L. leichmannii_ 2.0 Kb DNA insert from pSEC1**.

Key:

A	_Ava_I
M	_Mlu_I
HIII	_Hind_III
HII	_Hinc_II
B	_Bgl_II
E	_Eco_RV
P	_Pvu_II

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 *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 & M[C]Clure, 1983).
Comparison of the putative *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).

Figure 5.4   Nucleotide sequence of *L. leichmannii* 2.0 Kb genomic DNA insert.

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
TGGAGATGGTGATCGTGGGCCGAAAGATTTTAGGGATGCGTGTCAGTGTCCTTGCCAGCA

      370       380       390       400       410       420
GCAGCTCAGGTAACGCAACCTATATTGAAACGCCTGGTCACAAGGTGTTAGTGGATGCCG

      430       440       450       460       470       480
GTTTGTCAGGCAAGAAAATAGGAGCACTGATGAAGAGCATCGGTCGAGATCTAACCGATG
                          MetLysSerIleGlyArgAspLeuThrAspV

      490       500       510       520       530       540
TTGACAGTGTTTTTATCACGCATGAACATAGCGATCATGTGCGTGGTGTAGGCGTGTTGG
alAspSerValPheIleThrHisGluHisSerAspHisValArgGlyValGlyValLeuA
```

Figure 5.6   The nucleotide sequence of the transcriptional control region of ORF 2b.

```
        130       140       150       160       170       180
GTTTATGTCCCGGCTTGGTACATTTGGCGTCAAGAATCGTTCGCCGGAAATGTCACGGTA


        190       200       210       220       230       240
AAACGTGTCAATGCGATCACTCGAACAGTTATGAAAAACCGTAATGACAGTTAAGTGGTA


        250       260       270       280       290       300
GATGGACGGCAGTGAGAGCCGATTGCTTAGATCAGGAGGTTGACTAAATGGTAAAAAGTT
                                                  MetValLysSerL


        310       320       330       340       350       360
TGGAGATGGTGATCGTGGGCCGAAAGATTTTAGGGATGCGTGTCAGTGTCCTTGCCAGCA
euGluMetValIleValGlyArgLysIleLeuGlyMetArgValSerValLeuAlaSerS


        370       380       390       400       410       420
GCAGCTCAGGTAACGCAACCTATATTGAAACGCCTGGTCACAAGGTGTTAGTGGATGCCG
erSerSerGlyAsnAlaThrTyrIleGluThrProGlyHisLysValLeuValAspAlaG


        430       440       450       460       470       480
GTTTGTCAGGCAAGAAAATAGGAGCACTGATGAAGAGCATCGGTCGAGATCTAACCGATG
lyLeuSerGlyLysLysIleGlyAlaLeuMetLysSerIleGlyArgAspLeuThrAspV


        490       500       510       520       530       540
TTGACAGTGTTTTTATCACGCATGAACATAGCGATCATGTGCGTGGTGTAGGCGTGTTGG
alAspSerValPheIleThrHisGluHisSerAspHisValArgGlyValGlyValLeuA
```

Figure 5.7   The nucleotide sequence of the transcriptional control region of ORF 3.

```
       1210         1220        1230        1240        1250        1260
GCGTTTACTSTGTGGAGTCGATGTAAGCCCTTGGTCGCAATGGTAAGCTCGCTCATTACG


       1270        1280        1290        1300        1310        1320
TCCGAGGCCACTAATGTTCTGATTTACGCCCATACAGTGCACGGTGTAAGGACCTCAGTC


       1330        1340        1350        1360        1370        1380
GCAAGATCCAAGCCTGGTCATCACGCCTGAGGCAACTTACACTCCGATCCCTAAACGCGC


       1390        1400        1410        1420        1430        1440
CGGTTCGCGCTCACGAAAAAATACCGTAGTTGTTCATAGTTTCGTCAAAATCGTCACTTA


       1450        1460        1470        1480        1490        1500
CAATAATCTTTGTAAACAGGGTGAGTTAGAATGCCCGTAAGGAGGAAACCGTTATGGATG
                                                               Met

       1510        1520        1530        1540        1550        1560
AACGGAAGTCATAACTTTCAGGAGGAGCGCACCACCGAAGCGCAACGGTCGGCGGAAAAA
AsnGlySerHisAsnPheGlnGluGluArgThrThrGluAlaGlnArgSerAlaGluLys

       1570        1580        1590        1600        1610        1620
CCGAAGAAGATGGTTGAAGCAGACTGCAATTGTTGCTGTTGTGGCTGCCTTGATTGGTGG
ProLysLysMetValGluAlaAspCysAsnCysCysCysGlyCysLeuAspTrpTrp

       1630        1640        1650        1660        1670        1680
CGTGTTGGTGGTGGTACTGCCTATTGGGCGATCAACCATACGACAAGCCTTGGCGTGACC
ArgValGlyGlyGlyThrAlaTyrTrpAlaIleAsnHisThrThrSerLeuGlyValThr
```

Figure 5.8 **Compilation of promoter sequences from Gram-positive organisms**.

(adaption from Graves & Rabinowitz, 1986).

