EXPRESSION, PURIFICATION AND CHARACTERISATION OF ANTIMICROBIAL PEPTIDES OF HUMAN AND BOVINE LACTOFERRINS

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Venkata Siva Prasad Sarma Rallabhandi

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Abstract

Lactoferrin (Lf), a basic, ~80 kDa iron-binding glycoprotein, is a member of the transferrin family. It is present in the milk and other secretory fluids of many, but not all, mammalian vertebrates. Several biological functions have been ascribed to this protein. These include bacteriostastis, modulation of the inflammatory response, iron nutrition, a role as an anti-oxidant and regulation of myelopoiesis.

Full-length human lactoferrin has been expressed in BHK cells, many strains of *Aspergillus* and with limited success in *Saccharomyces cerevisiae*. The main aim at the start of this project was to express full-length human lactoferrin (hLF) cDNA in the yeast *Kluyveromyces lactis* on whey-based media. Yeasts of the genus *Kluyveromyces* have been used for many years in the food industry and are classified as "Generally Regarded As Safe" (GRAS) organisms. *K. lactis* has impressive secretion capabilities and can grow on whey-based media (which is abundantly available in New Zealand). Attempts were made to sub-clone full-length hLF cDNA into the *K. lactis* vector, pEPS1 and the *S. cerevisiae* vector, pYEXS1 and to express the protein. The establishment of stable insert-carrying constructs of these yeast vectors in *E. coli* turned out to be an unattainable goal. Direct transformation of the ligation mix into *K. lactis* produced transformants, which secreted human lactoferrin protein products into the media as assessed by the lactoferrin ELISA assay. The secretion of hLF protein products by recombinant *K. lactis* continued for few generations, but gradually stopped. There are no known reports on the use of these vectors for the expression of any mammalian proteins in yeast.

Lactoferrin has antimicrobial activity against a broad range of Gram-negative and Gram-positive bacteria and against fungi. Originally, the antimicrobial effect of lactoferrin was attributed to its ability to tightly sequester two atoms of iron and hence inhibit microbial growth through nutritional deprivation of iron. Recently, an N-terminal peptide called lactoferricin, isolated from the acid-pepsin hydrolysate of lactoferrin was

shown to have greater antimicrobial activity than the intact protein.

Currently, the only way to obtain native lactoferricins is to isolate the peptides from the acid pepsin-hydrolysate of lactoferrin, which gives very low yields, or to synthesise them by protein chemical methods, which is very expensive on a large scale. So, heterologous expression of both human and bovine lactoferricins in *E. coli* was attempted in this study. Synthetic DNA fragments encoding both human and bovine lactoferricins and 3′-end variants of these fragments were sub-cloned into *E. coli* expression vectors, pPROEXHTa, pET-15b and pGEX-4T1. The constructs were designed to express lactoferricins either as poly-His- or as GST-fusion proteins. In all cases the fusion proteins were expressed as inclusion bodies. The inclusion bodies were urea solubilised and purified on appropriate affinity resins. However, none of the recombinant proteins remained soluble after the urea was removed and therefore could not be further characterised. A similar situation was encountered by other investigators who attempted to express cationic peptides in *E. coli.*

Both lactoferrin and lactoferricin have been shown to bind to the lipid A portion of the bacterial cell wall lipopolysaccharide (LPS), induce the release of LPS and kill the bacteria. In this work, five different *E. coli* strains were shown to have different susceptibility to native lactoferricin B. Transmission electron microscopy studies of the *E. coli* strains treated with lactoferricin B revealed considerable differences in their membrane ultrastructure. The most susceptible *E. coli* strain showed a direct outer membrane dislocation and effect on the cytoplasmic contents. A relatively resistant *E. coli* strain showed an initial formation of 'membrane blisters'. However, after a long exposure to lactoferricin B, a few cells of this strain showed an outer membrane dislocation and effect on the cytoplasmic contents. The formation of 'membrane blisters' might allow the relatively resistant strain of *E. coli* to reduce the lethal action of lactoferricin B.

List of publications and presentations

This work has been published or presented in part in the following papers.

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Abbreviations

amp or Ap	ampicillin
ARS	autonomous replicating sequence
ATP	adenosine triphosphate
A_x or OD_x	absorbance at x nm
BHK	baby hamster kidney cells
bLF	bovine lactoferrin
bp or bps	base pairs
BRL	Bethedsa Research Laboratories
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
cpm	counts per minute
C-terminal	carboxy terminal
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double-stranded

DTT	dithiothreitol
dUTP	deoxyuridine triphosphate
EDTA	ethylene diamine tetra acetic acid
EEO	electroendosmosis
ELISA	enzyme-linked immunosorbant assay
GM	granulocyte macrophage
GM-CSF	granulocyte macrophage colony stimulating factor
GRAS	generally regarded as safe
HEPES	N-2-hydroxy ethyl piperazine-N'-2-ethane sulfonic acid
hGH	human growth hormone
hLF	human lactoferrin
HPLC	high performance liquid chromatography
hTf	Human transferrin
HSA	human serum albumin
Ig	immunoglobulin
IPTG	isopropyl-D-thiogalactopyranoside
Kan	kanamycin
L or l	litre
LB	Luria-Bertani
Lf	Lactoferrin
LFcin-B	Bovine lactoferricin
LFcin-H	Human lactoferricin
Lfn	the amino terminal half of human lactoferrin
LMP	low melting point
MCS	multiple cloning site
MES	2 (N-morpholino) ethane sulphonic acid
MM	minimal medium
MOPS	morpholinopropane sulphonic acid
MW	molecular weight
MWCO	molecular weight cut off
N-terminal	amino terminal
NTA	nitrilotriacetic acid

OD	optical density
ORF	open reading frame
ori	origin of replication
PAGE	polyacrilamide gel electrophoresis
PB	phosphate buffer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
psi or lb/in^2	pounds per square inch
RIA	radio immuno assay
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecylsulphate-polyacrilamide gel electrophoresis
ss	single-stranded
SSC	standard saline citrate
sTf	serum transferrin
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TCA	trichloroacetic acid
TE	Tris (10 mM) EDTA (1 mM), pH 8.0
TEMED	N,N,N′,N′-tetra methyl ethylene diamine
Tris	Tris- (hydroxy methyl) amino methane
TTP	thymidine triphosphate
UV	ultraviolet
YEPD	rich glucose medium
YEPL	rich lactose medium
X-gal	5-bromo-4-chloro-3-indolyl -D-galactopyranoside

Additional Abbreviations

APR	Acute Phase Reaction
EGTA	Ethylene Glycol-bis (β-aminoethyl Ether) N,N,N',N'-Tetraacetic Acid
GST	Glutathione S-Transferase
HCMV	Human cytomegalo virus
hrLF	human recombinant Lactoferrin
IL	Interleukin
KN	Korean Native
Lbp	Lactoferrin binding protein
LFcin	Lactoferricin
MIC	Minimum Inhibitory Concentration
NK	Natural Killer
OPD	O-Phenylene Diamine
PIPES	Piperazine N,N'-bis[2-ethanesulphonic acid]
PMN	Polymorphonuclear
PMNs	Polymorphonuclear leukocytes
PVDF	Polyvinylidene difluoride
RES	Reticuloendothelial system
RNAse	Ribonuclease
TB	Tris buffer
TNF	Tumour Necrosis Factor
YPD	Yeast extract Peptone Dectrose (rich glucose medium)

Abbreviations of units

°C	degrees Celsius
g	gram(s)
Hr or hr	hour(s)
kb	kilobases
kDa	kilodaltons
L	litre(s)
M	molar
min	minute(s)
m	metre(s)
rpm	revolutions per minute
% (w/v)	percentage weight by volume
%(v/v)	percentage volume by volume
µf	microfarads
Ω	resistance in ohms
s	second(s)
V	volts