```
            -45            -35                     -15    -10    -5
 1   TATAATAAACTA TTGACA CCGATATTACAATTGTAA TATTAT TGATTTA
 2   TATAGAAAAGTG TTGAAA ATTGTCG AACAGGGTGA TATAAT AAAAGAG
 3   TACAAAAAGGTA TTGACT TTCCCTA CAGGGTGTGT AATAAT TTAATTA
 4   TGCAAAAAGTTG TTGACT TTATCTA CAAGGTGTGG CATAAT AATCTTA
 5   TTTAAATTTTAT TTGACA AAAATGG GCTCGTGTTG TACAAT AAATGTA
 6   CATGAAGTCTCC TTGAAA TCAGAAG ATATTTAGGA TATATT TTTCTATG
 7   CCGAAAAAACGG TTGCAT TTAAATC TTACATATGT AATACT TTCAAAG
 8   CCCGATTTATCA TTGACA AAAAATACTGAATTGTAA TACGAT AAGAACA
 9   TCACTCATCATG TTCATA TTTATCA GAGCTCGTGC TATAAT TATACTA
10   TACATATAAAGG TTTATT CTAAAAT GAAAAGATGA TACAAT CATATTCAGTTA
11   AATAAAGGGGGG TTGTTA TTATTTT  ACTGATATG TAAAAT ATAATT
12   CAAAAAAGGGCA TTGATT TCAGAGCACCCTTATAAT TAGGAT AGCTTT
13   ACTTGGATTAGG TTGACG CTTTTTTGTCCATTACTG TATAAT ATTTCTATCTA
14   TTTAAAAAACTA TTGCAA TAAATAA ATACAGGTGT TATATT ATTAAACG
15   TGAAAAAGTTG TTGACA AAAAAGA ACGTGAATGT TATATT AGTAA
16   GTGTCATAACCC TTTACA GTCATAA  AAATTATGG TATAAT CATTTCTG
17   TTCAAAAAAGTA TTGACC TAGTTAA CTAAAAATGT TACTAT TAAGTAG
18   TGATATTATGTA TTGACT TAGACAA CTGAAGGTGT TATTCT AATATCG
19   GCTAAAAAGTTG TTGACA GTAGCGG CGGTAAATGT TATGAT AATAAAG
20   ATAAATTACACT TTTAAA AAGTTTA  AAAACATGA TACAAT AAGTTATG
21   GCACTAGTGAAG TTGACA ATGAATAGGTAACGCAAA TATAAT AAGTAAGACTG
22   AAAAAAATAAGG TTTCGA AAGTTGA  AAGGTATGG TATCCT ATTATGG
23   TTGTGACAACCA TTGCAA GCTCTCG TTTATTTTGG TATTAT ATTTGTG
24   AAATCCACAGGC CCTACT ATTACTTCTACTATTTTT TATAAA TATATA
```

25	CAAGTTTTCCCT TTCCCT	AATTCGT	TTTTTTTTAG	TACAAT TAGATATTAGT
26	GACAAAGAACTT ATACGG	TGGGACA	AAGACTATGC	TAAAAT AGAGGGA
27	TAGCAATTTAAT GTGTAT	AATCATA	AGTTTATTGA	TATAAT GGAGAATA
28	TACAGAACCAAA TTCAAA	AATTATA	TGGAGATCTG	TATAAT AAAGAATAA
29	TTAGAAATGGGC GTGAAA	AAAAGCG	CGCGATTATG	TAAAAT ATAAA
30	GTGTCAGTGTCC TTGCCAGCAGCAGCTCAGGTAACGCAACCTATAAT GAACG			
31	TCACTCGAACAG TTATGA	AAAACCG	TAATGACAG	TTAAGT GGTAGA
32	ATACCGTAGTTG TTCATAGTTTCGTCAA AATCGTCACT			TACAAT AATCTT

```
           -45          -35                      -15    -10  -5
```

Gram -	Ta AAAAA	TTGAcA	a A	a T TG	TATAAT AAtAt
E. coli	a	t TTGACa t t		t tg	TAtAAT

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 <u>small letters</u>, those more than 50% in <u>capital letters</u>, and those more than 75% are in <u>tall capital letters</u>.

Sequences are from:-

1.   *S. aureus* β-lactamase (McLaughlin *et al*, 1981)

2.   *B. subtilis* phage φ29G3b (Murray & Rabinowitz, 1982)

3.   *B. subtilis* spo1-15 (Lee & Pero, 1981)

4.   *B. subtilis* spo1-26 (Lee & Pero, 1981)

5.   *B. subtilis* veg (Moran *et al*, 1982)

6.   *B. subtilis* tms (Kroyer & Chang, 1981; Moran *et al.*, 1982)

7.   *B. licheniformis* penP (Kroyer & Chang, 1981)

8.   *B. subtilis* trp operon (Shimostu & 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* rrnO p1 (Ogasawara *et al*, 1983)

17.   *B. subtilis* rrnO p2 (Ogasawara *et al*, 1983)

18.   *B. subtilis* rrnA p1 (Ogasawara *et al*, 1983)

19.   *B. subtilis* rrnA p2 (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)

29.   *B. subtilis* P43 (Wang & Doi, 1984)

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.