All living organisms require transition metal ions for essential metabolic functions. This is because of the interaction these metals have with oxygen and their ability to participate in donor-acceptor complex formation (co-ordination). These metals are essential micronutrients utilised in almost every aspect of normal cell function. In particular, the iron group consisting of iron, copper and zinc are involved in a variety of biological reactions. Out of the elements of this group of transition metals, Iron (Fe) is undoubtedly the most biologically significant element, because of its abundance in nature. Iron has two stable oxidation states in aqueous solution. They are the ferrous, Fe (II) or Fe^{2+} state and the ferric, Fe (III) or Fe^{3+} state and many biochemical pathways of living cells require one of these two states for electron transport, detoxification, biosynthesis and oxygen transport. Ferric ion, Fe (III) is essentially the favoured state of iron under the physiological conditions that prevail inside the cell. However, the Fe (III) form of iron is practically insoluble at physiological pH and if the concentration of uncomplexed Fe (III) ion exceeds 10^{-17} M in neutral solution, it is prone to rapid hydrolysis and ultimately forms insoluble ferric hydroxides (Aisen and Listowsky, 1980). On the other hand, ferrous (Fe^{2+}) ions are capable of catalysing the production of highly reactive species, such as the hydroxyl radical (.OH) (Haber and Weiss, 1934), which are capable of rapidly destroying cellular functions and reducing cell viability. Consequently, living cells face the problem of maintaining adequate amounts of iron in a non-toxic and bio-available form. To achieve this, most organisms, from bacteria to mammals, produce specialised iron-binding proteins. Most bacteria have evolved an iron chelation system based on low molecular weight chelate compounds called siderophores (Neilands, 1981), animals instead use proteins of the transferrin family for the solubilisation, sequestration and transport of ferric ion.

There are three functionally distinct groups of iron-containing proteins in vertebrates. Table1.1 provides a comprehensive list of the iron compartments in normal humans.

Table 1.1 ***Iron compartments made by groups of iron binding (containing) proteins in normal humans*** (Smith *et al.*, 1983).

Iron compartment	Iron content (mg)	Total body iron (%)
Group I		
Haemoglobin	2500	67
Myoglobin	130	3.5
Iron in the tissues (eg. Enzymes)	8	0.2
Group II (Iron storage)		
Ferritin and Haemosiderin	1000	27
Group III		
Iron transport (eg. Transferrin & Lactoferrin)	3	0.08
Labile pool	80	2.2

Group I ***The haem-containing proteins with catalytic or carrier functions*** **(haemoglobin, myoglobin and cytochromes)**

In normal humans, approximately 70% of the iron is associated with this group of iron-containing proteins, which constitutes the largest compartment of iron. They include the haem-containing cytochromes, oxygen carriers such as haemoglobin and myoglobin.

Group II ***The iron storage proteins*** **(eg. ferritin and haemosiderin)**

In normal humans, approximately 27% of the total body iron is complexed with ferritin which is the main iron storage protein. Consequently, the ferritin bound pool is the second largest compartment of iron after haemoglobin and myoglobin (Smith *et al.*,

1983). Iron in ferritin exists as an iron-oxide-phosphate complex containing twenty-four ferritin molecules which combine to form an almost spherical protein complex capable of binding approximately 4,500 iron atoms. Ferritin is generally abundant in mammalian liver, spleen and bone marrow cells and has a role in the recycling of iron for haemoglobin synthesis. Haemosiderin is an insoluble iron binding protein found predominantly in spleen, bone marrow and Kupffer (macrophage) cells of the liver. It is a partially denatured form of ferritin and may function as a long-term iron-storage compartment.

The first two groups of iron-binding proteins (Haemoglobin/ Myoglobin group and ferritin group) comprise greater than 97% of the iron stores in human body.

Group III ***Iron transport or iron-regulatory proteins***

The smallest group of iron-binding proteins is the transferrin family, characterised by their ability to reversibly bind iron with great avidity. In normal humans, only ~ 0.1% of the total body iron is found complexed with transferrins. Despite the low proportion of total iron in transferrins, these proteins are believed to play an important role in the transfer of iron between sites of storage and all other tissues. The transferrin family includes serum transferrin found in the blood of vertebrates, ovotransferrin found in egg-white and in the serum of a variety of birds, melano-transferrin in melanocytes and lactoferrin (Lf), which is found in mammalian milk and other secretions such as tears, saliva, mucosal and genital secretions (Masson and Heremans, 1966; Masson *et al.*, 1966a; Masson *et al.*, 1996). Lactoferrin is also found in some classes of white blood cells (Baggiolini *et al.*, 1970).

1.1 General properties of transferrin family of proteins

All of the secreted transferrins of higher vertebrates are typically monomeric glycoproteins with a single polypeptide chain of 650-700 amino acids and a molecular weight of ~80 kDa. They are divided into four classes based on their homology in amino acid sequence. These four classes of transferrins are homologous with between 40 and 50% identity (similarity) of amino acid sequence (Metz-Boutigue *et al.*, 1984).

Transferrins also are internally homologous with at least 40% identity between the N- and C-terminal halves. This homology is reflected in their characteristic bilobal structure (Anderson *et al.*, 1987). Transferrin binds very tightly but reversibly two Fe^{3+} ions together with two CO_3^{2-} ions. The relationship between the binding of the metal ion and the anion is synergistic in that neither is bound strongly in the absence of other.

Each lobe contains one iron-binding site, which binds one Fe (III) ion concomitantly with one CO_3^{2-} anion (Anderson *et al.*, 1989). The ligand binding amino acid residues are identical in both lobes of transferrin and are conserved in all members of the family. The single exception is melano-transferrin (formerly called p97 antigen) which, while homologous to the other transferrins, binds only one iron atom while all the other members bind two. Melano-transferrin is slightly larger due to the presence of a 25 amino acid C-terminal hydrophobic extension. This extension is believed to be involved in anchoring melano-transferrin to the plasma membrane of melanocytes (Rose *et al.*, 1986). In addition melano-transferrin does not have Fe^{3+}/CO_3^{2-} binding ligands in its C-lobe (Baker and Lindley, 1992).

The bilobal structure and homology between the two lobes suggests that the transferrin protein arose by gene duplication. The extent of identity and similarity between the transferrin proteins implies that these proteins have evolved from an ancestral 40 kDa protein to give a duplicated 80 kDa protein. Lactoferrin is a member of the transferrin family of proteins, but besides the many similarities in the functional and structural characteristics of lactoferrin and transferrin, there are also important differences between these two proteins.

1. Both proteins have an extremely high affinity for Fe^{3+} (K_{app} ~10^{20}) (Aisen and Listowsky, 1980; Brock, 1985), but the binding constant of lactoferrin for iron is 50-90 times greater than that of transferrin at pH of 6.4 (Harris, 1986).

2. The pH dependency for the release of iron is quite different between these two proteins. Complete release of iron from lactoferrin requires more acidic conditions than

does iron release from transferrin. Transferrin begins to release iron at around pH 6.0 whereas, lactoferrin does not release iron until a pH of less than 4.0 under most conditions (Mazurier and Spik, 1980). Also lactoferrin has been shown not to bind to receptors for transferrin and *vice versa* (Cox *et al.*, 1979; van Bockxmeer and Morgan, 1982). This suggests that different protein-receptor interactions may occur with these two proteins and the iron release may be stimulated by factors other than pH. Zak *et al.*, (1995) have studied the influence that mutations of kinetically active residues, involved in iron binding, have on iron release in the recombinant N-lobe of human transferrin. They have compared the release of iron from unaltered native transferrin to six N-lobe mutants of recombinant N-lobe half- transferrin (R124S, R124K, K206R, H207E, H249Y and Y95H). They showed that mutation of R124, which serves as the principal anchor for the synergistic carbonate anion accelerates iron release. They also showed that this effect was most marked at pH 5.6 (endosomal pH) and was also evident at pH 7.4 (extracellular pH). This points to a critical and perhaps initiating role of carbonate in the release process (Zak *et al.*, 1995). They have also shown that mutation of K206 to arginine or of H207 to glutamine, each lying in the interdomain cleft of the N-lobe of transferrin, gave products mimicking the arrangements in lactoferrin. Release of iron from these two mutants was substantially slower (as in the case of lactoferrin) than from unaltered recombinant N-lobe. Interdomain residues that are not directly involved in iron or anion binding might therefore participate in the control of iron release within the endosome. The H249Y mutant released iron much more rapidly than its wild-type parent or any other mutant, possibly because of steric effects of the additional phenolic ring in the binding site.