```
     -45         -35                                    -10     -5
1.  GCGTCAAGC TGGTCA CAACAACT      GGTCGGCAA TACATT GAATTA
2.  AATAAAACG CTTTAC TTTTTTAT      TTTTCAAGGT TATAAT GAAAAT
3.  TTTTCGACT TTGACA TGAAGGAT      ATTAAGTTGC TTAATT ACGATC
4.  AGATGCTTT TTCACA GAGGCA        TCTTGTA TACGGT GAGTGG
5.  ACGGTCAAA TTAAAC GGGACTGGC     TTTGTCAGC TATGTT GAAGAG
6.  TCAGTGTCC TTGCCA GCAGCAGCTCAGGTAACGCAACC TATATT GAAACG
7.  CTCGAACAG TTATGA AAAACCG       TAATGACAG TTAAGT GGTAGA
8.  CCGTAGTTG TTCATA GTTTCGTCA  AAATCGTCACT TACAAT AATCTT
9.  CAATAATTT TAAACA GAATCATT      TATACTATA ATTAAT AAAAAC
Gram +ve        TTGACA                    T    TATAAT AAtat
```

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:-

1. *L. casei* pbg gene (Porter & Chassy, 1988).
2. *Lactobacillus* 30a histidine decarboxylase gene (Vanderslice *et al*, 1986).
3. *L. casei* dihydrofolate reductase gene (Andrews *et al*, 1985).
4. *L. casei* D-2-hydroxyisocaproate dehydrogenase gene (Lerch *et al*, 1989).
5. *L. bulgaricus* β-galactosidase gene (Schmidt *et al*., 1989).
6. *L. leichmannii* ORF 2a (this work).
7. *L. leichmannii* ORF 2b (this work).
8. *L. leichmannii* ORF 3 (this work).
9. *L. helveticus* subsp. *jugurti* cryptic plasmid pLJ1 (Takiguchi *et al*., 1989)

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 analagous 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.
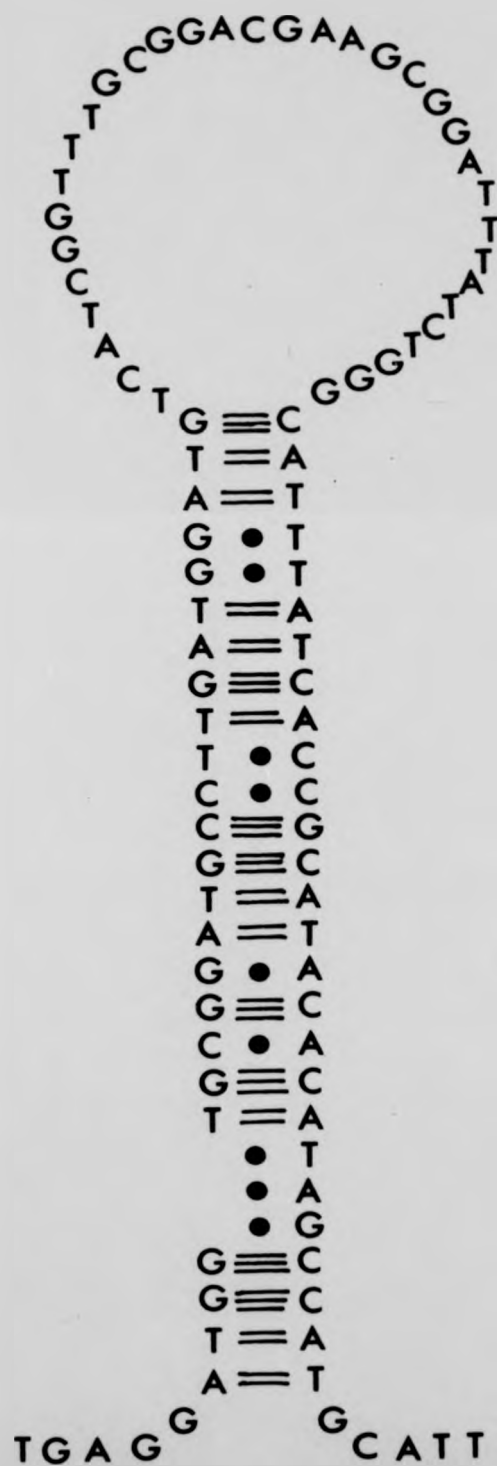
Figure 5.10b <u>Structure of a second putative Rho-independent terminator of ORF 2.</u>

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.

		1	2	3	4	5	6	7	8	9	10
UUU	Phe	26	7	7	0	7	7	3	17	4	10
UUC	Phe	23	1	1	1	1	1	0	8	0	3
UUA	Leu	14	7	7	1	3	1	1	3	4	8
UUG	Leu	24	3	7	1	7	5	5	12	2	8
CUU	Leu	12	0	4	1	2	1	5	4	1	4
CUC	Leu	12	0	1	0	1	1	0	3	1	1
CUA	Leu	4	0	0	0	2	2	0	13	1	3
CUG	Leu	42	3	0	1	2	1	1	7	1	3
AUU	Ile	14	3	15	3	3	1	2	16	1	4
AUC	Ile	13	2	5	2	5	4	2	7	1	6
AUA	Ile	0	0	0	0	1	0	5	1	0	3
AUG	Met	12	3	12	2	8	5	6	11	2	3
GUU	Val	13	8	14	2	7	7	8	11	2	2
GUC	Val	30	5	0	4	3	1	4	4	0	4
GUA	Val	9	1	2	1	3	2	3	2	1	3
GUG	Val	11	2	1	1	9	5	3	12	0	4
UCU	Ser	9	0	5	0	0	0	1	4	2	1
UCC	Ser	16	1	2	1	2	2	1	2	2	3
UCA	Ser	5	0	9	0	5	3	4	5	1	5
UCG	Ser	1	0	0	2	1	1	1	4	0	0

CCU	Pro	8	3	4	1	5	4	0	3	1	0
CCC	Pro	6	1	0	0	0	0	0	3	0	2
CCA	Pro	18	2	14	0	1	1	2	7	1	2
CCG	Pro	16	2	0	1	1	1	1	9	1	3
ACU	Thr	10	1	12	1	2	2	1	2	0	1
ACC	Thr	18	7	1	0	3	2	7	10	2	2
ACA	Thr	3	2	3	2	1	1	3	8	1	5
ACG	Thr	8	4	0	2	7	6	2	5	0	5
GCU	Ala	28	6	17	4	1	1	2	9	2	10
GCC	Ala	39	1	0	1	7	5	3	17	8	8
GCA	Ala	8	3	9	1	6	4	5	5	1	4
GCG	Ala	6	5	1	2	4	4	6	10	1	4