3. Lactoferrin and transferrin have a marked difference in their isoelectric point (pI). Serum transferrin has a pI between 5 and 6, while lactoferrins are strongly basic with a pI between 8.7 and 9.6 (Kinkade *et al.*, 1976).

4. Lactoferrin is more stable to denaturation by guanidine hydrochloride and other chemical denaturants than transferrin (Krysteva *et al.*, 1976). It is also more stable to heat denaturation than transferrin.

1.2 Location of lactoferrin in mammalian vertebrates

In 1939, Sørensen & Sørensen first documented the presence of a 'red protein' in bovine milk (Sorensen and Sorensen, 1939). Later in 1960, Groves isolated this protein from bovine milk and called it 'red protein' due to its distinctive salmon pink colour when saturated with iron (Groves, 1960). At the same time, Johansson reported the isolation of the 'red protein' from human milk (Johansson, 1960). Since then this red protein, now called lactoferrin or lactotransferrin or lactosiderophilin has been identified in the milk of many but not all mammalian vertebrates. Besides milk, lactoferrin has been shown to be present to a lesser extent, in other secretory fluids such as saliva, tears, seminal fluid, urine and gastric and cervical mucus. Plasma also contains lactoferrin, but at a very low concentration compared to milk (Scott, 1989). Lactoferrin was also shown to be present in neutrophils (Masson *et al.*, 1969). In these polymorphonuclear granulocytes, lactoferrin has been shown to be localised in the secondary (specific) granules (Baggiolini *et al.*, 1970). Lactoferrin has also been identified as the major oestrogen inducible uterine protein in mice (Pentecost and Teng, 1987). All known sources of lactoferrin are secretory cells. In the case of neutrophils, lactoferrin synthesis follows the same secretory pathway, but the mature protein is sequestered in specific secondary granules. The fact that lactoferrin is synthesised by a wide variety of cells suggests that this is a protein of critical importance to the organism.

There are considerable differences in the concentration of lactoferrin in the mammary secretions of different species. For example, human milk, and milk from other primates, pigs, and mice are high in lactoferrin, whereas milk from the cow and other ruminants is very low in lactoferrin. There is no lactoferrin present in the milk of rats. Species that have low lactoferrin concentrations in their milk usually have high milk transferrin levels and *vice versa* (Masson and Heremans, 1971). Also, there are differences in the concentrations of lactoferrin throughout the lactational cycle within the same species. For example, human colostrum contains concentrations of lactoferrin up to 6 mg/ml but the concentration falls to 1-2 mg/ml later in lactation (Lonnerdal *et al.*, 1976). Bovine colostrum contains lactoferrin concentrations from 1 to 5 mg/ml but this subsequently falls to concentrations as low as 0.1 mg/ml in later lactation (Smith and

Schanbacher, 1977).

The following table 1.2 details both human lactoferrin (hLF) and bovine lactoferrin (bLF) concentrations in various secretions of respective mammary glands.

Table 1.2 ***Lactoferrin concentrations in various secretions of both human and bovine mammary glands*** (Lonnerdal *et al.*, 1976) (Smith and Schanbacher, 1977).

Type of mammary secretion	**Lactoferrin concentration (mg/ml)**	
	Human	**Bovine**
Colostrum	1 to 6	1 to 5
Normal milk	1 to 2	0.1 to 0.35
Early involution	n/d	1 to 8
Thirty days of involution	n/d	20 to 30
Clinical mastitis	-----	1 to 8

(n/d = not determined)

Immunological evidence together with protein and cDNA sequence data suggest that lactoferrins from different tissues within the same species are the same (Masson *et al.*, 1966a; Moguilevsky *et al.*, 1985; Powell and Ogden, 1990; Rado *et al.*, 1987). However, there may be tissue-specific differences in glycosylation of lactoferrin, which may have implications for the function of lactoferrin in different tissues and may possibly be related to receptor recognition (Spik *et al.*, 1988).

The main focus of the work to be described in this thesis has been to examine the antimicrobial properties of the N-lobe cationic peptide fragment of lactoferrin viz. lactoferricin. Because of this focus, the literature review will concentrate on the various putative functions, which have been suggested for lactoferrin, with a particular emphasis on the bacteriostastic and bactericidal properties of the protein.

1.3 Biological functions of lactoferrin

There has been a considerable amount of work done over many years to elucidate the biological functions of lactoferrin and many physiological functions have been attributed to this protein. Although there are many contrasting reports regarding some of these biological functions, six putative functions have been identified and these will be discussed under separate headings.

1.3.1 Lactoferrin in iron absorption and nutrition

Human breast milk contains an unusually high concentration of lactoferrin, and a major proportion of iron in human milk is bound to lactoferrin. However, lactoferrin in milk is only 1- 4% iron saturated (Fransson and Lonnerdal, 1980). Though most of the evidence for lactoferrin's role in iron nutrition is indirect, based on the observation that 30 to 35% of milk iron is in lactoferrin, it has been proposed that lactoferrin has a role in iron absorption in small intestine (Lonnerdal, 1985). Later it was reported that iron in breast milk is more readily assimilated by infants than iron in cow's milk (McMillan *et al.*, 1977). Despite a relatively low concentration of iron in human milk, the human-milk fed infants maintain adequate levels of iron up to at least six months of age, longer than the infants fed either cow's milk or infant milk formulas based on bovine milk (Saarinen *et al.*, 1977). This suggests that the bioavailability of iron in human milk is much greater than cow's milk or formula milk. Although the concentration of iron in both human and cow's milk is in the range 0.2 to 0.5 μg/ml, the concentration of lactoferrin in human milk is greater than in cow's milk (Lonnerdal *et al.*, 1976). Stable radioisotope experiments in infants showed that iron absorption is higher from breast milk than from infant formula (Saarinen *et al.*, 1977). Lactoferrin in milk is mainly secreted in the iron-free or apo-form. Also, lactoferrin retains most of its bound iron at pH 4.0 (Mazurier and Spik, 1980). This led to the proposal that lactoferrin could be involved in the sequestering of free iron and retention in the infant's stomach (pH 4 to 5) for uptake in the duodenum and small intestine for the purpose of iron nutrition.

In 1979 Cox *et al.,* reported the presence of specific receptors for lactoferrin on the surface of human mucosal cells. They suggested that lactoferrin may interact with

specific binding sites on the brush-border membranes and release iron to these cells. Experiments involving ^{59}Fe and ^{125}I lactoferrin showed that iron was transported across the brush-border while intact lactoferrin was excluded (Cox *et al.*, 1979). This was in contrast to the work done by Brock and coworkers on the interaction of lactoferrin *in vitro* with the promonocytic cell line U937 and with differentiated human colon carcinoma cells. They reported that U937 cells bound more lactoferrin than transferrin but most of the binding was non-specific. Uptake of iron from transferrin by U937 cells was rapid but uptake from lactoferrin was slow. This might have been due to prior transfer of iron to transferrin in the culture medium as a result of labilisation of iron from membrane-bound lactoferrin. They also reported that unlike transferrin, lactoferrin was not internalised by U937 cells. Lactoferrin significantly reduced uptake of non-transferrin-bound iron by the cells, but had no effect on uptake of transferrin-bound iron. They also had shown that the transport of lactoferrin-bound iron across monolayer cultures of differentiated Caco-2 cells in bicameral chambers was similar to that of ferric citrate, while transport of transferrin-bound iron was detectable but, lower than in U937 cells. Lactoferrin and transferrin themselves were not transported, although some proteolytically degraded material did cross the monolayer. Thus lactoferrin, unlike transferrin, was not an important iron donor to monocytic cells, but may instead serve to regulate iron uptake from other sources (Brock *et al.*, 1994).

Lactoferrin receptors in the small intestinal mucosa in rabbit brush-border membranes have been demonstrated using a ligand blotting technique (Mazurier *et al.*, 1985). Davidson & Lönnerdal reported that lactoferrin has a specific and saturable interaction with rhesus monkey brush-border membranes. These membranes are the closest model to the human system (Davidson and Lonnerdal, 1988). The lactoferrin receptors on these membranes bound diferric monkey and human lactoferrin tightly (K_d ~9×10^{-6}), but did not bind either bovine lactoferrin or human serum transferrin. This specific binding between lactoferrin and small intestine mucous membranes was observed regardless of the age of donor monkey. The authors suggest that lactoferrin may be involved in iron uptake not only during infancy but also throughout the life span. Subsequently, putative lactoferrin receptors were identified in the brush-border

membrane vesicles from suckling pigs (Gislason *et al.*, 1994) and mice (Hu *et al.*, 1988). A putative lactoferrin receptor has been isolated from the human foetal intestinal brush-border membranes and was shown to be a glycoprotein of ~114 kDa size composed of ~ 37 kDa subunits (Kawakami and Lonnerdal, 1991). These authors also reported that the binding was pH dependent, with optimum binding occurring at pH 6.5 - 7 and also that the enzymatic deglycosylation of lactoferrin did not inhibit its binding to the receptor.

Clinical studies involving breast-fed term infants (2-10 months; mean age 5 months) fed with either whole human breast milk (with its native content of lactoferrin) or the same milk from which lactoferrin had been specifically removed (> 97%) by treatment with heparin-Sepharose showed slightly higher iron-absorption from the lactoferrin-free milk (Davidsson *et al.*, 1994). These results did not support a direct role for lactoferrin in the enhancement of iron absorption from human milk at this age. Only the youngest infant (less than 3 months old) in this study showed greater iron absorption from the complete milk. This suggests that lactoferrin may not promote iron absorption from breast milk and instead supports an earlier finding that lactoferrin inhibits iron absorption depending upon the age from early to late infancy (Brock, 1985). The effect of bovine lactoferrin on iron absorption in formula-fed human infants was also studied. No significant difference could be seen in the iron status between infants fed formula supplemented with bovine lactoferrin and those fed formula supplemented with ferrous sulfate (Chierici *et al.*, 1992; Lonnerdal and Hernell, 1994). The validity of this work could be questioned in light of the reported species specificity of the lactoferrin receptor (Davidson and Lonnerdal, 1988).

Keeping all these contradictory observations in mind, a conclusive role can not be attributed to lactoferrin in iron absorption and nutrition. In summary, lactoferrin may act as a regulator rather than an enhancer of iron absorption in infants (Brock, 1980). Also, lactoferrin may possibly help in keeping iron in solution at the neutral pH of the small intestine (Kawakami *et al.*, 1993).

1.3.2 Role of lactoferrin in growth promotion

It has been shown that iron is required for cell growth and transferrin has been characterised as an essential growth factor in serum-free systems (Dillner-Centerlind *et al.*, 1979). Later it was confirmed that transferrin promotes cell proliferation as transferrin receptors are expressed on proliferating cells both *in vitro* and *in vivo* (Casey *et al.*, 1989; Huebers and Finch, 1987). In similar studies, lactoferrin was shown to have greater growth promoting ability than transferrin in the proliferation of several human B- and T- lymphocytic cell lines and the level of lactoferrin concentration was related to the level of cell proliferation (Hashizume *et al.*, 1983). These authors also reported that human lactoferrin was an essential growth factor for the cell line and it has higher growth stimulator activity than human transferrin, and was sensitive to heat. Also long-term cultivation of the cells was achieved in a defined medium (developed by these authors) supplemented with human lactoferrin only. In these cultures bovine lactoferrin could be substituted for human lactoferrin. Milk, in particular colostrum, has been shown to stimulate proliferation of the small intestine of new born beagle puppies (Heird *et al.*, 1984) and lactoferrin is the major whey protein present in milk. This is the only report where dog milk has been reported to contain lactoferrin. Nichols *et al.*, (1990), reported that thymidine incorporation into DNA of rat crypt enterocytes was enhanced in the presence of human lactoferrin (Nichols *et al.*, 1987). This stimulation appeared to be independent of the presence of bound iron in human lactoferrin (Nichols *et al.*, 1990). Since most of the lactoferrin present in the milk is in the apo-form, it may have a role in the maturation of the small intestine in the infants. The presence of high concentrations of lactoferrin in some tumours has been suggested as supporting the role of lactoferrin in growth stimulation (Tuccari *et al.*, 1989). Mazurier *et al.,* (1989) have shown that lymphocytes stimulated by phytohaemagglutinin expressed a specific receptor for human lactoferrin and that these receptors were only expressed following a mitogenic stimulus as the resting peripheral lymphocytes are devoid of surface or intracellular receptors for human lactoferrin (Mazurier *et al.*, 1989).

In contrast, in a non-human cell line (BALB/c 3T3- mouse embryo), only iron-saturated lactoferrin, both human and bovine, stimulated growth (Azuma *et al.*, 1989).

Lactoferrin could not substitute for transferrin or allow cell proliferation of a human adenocarcinoma cell line (HT-29) in a defined serum-free medium (Amouric *et al.*, 1984) and was growth inhibitory to MAC-T bovine mammary epithelial cells (Rejman *et al.*, 1992). With Caco-2 cells (colon carcinoma), lactoferrin present in the medium has little or no effect on cell number or DNA synthesis (Brock *et al.*, 1994).

More studies are required to establish the role of lactoferrin as a growth promoter as the present body of knowledge incudes a lot of contrasting observations.

1.3.3 Lactoferrin's role as a regulator of myelopoiesis

The proliferation and differentiation of granulocyte-macrophage progenitor cells (the precursors to the phagocytic blood cells that include neutrophils and macrophages) is a dynamic process. The granulocyte and macrophage numbers are tightly regulated *in vivo* by both positive and negative feedback mechanisms and this process is dependent upon neutrophil derived molecules. Since lactoferrin is a component of secondary granules of neutrophils, it has been proposed to have a role in the regulation of myelopoiesis. Broxmeyer *et al.,* (1978) have shown that iron-saturated lactoferrin can suppress the production of granulocytes and macrophages *in vivo* in mice and also *in vitro* through negative feedback regulation of the maturation of white blood cells (Broxmeyer *et al.*, 1978). Although lactoferrin released by neutrophils is in the apo- or iron-free form, iron-saturated lactoferrin was used by Broxmayer and co-workers in all their experiments (Broxmeyer *et al.*, 1980). They suggested that the iron from serum binds the apo-lactoferrin released by neutrophils and induces a conformational change enhancing the binding of iron-saturated lactoferrin to monocytes and the suppression of granulocyte-macrophage colony stimulatory factor (GM-CSF), a 23 kDa glycoprotein present in the serum. These authors also reported that lactoferrin bound to specific receptors on monocytes and macrophages and suppressed the production of GM-CSF which is necessary for stimulation of proliferation and differentiation of myeloid progenitor cells in the bone marrow (Broxmeyer *et al.*, 1987). The same inhibitory effect of lactoferrin on myelopoiesis could not be reproduced *in vitro* (Winton *et al.*, 1981). Zucali *et al.,* (1989) observed that the synthesis and release of interleukin-1

(IL-1), a cytokine known to stimulate the production of GM-CSF is inhibited by lactoferrin (Zucali *et al.*, 1989). It was further reported that lactoferrin inhibits the production of TNF-α, an activator of IL-1 (Crouch *et al.*, 1992), and increases the synthesis and secretion of IL-6, an inhibitor of TNF-α (Machnicki *et al.*, 1993). Even though there is a general consensus that lactoferrin has a role in the regulation of myelopoiesis, the data is conflicting. Although the ability of monocytes and macrophages to bind lactoferrin has been known for many years, the nature of the interaction involved is unclear. A 43 kDa protein present in the membranes of monocytes and macrophages was suggested to be a possible lactoferrin receptor (Britigan *et al.*, 1991).