UAU	Tyr	18	5	7	3	3	2	1	19	3	8
UAC	Tyr	36	0	8	2	3	3	0	6	2	5
UAA	ocher	1	1	1	1	0	0	0	1	1	0
UAG	amber	0	0	0	0	0	0	0	0	0	1
CAU	His	3	6	1	0	7	7	3	15	1	5
CAC	His	15	1	1	0	1	0	0	6	0	4
CAA	Gln	11	3	9	1	2	2	4	18	2	17
CAG	Gln	27	6	0	2	4	4	2	6	1	3
AAU	Asn	19	2	4	6	1	1	3	13	3	5
AAC	Asn	30	1	8	1	1	0	5	1	2	7
AAA	Lys	22	5	3	3	4	2	6	12	4	13
AAG	Lys	43	4	17	0	7	4	7	13	6	5

GAU	Asp	25	14	15	3	12	11	1	25	3	8
GAC	Asp	46	1	10	2	5	5	2	12	1	5
GAA	Glu	62	5	22	2	7	5	3	23	6	7
GAG	Glu	16	2	0	0	1	0	3	10	2	4
UGU	Cys	1	0	1	0	1	1	3	1	0	2
UGC	Cys	4	0	1	0	0	0	3	1	0	3
UGA	opal	0	0	0	0	1	1	1	0	0	0
UGG	Trp	2	4	6	2	0	0	5	10	1	5
CGU	Arg	2	2	10	4	4	3	1	5	4	9
CGC	Arg	12	3	0	0	1	1	1	3	2	4
CGA	Arg	1	1	0	1	6	5	1	4	0	3
CGG	Arg	23	2	1	0	2	2	1	5	1	0
AGU	Ser	5	2	4	4	4	2	2	6	3	3
AGC	Ser	24	1	2	0	5	2	2	3	0	7
AGA	Arg	5	0	0	0	1	1	2	0	0	2
AGG	Arg	1	0	0	0	1	1	3	0	0	4
GGU	Gly	11	5	25	0	6	3	4	10	1	6
GGC	Gly	37	5	3	1	7	5	6	13	0	2
GGA	Gly	9	0	0	1	0	0	2	4	1	3
GGG	Gly	19	0	0	0	4	3	0	6	0	1

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

2. dihydrofolate reductase gene from *L. casei* (Andrews *et al*, 1985).

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).

8. β-D-phosphogalactoside galactohydrolase (pbg) gene from *L. casei* (Porter & Chassy, 1988).

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 *Hind*III *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

	ORF 2a	ORF 2b	ORF 3	ORF 1
Acidic (Asp + Glu)	21	25	9	7
Basic (Arg + Lys)	19	26	22	8
Aromatic (Phe + Trp + Tyr)	13	14	9	8
Hydrophobic (Aromatic + Ile + Leu + Met + Val)	49	70	54	27

This data is backed up by the hydropathy profiles, presented in Figure 5.11. These plots were performed, using a sub-routine 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 *Hind*III fragment and the *btu* genes (*btu*B and *btu*CED) 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* *btu*B 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 _Hind_III 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

ORF2

ORF3

HYDROPATHIC INDEX
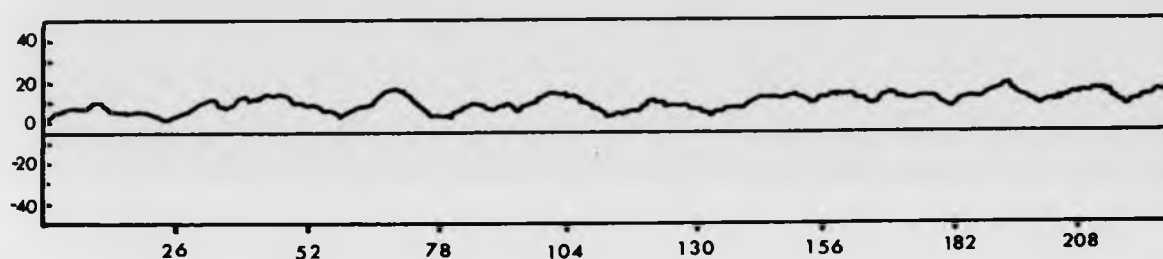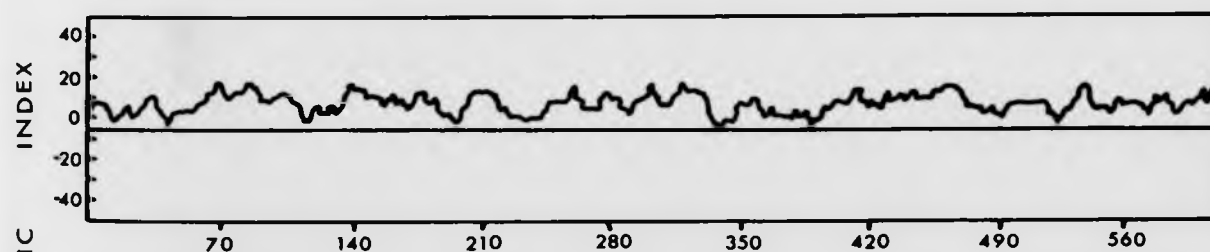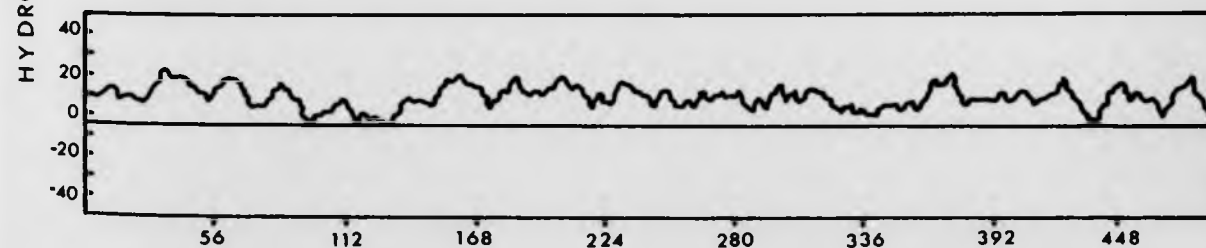
SEQUENCE NUMBER

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 *btu*B dependent system, but also another cobalamin uptake system not present in *E. coli* (Rioux & Kadner, 1989b). It is unlikely that this system is *ton*B 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 *btu*B 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 <u>Comparison of amino acid sequences</u>.

		Residue							