It has been reported that human apo-lactoferrin was inhibitory to the proliferation of granulocyte-macrophage progenitor cells at concentrations of 10^{-7} M whereas fully iron-saturated lactoferrin was inhibitory at concentrations as low as 10^{-17} M (Broxmeyer *et al.*, 1978). It is surprising to note that this value is much lower than the lowest concentrations of lactoferrin found in normal plasma. Also at physiological pH, transferrin and lactoferrin have essentially similar binding affinity for iron (Van Snick *et al.*, 1974) and serum transferrin is only 30% iron-saturated (Halliwell and Gutteridge, 1990) and the amount of free iron available in plasma is limited. Under these conditions lactoferrin released by neutrophils would not have an edge over transferrin in sequestering the iron. In addition Broxmeyer and coworkers used human lactoferrin with mouse cells in all their experiments which is questionable as the human lactoferrin receptors are reported to be species specific (Davidson and Lonnerdal, 1989).

Though lactoferrin may not have a direct effect on the progenitor cells themselves, since myelopoeisis is known to involve many factors, lactoferrin may be considered as one of these factors as evidenced by the available literature.

1.3.4 Lactoferrin in inflammation and immunomodulation

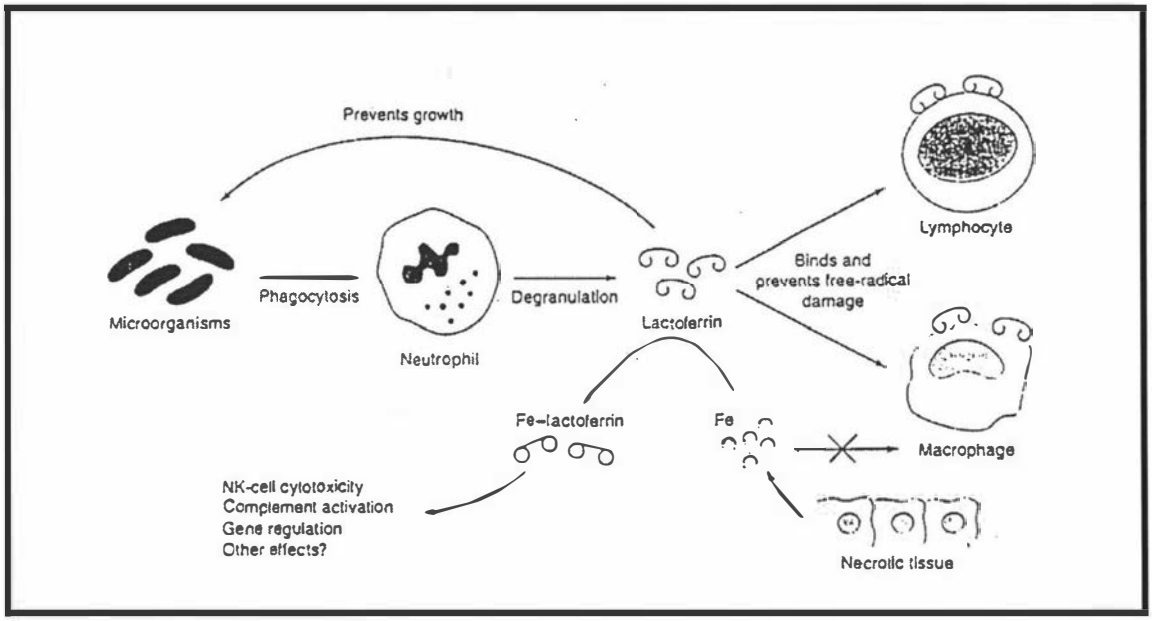
Inflammation is the basic response of the body to injury of tissues caused by physical, chemical and infective agents. Anaemia of inflammation (AoI) (previously

called anaemia of chronic disease) is seen in the setting of infectious, inflammatory, and neoplastic disease. When excess iron becomes available under certain clinical circumstances like haemolysis, trauma, haemochromatosis, the use of deferoxamine, iron supplementation or transfusion, it becomes possible for microorganisms to proliferate and cause disease (Jurado, 1997). Iron-binding proteins like lactoferrin, transferrin and ferritin are proposed to have a role in withholding this excess iron and providing non-specific immunity by sequestering iron during the pathogenesis of anaemia of inflammation. Consequently, microorganisms do not proliferate because of iron deprivation and hence no infection is caused.

It has been known for a long time that the concentration of iron in the plasma decreases with an increase in ferritin levels during inflammation. The decrease in the serum iron levels (hyposideraemia) and its accumulation in the form of ferritin in the inflamed tissues and in the reticuloendothelial system (RES) may be a non-specific defence mechanism of the host to prevent the proliferation of disease-causing micro-organisms. These iron-withholding mechanisms make the amount of iron available to bacteria extremely small, in the order of 10^{-15} M, which may be compared to the requirement levels of microorganisms which are on the order of 10^{-6} M (Jurado, 1997). Since neutrophilic leukocytes are required for the development of inflammatory hyposideremia, Van Snick *et al.*, (1974) proposed that the development of inflamatory hyposideremia is mediated by lactoferrin present in the specific granules of neutrophilic leukocytes (Van Snick *et al.*, 1974). Lactoferrin rather than transferrin can act as an efficient iron scavenger at the sites of inflammation or infection because, in an acidic environment (pH< 7.0) as found at sites of inflammation or infection, affinity of lactoferrin for ferric ions is approximately 300 times higher than the affinity of transferrin (Aisen and Leibman, 1972). Normally, the plasma concentration of lactoferrin is very low but it increases during inflammation with a concurrent decrease in neutrophil lactoferrin content (Oseas *et al.*, 1981). Lactoferrin released from the secondary granules of activated neutrophils in response to inflammation, is in the iron-free or apo-form. For these reasons, it has been proposed that the apo-lactoferrin released from degranulating neutrophils during inflammation removes the iron from transferrin and,

together with its load of iron, is selectively taken up by the RES causing accumulation of iron within ferritin (Van Snick *et al.*, 1974). Patients with lactoferrin-deficient polymorphonuclear (PMN) leukocytes have an increased susceptibility to infections (Boxer *et al.*, 1982a; Breton-Gorius *et al.*, 1980). Fig.1.1 is a schematic representation of the proposed immunoregulatory activity of lactoferrin.

Fig. 1.1 **Schematic representation of the immunoregulatory activity of lactoferrin** [adapted from (Brock, 1995)].



(Abbreviation: NK, natural killer)

On the basis of these studies, it might appear that the role of lactoferrin in inflammation is convincing. However, there are other reports that contradict this. Van Snick *et al.*, (1977) have proposed that human lactoferrin interacts with mouse peritoneal macrophages when the protein is ingested (van Snick *et al.*, 1977). During acute-phase reaction (APR), macrophages are activated and the activated macrophages exhibit an increase in the concentration of lactoferrin receptors, which increases the internalisation of iron. Thus, lactoferrin-bound iron is taken up by macrophages preferentially compared to erythroid precursors. Within the macrophage, the lactoferrin-bound iron is transferred to the iron-storage molecule ferritin, which stores iron in a

more stable form (Jurado, 1997; van Snick *et al.*, 1977). In contrast, it has been shown that iron uptake by macrophages is extremely slow (Oria *et al.*, 1988).