E. coli BtuB	507	Arg		Asn	Ala	Ile	Thr	Asp	Thr
L. leichmannii ORF 1	61	Arg	Val	Asn	Ala	Ile	Thr	Arg	Thr

significant as Moir *et al.* (1987) stated that, removal of the C-terminus of the *E. coli* *btu*B 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

Chapter 6   Expression analysis of the cloned *L. leichmannii* genomic DNA 2.0 Kb *Hind*III 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 (μ = 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 btu*B$^-$ 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 btu*B mutant L1-431 (i.e. no plaques formed upon infection of transformants phage with BF23). The 2.0 Kb *Hind*III insert of pSEC1 was subsequently recloned in the opposite orientation in pBR325 by restriction of pSEC1 with *Hind*III, 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 *btu*B 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 *btu*B 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 leu*B 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 *leu*B allele occurred by missense suppression. Also, the *Streptococcus pneumoniae pol*A gene can functionally substitute for the *E. coli pol*A gene (López *et al.*, 1987).

6.2.2 Purification of the Vitamin B_{12} binding protein from the cell walls of *L. leichmannii*.

A Vitamin B_{12} 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.
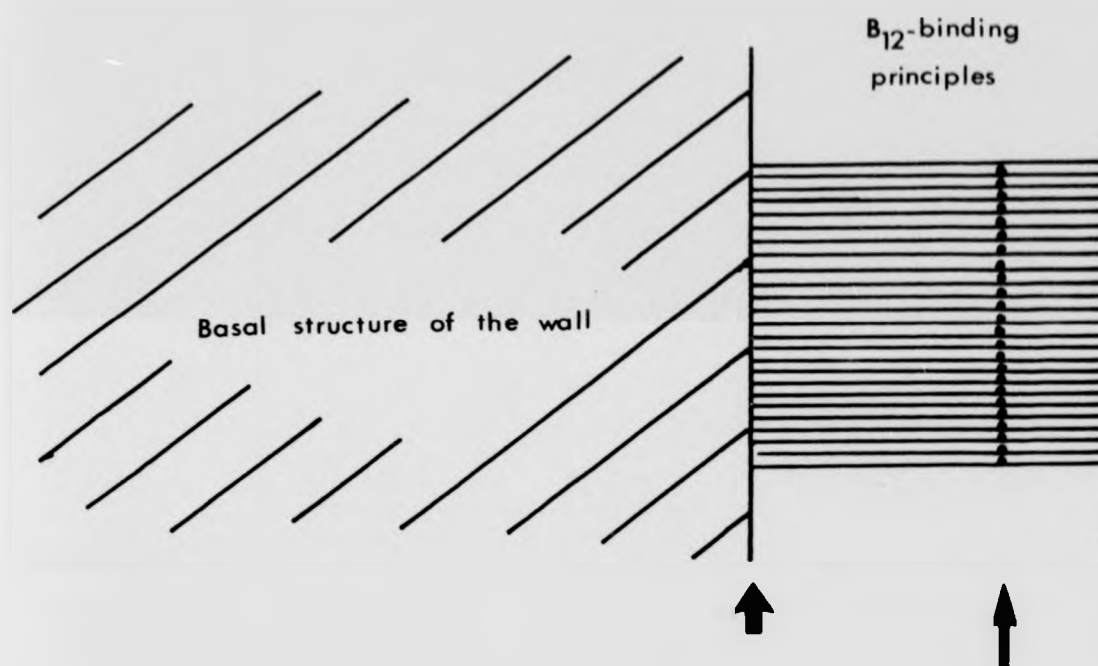
A dot represents one molecule of B$_{12}$. Arrows indicate the sites where 0.2 M HCl (➡) and pepsin (➝) acts, respectively.

Figure 6.2   **CM-cellulose column chromatography of the B_{12}-complex of the cell wall from _L. leichmannii_ 4797.**
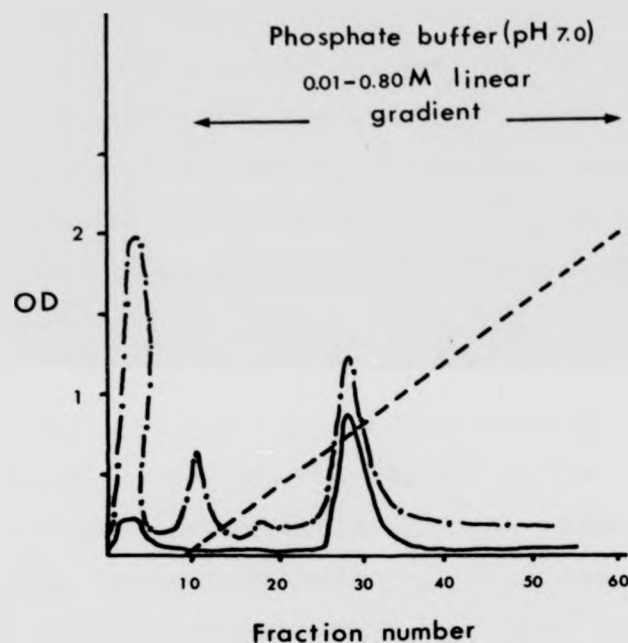
A B_{12} complex solution in 0.01 M phosphate buffer (pH 7.0) containing 0.1 M NaCl (PBN). 200 ml with optical densities : 280 nm = 1.44 and 361 nm = 0.62 was placed on a CM-cellulose column (1.5 by 20 cm) equilibrated with PBN. Elution was performed as described in Methods and Materials.

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   **Polyacrylamide gel electrophoresis of the purified B$_{12}$ complex of the cell walls from _L. leichmannii_.**
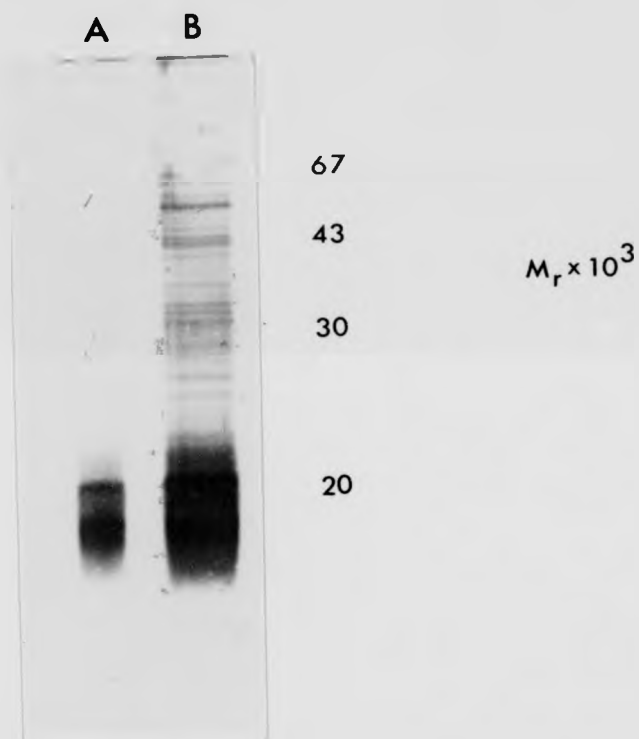
Key to tracks:-

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 B$_{12}$ complex
                of the cell wall.
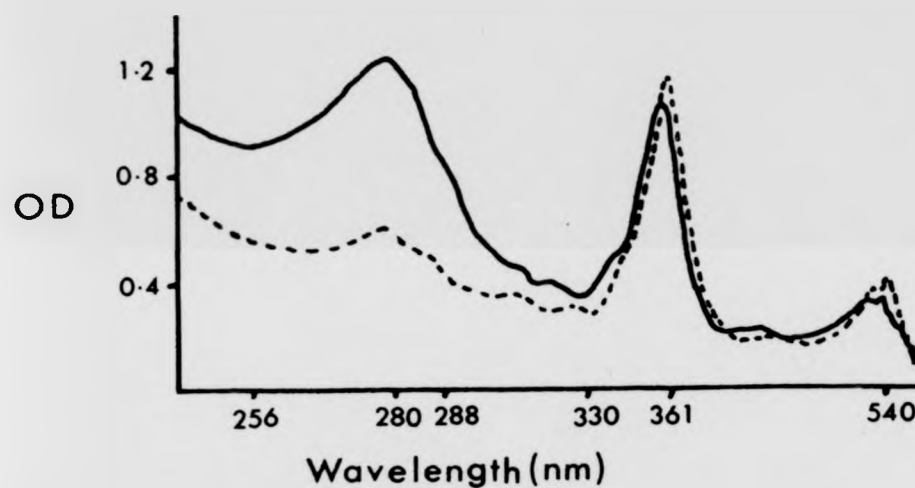
Key:-

(————) B$_{12}$ complex

(------) B$_{12}$

Absorption spectra were recorded using a Beckman DU-70
spectrophotometer.

Figure 6.5   <u>Gel-filtration on sephadex G-75 of the purified</u>