Birgens and co-workers confirmed the presence of specific lactoferrin receptors on the surface of monocytes. Lactoferrin receptors were found to be present on the surface of the macrophages but not on the surface of erythroid precursors (Birgens *et al.*, 1983). Once monocytes bind lactoferrin, it is not released and hence no recycling of lactoferrin occurs (Birgens and Kristensen, 1990). Smith *et al.*, (1985) reported that lactoferrin synthesis and release from the secondary granules of neutrophils is increased by IL-1, as a part of the APR (Smith *et al.*, 1985). This was contradicted by the studies of Gordeuk *et al.*, (1988), who reported that interleukin-1 (IL-1) administered to mice induces hyposideremia even in the presence of neutropenia (a deficiency of granulocytes) (Gordeuk *et al.*, 1988). This finding suggests that lactoferrin may not have a significant role in hyposidereramia resulting from acute inflammation. It was also reported that at concentrations lower than 10^{-8} M, lactoferrin has an inhibitory effect on the cytokine responses *in vitro*, suppressing the release of IL-1, IL-2 and tumour necrosis factor (TNF) (Crouch *et al.*, 1992). This indicates a regulatory role for lactoferrin in immunomodulation. This finding could be questioned as the concentration of lactoferrin (10^{-8} M) shown to have an inhibitory effect is well within the concentration range in normal plasma (10^{-9} M) (Lonnerdal and Iyer, 1995).

Machnicki *et al.*, (1993) demonstrated that feeding mice with bovine lactoferrin prior to challenging with *E. coli* induced an increase in IL-6 in serum, an inhibitor of TNF-α production (Machnicki *et al.*, 1993). Later it was reported that both human and bovine lactoferrins and lactoferricin, a cationic peptide derived from the N-terminal region of lactoferrin, have the ability to stimulate the release of neutrophil-activating polypeptide interleukin-8 (IL-8) from human polymorphonuclear leukocytes (neutrophils, PMNs) (Shinoda *et al.*, 1996). This finding suggests that lactoferrin and l actoferricin may both function as immunomodulators of the host defence system. Shau *et al.*, (1992) have reported that the natural killer (NK) and lymphokine-activated killer (LAK) cell cytotoxic functions can be strongly augmented by lactoferrin (Shau *et al.*,

1992). Iron appeared to be non-essential for this action. Natural killer cells have diverse biological properties including effects on myelopoiesis and inflammation. Recently, it has been reported that bovine lactoferrin could induce both mucosal and systemic immune response in mice (Debbabi *et al.*, 1998). From their experiments using groups of ten BALB/c mice either fed with bovine lactoferrin or not and also immunized intramuscularly with bovine lactoferrin, they suggested that lactoferrin could act as an immunostimulating factor on the mucosal immune system and that activation of the mucosal immune system was dependent on the ability of lactoferrin to bind to the intestinal mucosa. Human lactoferrin has also been shown to have anti-tumour activity (Bezault *et al.*, 1994). They reported that human lactoferrin reduced solid tumour growth and strongly inhibited experimental metastasis (lung colonisation). The effects of lactoferrin on experimental metastasis appeared to be mediated through NK cells and were independent of iron saturation.

1.3.5 Lactoferrin as an anti-oxidant

Transition metals carry out a number of biological functions in the living cell by interacting with molecular oxygen. Due to its ability to interact with oxygen and also its abundance in nature, iron has a greater participation in living systems compared to other transition metals. Though iron acts as a catalyst in numerous reactions involving molecular oxygen, it also makes these reactions a potential threat to cells by the generation of oxygen-based free radicals as follows:

$$Fe^{2+} + O_2 \rightleftharpoons Fe^{3+} + {}^{\bullet}O_2^{-}$$

$$Fe^{2+} + {}^{\bullet}O_2^{-} + 2H^{+} \rightleftharpoons Fe^{3+} + H_2O_2$$

$$Fe^{2+} + H_2O_2 \rightleftharpoons Fe^{3+} + .OH + OH^{-}$$

The free radicals ${}^{\bullet}O_2^{-}$ and .OH generated by transition metals are highly toxic to cells and have capacity to damage almost all known bio-molecules. It had been shown that lipid peroxidation and consequently hydroxyl radical formation require activated iron (Gutteridge *et al.*, 1981). In order to limit oxidative damage, the host sequesters the

available transition metals (especially iron) and stores them in a chemically less reactive form. Macrophages, for example sequester iron intracellularly and prevent cellular damage by limiting its potential to generate free radicals (Olakanmi *et al.*, 1993). Anti-oxidants are also present intracellularly as well as in extracellular fluid to prevent oxidative damage. Because of its ability to sequester iron with high affinity, lactoferrin has also been suggested to have an anti-oxidant role.

Lactoferrin is commonly found in human mucosal secretions like milk, tears, semen and plasma and also in the specific granules of PMN. Since these are the sites of interaction between the host and its external environment, lactoferrin has been implicated in the detoxification of metal chelates (Ghio *et al.*, 1998). These authors have hypothesised that the anti-oxidant function of intracellular ferritin, due to its ability to sequester transition metals, should be supported in some way by the transportation of the metal across the cell membrane in these cells and this is done by lactoferrin but not by transferrin. Upon the exposure of the respiratory epithelial cells *in vitro* to catalytically active metals in the form of residual oil fly ash (a known oxygen radical generator), it was observed that ferritin but not its mRNA concentration increased. Lactoferrin and its mRNA levels increased whereas transferrin and its mRNA levels decreased (Ghio *et al.*, 1998). Deferoxamine, a metal chelator, inhibited this process. Lactoferrin mRNA levels also increased when respiratory epithelial cells were exposed to Vanadium metal, suggesting that this process is activated by metal itself, rather than by oxidative stress (Ghio *et al.*, 1998). In contrast, there are reports that lactoferrin could efficiently provide iron to the oxygen radical generating system and that it was a good catalyst of oxygen radical production in the presence of EDTA and NTA (Ambruso and Johnston (Jr), 1981; Bannister *et al.*, 1982). But lactoferrin is a very poor catalyst for oxygen radical formation except in the presence of the strong metal chelators like EDTA and NTA. These metal chelators complex with lactoferrin and effect its properties by releasing the bound iron (Winterbourn, 1983).

From the available literature, it is likely that lactoferrin has an extracellular anti-oxidant role. However the evidence for this is not conclusive.

1.3.6 Antimicrobial activity of lactoferrin

It has been known for a long time that breast-fed infants are less susceptible to infantile diarrhoea and vomiting compared to those fed artificial milk formulas. In 1941, David Levi reported a striking difference in mortality due to congenital pyloric stenosis in breast-fed and bottle-fed infants. Gastro-enteritis is a danger to be feared in a child with pyloric stenosis whether medically or surgically treated. He concluded that breast-fed infants showed 100% cure after the operation, which was speedy, safe and certain with a total elimination of anaesthetic risk during the operation. He also concluded from his observations as follows: "A child appears to lose immunity to gastro-enteritis if once it is taken-off the breast. Never take a young infant off the breast, provided the mother is well, till the presence of pyloric stenosis has been definitely excluded. It should be regarded as axiomatic that breast milk never disagrees with an infant. This should be taught to students, nurses, midwives, and welfare and social workers of all kinds " (David Levi, 1941).

Later in 1948, Alexander, after treating a large number of infants with gastro-enteritis reported and reconfirmed that "few breast-fed infants were admitted with enteritis. Maintenance or re-establishment of breast-feeding was usually all that was required for their recovery. The majority of infants with enteritis and almost all the severe cases had been artificially fed before admission" (Alexander, 1948). This study clearly proves that human breast milk possesses some resistance factors that reduce the frequency and severity of these infections in human infants. The gut micro-flora of the breast-fed infant consists mainly of bifidobacteria, lactobacilli and staphylococci. This differs considerably from the gut micro flora of the formula-fed infant, which contains enterococci, coliforms and bacteroides (Balmer and Wharton, 1989).