B_{12} <u>complex from the cell wall of</u>
<u>L. leichmannii</u>.
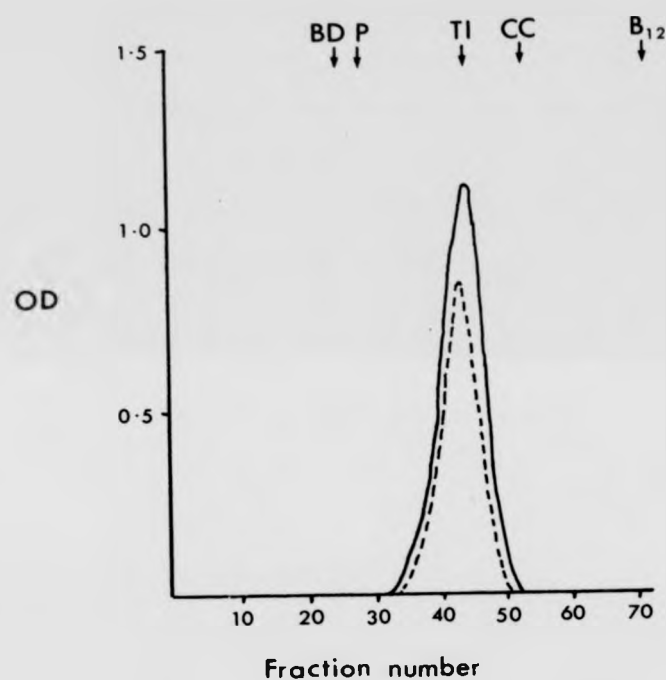
Key:-

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 B_{12} complex was required for the production of antibodies. Preparation of the free binding protein was achieved by dissociation of the Vitamin B_{12}-binding protein complex. Gräsbeck had found dissociation to occur at high pH (12.9). When the pH of the 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 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_2PO_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. coli* Vitamin 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$_{12}$ 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$_{12}$ binding protein was able to detect the *E. coli* Vitamin B$_{12}$ receptor (BtuB), shown quite clearly in (b). However, antisera raised against *E. coli* BtuB could only just detect the *Lactobacillus* B$_{12}$-binding protein. This difference in specificity could be because the antisera raised against *L. leichmannii* B$_{12}$ 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 *Hind*III fragment from *L. leichmannii* in an *E. coli* system.

There are many examples of *Lactobacillus* genes being efficiently expressed in *E. coli*; for example, β-D-

Figure 6.6   Western Blot Analysis.

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

b)   Probed with antisera
     raised against
     L. leichmannii B_{12}
     binding protein.

$M_r \times 10^3$

66

21
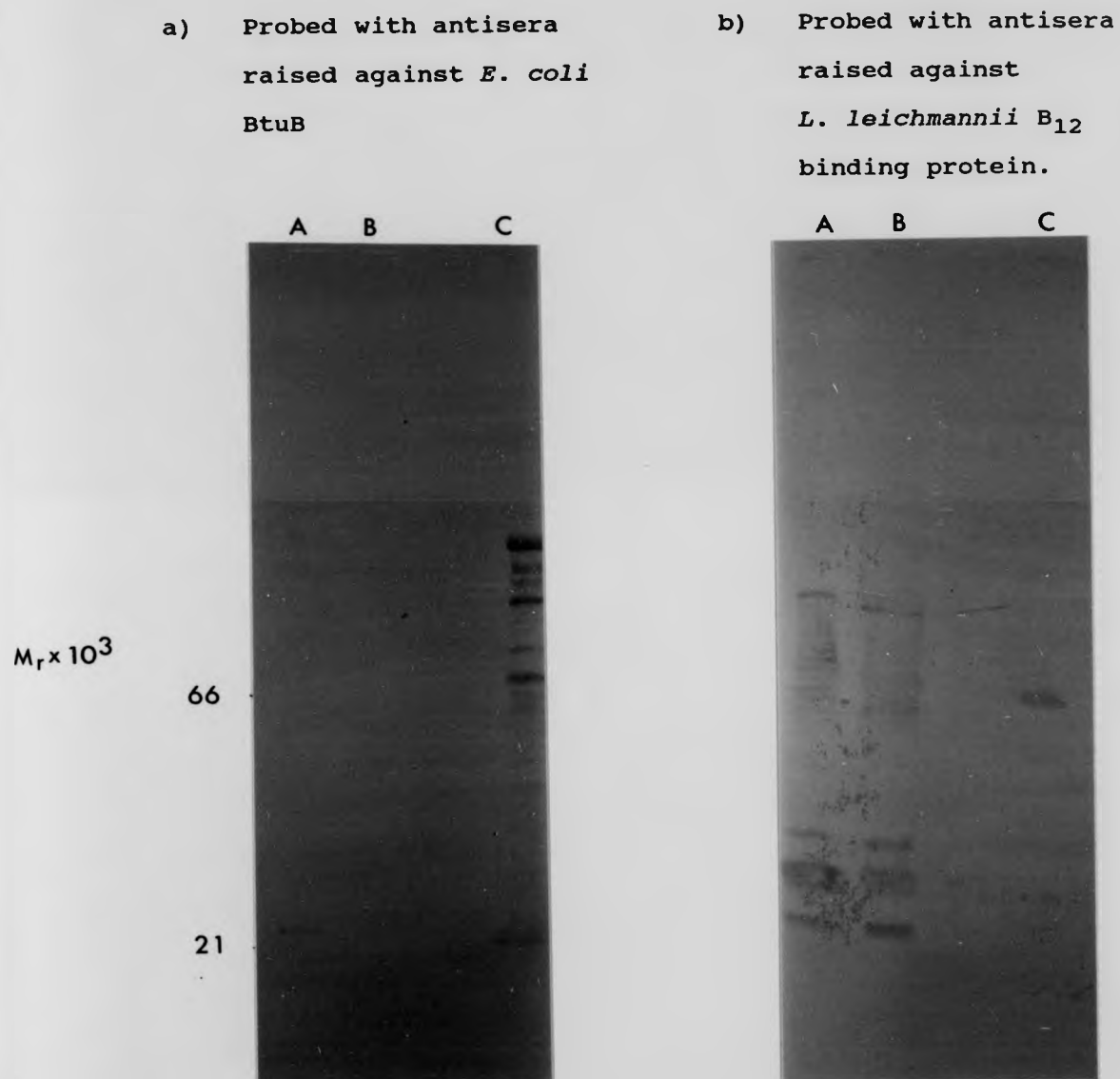
Key to tracks:

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 *Hind*III 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 M_rs of 17,388 and 17,792 respectively, whereas ORF 2b had a predicted M_r 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 *Hin*dIII 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 *Hin*dIII site.
However, since the *Hin*dIII 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 B_{12}-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 B_{12}-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 M_r-66,400 polypeptide on track A

Figure 6.7 <u>**Western blot analysis of Zubays produced**</u>
<u>**polypeptides**</u>.

a)   Western blot probed
      with antisera raised
      against *E. coli* BtuB.
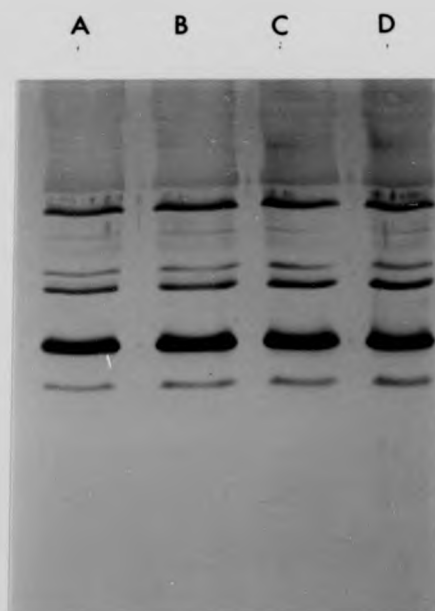
b)   Western blot probed
      with antisera raised
      against *L. leichmannii*
      B_{12}-binding protein.
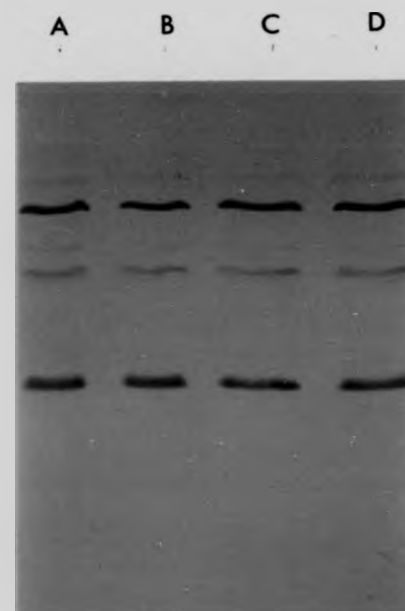
Key to tracks:

A   plasmid pNF48 (harbouring *E. coli* *btu*B 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 *Hind*III 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 *Hind*III 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 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 *Hind*III 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* 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 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 B_{12} binding protein by this method does not suggest that the 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* 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

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_o), 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).
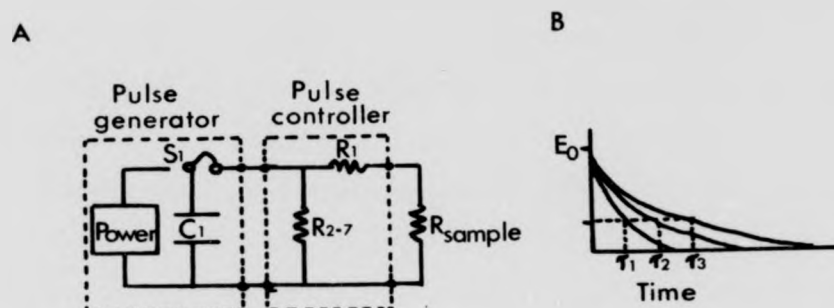
A capacitor (C_1) is charged to the desired voltage when switch S_1 places it in series with the power supply. Discharge of the capacitor across the pulse controller and sample results in an electric field that decays exponentially as shown in part B. The rate of decay is inversely proportional to the time constant (T). T (in seconds) is defined as the product of the total resistance (R_T, in ohms) and capacitance (C_1, in farads). T is also a measure of pulse duration and is equal to the time required for E_o (the initial electric field) to decline to E_o/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_o) 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 (4×10^{10} ml^{-1}) and small volumes (40 μl), 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 *Lactobacillus* and *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 *S. lactis* and *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 *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
4×10^7 CFU ml^{-1}). Electroporation of *L. leichmannii* was
carried out using a 'Gene Pulser'^{TM}, supplied by Bio-Rad
Laboratories U.S.A., set at 25 μF. 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 Ω resistor in series with the
sample, and a selection of resistors of 100 to 1000 Ω 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 Ω for these studies.

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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 pAMβ1 (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 7.2   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 *Hin*dIII DNA markers

Table 7.1 Transformation of *L. leichmannii* by electroporation.