Gastro-enteritis in infants is caused by some specific serotypes of *E. coli* (coliforms like 0111). These serotypes of *E. coli* identified in infants suffering from

gastro-enteritis can also be found in healthy babies, which suggests that the disease is caused by an increase in the population of *E. coli* and not by a new infection and that lactoferrin protects infants from this condition (Bullen *et al.*, 1972). *In vitro*, the growth of *E. coli* serotype 0111 (NCTC 8007), responsible for gastro-enteritis in infants, was inhibited by milk. Addition of iron to the milk permitted growth of bacteria. An extensive study of the components of human milk that confer resistance to infants against gastro-enteritis showed that purified lactoferrin is the main anti-microbial component of both colostrum and milk, that protects the infant from this condition (Brock, 1980; Bullen *et al.*, 1972; Masson and Heremans, 1966). Purified lactoferrin had an anti-microbial effect, almost equal to that of whole milk. It has been reported that patients with neutrophil lactoferrin deficiency are prone to recurrent infections (Boxer *et al.*, 1982b; Breton-Gorius *et al.*, 1980).

Recently, it has been demonstrated that the anti-bacterial activity of Korean Native (KN) goat lactoferrin was greater compared to Sannen goat lactoferrin (Lee *et al.*, 1997). This elevation in the anti-bacterial activity of KN goat lactoferrin was attributed to the six amino acid substitutions it contains when compared to the earlier published goat lactoferrin gene sequence. Lactoferrin has also been shown to have anti-viral activity (Harmsen *et al.*, 1995; Hasegawa *et al.*, 1994). Hasegawa *et al.*, (1994) have shown that both hLF and bLF inhibited the infection of tissue-cultured cells by human cytomegalovirus (HCMV) and human herpes simplex virus-1 (HSV-1). They found that lactoferrin prevented virus absorption and/or penetration into host cells by interfering with the early events of virus infection (Hasegawa *et al.*, 1994). Maximum inhibition, for both viruses, was obtained with 0.5 to 1 mg/ml of lactoferrin. The antiviral activity of lactoferrin was associated with its protein moiety, not with its iron molecule. Harmsen *et al.*, (1995) extended this work by showing antiviral activity of native lactoferrin *in vitro*, against human immuno-deficiency virus (HIV) and HCMV (Harmsen *et al.*, 1995).

Expression of bovine lactoferrin increases significantly during involution and mammary infections and the lactoferrin has an important role in the inhibition of the

growth of coliform bacteria associated with bovine mastitis (Bishop *et al.*, 1976). It was reported that the onset of involution (mainly at the beginning of the dry period) was the period of increased risk of mammary infection in cows(Neave *et al.*, 1950). It also has been reported that lactoferrin concentrations in excess of 100 mg/ml could be seen in the dry secretions of some cows (Welty *et al.*, 1976). Lactoferrin is secreted in the apo- or iron-free form, rarely exceeding 20% iron saturation (Mazurier and Spik, 1980). The anti-microbial activity of lactoferrin was thought to be dependent on its iron-free state and was commonly attributed to its ability to tightly bind and sequester iron producing an iron-deficient environment that limits microbial growth (Bullen, 1981; Bullen *et al.*, 1978). Later it was shown that iron-saturated lactoferrin or exogenous iron and apo-lactoferrin together lacked this anti-microbial property (Bishop *et al.*, 1976; Bullen, 1985; Reiter, 1983).

Citrate also appeared to reverse the growth-inhibition by lactoferrin (Reiter *et al.*, 1975). These authors have suggested that citrate competes with the iron-binding proteins for iron and makes it available to the bacteria. Also addition of bicarbonate, which is required for the binding of iron by transferrin and lactoferrin could overcome the effect of citrate. Hence, the bacteriostatic effect of cow serum and precolostral calf serum was shown to be due to the presence of both transferrin and bicarbonate as well as the low levels of citrate. (Reiter *et al.*, 1975). Lactoferrin and citrate concentrations vary significantly during involution and lactation (Smith and Schanbacher, 1977). Hence the molar ratio of citrate to lactoferrin would determine the protective role of lactoferrin during the infection of the bovine mammary gland. Table 1.3 shows the molar ratio of citrate to lactoferrin in different secretions of the bovine mammary gland. The concentration of citrate decreases as involution progresses. Conversely, a direct increase in the concentration of lactoferrin is seen in the lacteal secretion from the involuting mammary gland. This led several investigators to suggest that citrate can reverse the inhibition of bacterial growth by bovine lactoferrin (Bishop *et al.*, 1976; Smith and Oliver, 1981; Smith and Schanbacher, 1977). It has also been reported that a molar ratio of citrate : apo-lactoferrin of 75 resulted in approximately 50% growth inhibition in an *in vitro* assay and a molar ratio of 300 or greater resulted in less than 10% growth

inhibition (Smith and Schanbacher, 1977).

Table 1.3 ***Citrate to lactoferrin molar ratios in the secretions from the bovine mammary gland*** (Smith and Schanbacher, 1977)

Bovine mammary secretion	**Molar ratio of citrate to lactoferrin**
Colostrum	300
Normal milk	3000
Fully involuted dry secretion	10

There is now considerable evidence that efficient iron acquisition is a very important factor in bacterial virulence. Human pathogens like *Neisseria meningitides, Neisseria gonorrhoea* and *Haemophilia influenzae* have the capacity to use human lactoferrin and transferrin as sources of iron (Schryvers, 1988; Schryvers, 1989; Schryvers *et al.*, 1998; Schryvers and Gonzalez, 1989; Schryvers and Lee, 1989; Schryvers and Morris, 1988a; Schryvers and Morris, 1988b). These pathogens have been found to have specific iron-regulated receptors for human lactoferrin and transferrin. The bacterium *N. meningitides* has been shown to have a single ~105 kDa outer membrane protein that can bind lactoferrin independent of its iron saturation level (Yu and Schryvers, 1993). Even though both *E. coli* and *Neisseria* appear to be able to bind lactoferrin, *E. coli* acquires iron from the environment by secreting siderophores, whereas *Neisseria* utilises lactoferrin bound iron. Recent studies on isogenic mutants of *N. meningitides* deficient in either or both lactoferrin-binding proteins, LbpA and LbpB showed that LbpA, but not LbpB was essential for iron acquisition *in vitro* (Bonnah and Schryvers, 1998).

Miehlke *et al.*, (1996), have shown that recombinant human lactoferrin exhibited a direct and potent bactericidal activity in a dose-dependent manner against 8 of 13 clinical isolates of *Helicobacter pylori* proving its potential therapeutic use (Miehlke *et al.*, 1996). They also suggested that the iron concentration of the culture media does not influence the bactericidal effect of recombinant human lactoferrin (rhLF) on *H. pylori.* Recently, a 70 kDa lactoferrin binding protein (Lbp) was identified from outer membrane proteins of *H. pylori* (Dhaenens *et al.*, 1997). They demonstrated that human lactoferrin supports growth of *H. pylori* in media lacking other iron sources and that the ability of *H. pylori* to use hLF as an iron source is dependent on cell-to-protein interaction. This 70 kDa Lbp was present only when *H. pylori* was grown in an iron-deficient medium and it mediates iron uptake from lactoferrin as *H. pylori* does not produce siderophores. In direct binding assays with increasing concentrations of biotinylated hLF, they observed a saturable lactoferrin interaction with the outer membrane of *H. pylori* grown on iron-restricted medium. This Lbp appeared to be highly specific for hLF. It did not bind bLF or transferrins from non-human origin. This could be one explanation for the very strict human specificity of *H. pylori* (Dhaenens *et al.*, 1997). It has also been shown that *H. pylori* synthesised three iron-repressive outer membrane proteins (IROMPS) (77, 50, and 48 kDa) that might be involved in haem-binding and/or uptake (Worst *et al.*, 1995).