DNA	Voltage $(KVcm^{-1})$	% Survival $\dfrac{CFU_{rec} \times 100}{CFU_{tot}}$	Frequency $\dfrac{CFU_{obs}}{CFU_{rec}}$	Efficiency $\dfrac{CFU_{obs}}{\mu g\ DNA}$
pSA3	2.50	83	6.02×10^{-8}	0.4×10^2
	5.00	74	1.01×10^{-7}	0.6×10^2
	6.25	68	2.21×10^{-7}	1.2×10^2
	7.50	50	7.00×10^{-7}	2.8×10^2
	10.00	53	-	-
	12.50	58	-	-
pC194	2.50	85	single isolate	0.5×10^1
	5.00	77	6.49×10^{-8}	1.0×10^1
	6.25	67	1.86×10^{-7}	2.5×10^1
	7.50	51	6.37×10^{-7}	6.5×10^1
	10.00	56	-	-
	12.50	60	-	-
pCK1	2.50	83	6.02×10^{-8}	0.2×10^2
	5.00	75	1.66×10^{-7}	0.5×10^2
	6.25	67	2.98×10^{-7}	0.8×10^2
	7.50	50	1.05×10^{-6}	2.1×10^2
	10.00	52	-	-
	12.50	54	-	-

DNA	Voltage $(KVcm^{-1})$	% Survival $\dfrac{CFU_{rec} \times 100}{CFU_{tot}}$	Frequency $\dfrac{CFU_{obs}}{CFU_{rec}}$	Efficiency $\dfrac{CFU_{obs}}{\mu g\ DNA}$
pAMβ1	2.50	-	-	-
	5.00	-	-	-
	6.25	-	-	-
	7.50	-	-	-
	10.00	-	-	-
	12.50	-	-	-

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. casei*. They suggested this may be due to electroporation-induced 'dechaining' of *L. 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. 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.**
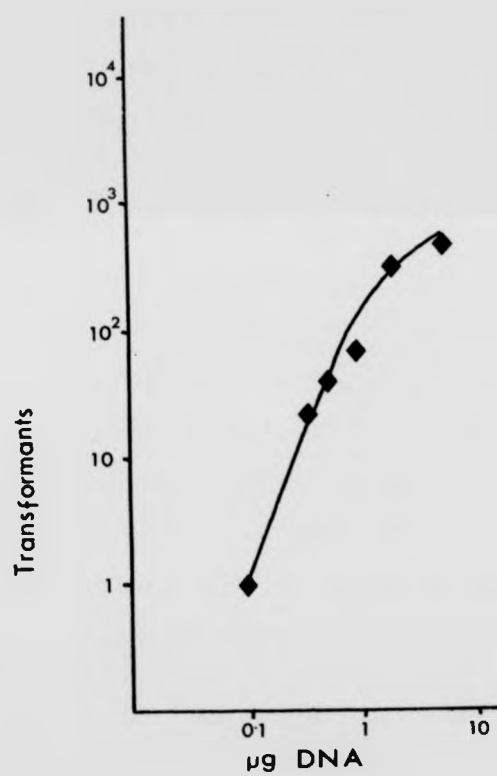
ranging from 3-5.3 msec and the higher-resistance buffer
(HEB) produced time constants ranging from 5.3-14.4 msec.

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 $CaCl_2$ (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).
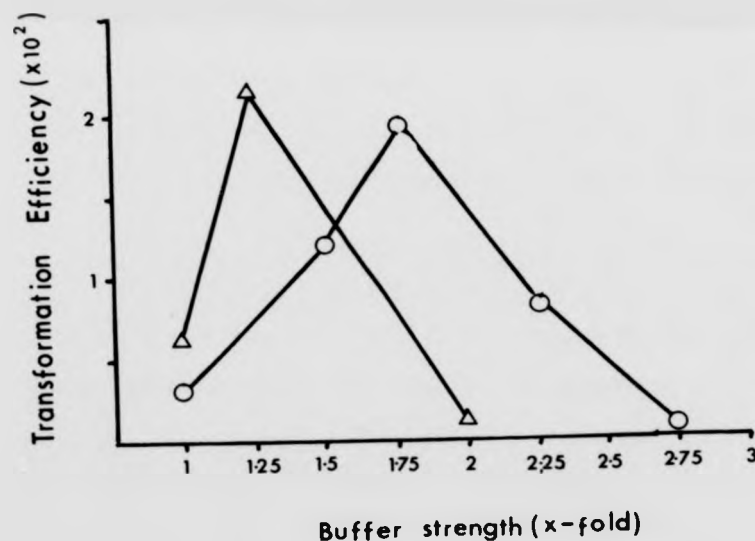
(200 Ω) were kept constant, since these particular values have been used in several successful bacterial electroporations to date.

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* (*btu*B) 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 btu*B 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 btu*B 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 *Hind*III *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 btu*B 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 *btu*B 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 B_{12} binding protein in *L. leichmannii* can act as a receptor for phage BF23, as does BtuB in *E. coli*.

The B_{12}-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 B_{12} 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 B_{12} 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 B_{12} deficiency assay system in current use.

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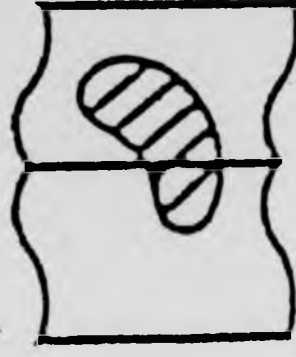
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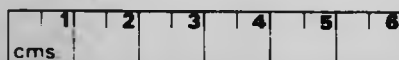
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