Lactoferrin (< 50 μM) has a direct bactericidal effect on strains of *Streptococcus mutans*, *Escherichia coli*, *Actinobacillus actinomycetemcomitans*, *Vibrio cholerae* and *Legionella pneumophila* (Arnold *et al.*, 1980; Arnold *et al.*, 1977; Arnold *et al.*, 1978; Arnold *et al.*, 1982; Arnold *et al.*, 1981). Saturating the lactoferrin with iron abolished this bactericidal property. The anti-microbial activity of lactoferrin against some *E. coli* strains was enhanced by concurrent exposure of bacteria to immunoglobulin G or secretory immunoglobulin A (Rainard, 1986; Stephens and Dolby, 1978). Hepatitis C Virus envelope proteins also bind both human and bovine lactoferrins (Yi *et al.*, 1997). Human lactoferrin and lysozyme have individually been shown to be fungicidal against oral isolates of *Candida albicans* and *Candida krusei,* the most common fungal pathogens encountered in the mouth (Samaranayake *et al.*, 1997). But, when they are

used in combination, no synergistic antifungal activity of the two proteins on either *Candida* species was noted. The fungicidal effect of lactoferrin was dose-dependent and observable only with the iron-free form of the molecule (apo-lactoferrin) on five oral isolates of *Candida albicans* and *Candida krusei* (Nikawa *et al.*, 1993) and iron-saturated lactoferrin was ineffective against all isolates. Bovine lactoferrin and its antimicrobial peptides have been shown to inhibit hyphal growth of azole-resistant strains of *Candida albicans* in the presence of triazole antifungal agents (Wakabayashi *et al.*, 1998a; Wakabayashi *et al.*, 1998b). Recently, it has been shown that human milk lactoferrin could attenuate the pathogenic potential of *Haemophilus influenzae* (Qiu *et al.*, 1998). Human lactoferrin exerted its antibacterial property against *Haemophilus influenzae* by removing IgA1 protease from the bacterial cell wall and also proteolytically degrading the Hap adhesin, which are the two auto-transported proteins that are presumed to be essential for *Haemophilus influenzae* colonisation. The capacity to extract IgA1 protease and degrade Hap adhesin was shown to be localised to the N-lobe of the bilobed lactoferrin and these functions were inhibited by serine protease inhibitors, suggesting that lactoferrin N-lobe might contain serine protease activity.

In 1988, Ellison *et al.*, showed that *in vitro*, human apo-lactoferrin has direct bactericidal activity by binding to sensitive Gram-negative bacteria, damaging their outer membrane by the release of lipopolysaccharide (LPS) and altering the permeability properties of the outer membrane (Ellison III *et al.*, 1988). This was quite distinct from its iron-sequestering property. They also observed that release of LPS from the outer membrane by lactoferrin enhances the susceptibility of bacteria to hydrophobic antibiotics like rifampin (an antibiotic normally excluded by the Gram-negative outer membrane at its sub-inhibitory concentration) and to lysozyme. Recently, it has been shown that human lactoferrin at the concentration found in cystic fibrosis sputum (0.9 mg/ml), lowered the Minimum Inhibitory Concentrations (MICs) of rifampicin and chloramphenicol for mucoid strains of *Pseudomonas aeruginosa* in *Pseudomonas* infections (Fowler *et al.*, 1997). They reported that MICs in the presence of human lactoferrin for some strains of *Pseudomonas* were lowered to levels comparable to clinically achievable levels of the antibiotics.

Lactoferrin binds a wide variety of metal ions with a high affinity. For this reason lactoferrin was thought to have a similar activity to chelating agents such as EDTA in altering Gram-negative outer membranes. Additionally, because of its high pI (~8.5 to 9), lactoferrin could also have an effect similar to the membrane-active polycationic compounds like defensins and magainins. As Ca^{+2} appears to be one of the major cations present in the bacterial outer membrane (Coughlin *et al.*, 1983) and since lactoferrin can not chelate Ca^{+2} (Ellison III and Giehl, 1991), it is unlikely that lactoferrin's effect on the membrane is due to its ability to act as an analogue of EDTA (Ellison III *et al.*, 1990). Lactoferrin influences the Gram-negative bacteria through a direct interaction with the bacterial outer membrane in a manner similar to poly-cationic agents, which bind directly to LPS and alter the conformation and permeability characteristics of the outer membrane. The damage to the Gram-negative bacterial outer membrane by lactoferrin (or transferrin) is modulated by Ca^{+2} and Mg^{+2}. The ions, Ca^{+2} and Mg^{+2} block the release of LPS by lactoferrin from the outer membrane of Gram-negative bacteria and also block the ability of lactoferrin to increase the susceptibility of *E. coli* to rifampin (Ellison III *et al.*, 1990).

Lactoferrin can bind directly to bacterial cells and its ability to bind relates to its anti-microbial activity. Naidu and co-workers (Kishore *et al.*, 1991; Naidu *et al.*, 1991a; Naidu *et al.*, 1991; Tigyi *et al.*, 1992) used a ^{125}I- labelled protein binding assay and showed that lactoferrin binds to strains of *E. coli*, *Staphylococcus aureus, Aeromonas hydrophila* and *Shiegella flexineri.* Lactoferrin binding was found to vary between species and also between different strains within a species. This variation could also be seen within the same species under different growth conditions, growth stage and cell density. These variations could be due to changes in accessibility of the lactoferrin-binding site on the bacterial outer membrane. Such changes could account for differences in bacterial susceptibility to lactoferrin. It appears that there may be a relationship between virulence and resistance to lactoferrin (Arnold *et al.*, 1980).

Lactoferrin has also been consistently observed to interact avidly with nucleic acids. Lactoferrin enhancement of the activity of natural killer and lymphokine-activated killer cells *in vitro* is inhibited by RNA and DNA (Shau *et al.*, 1992). Lactoferrin has been shown to be taken up by K562 human myelogenous leukaemia cells and appeared in the nucleus where it is apparently bound to DNA (Garre *et al.*, 1992). This group has shown that lactoferrin, after binding at the cell surface, was internalised in a temperature dependent manner and could be detected immunologically as a DNA-linked protein in nuclear extracts. He *et al.*, (1995) reported that lactoferrin binds to specific sequences of DNA under stringent conditions and suggested that, in the nucleus the interaction between lactoferrin and these sequences leads to transcriptional activation (He and Furmanski, 1995).

Few *in vivo* studies have addressed the anti-bacterial function of lactoferrin. Although the available data support the hypothesis that lactoferrin contributes to the host's antimicrobial defences, there are conflicting observations between studies because of methodological differences (Ellison III, 1994). Initially the antibacterial activity of iron-free lactoferrin was attributed to its ability to bind and sequester iron. Later it was shown that apo-lactoferrin has bactericidal activity and kills sensitive bacteria by a mechanism that involves its direct interaction with the bacterial cell membrane distinct from iron-sequestration. Further studies addressed the question of whether the entire lactoferrin molecule is required for the bactericidal activity or only a part of it. Tomita *et al.*, (1991) examined enzymatic hydrolysates of bovine lactoferrin. They showed that the cleavage of lactoferrin by porcine pepsin generated low molecular peptides, which have broad-spectrum antibacterial activity, inhibiting the growth of a number of Gram-negative and Gram-positive species, including strains that were resistant to native lactoferrin (Tomita *et al.*, 1991). With all the strains tested, the hydrolysate had at least eight-fold higher antibacterial potency compared to the undigested lactoferrin and unlike native lactoferrin the active peptides retained their activity in the presence of iron. The undigested lactoferrin exhibited no antibacterial activity at 0.5 mg/ml iron whereas, at the same concentration of iron lactoferrin hydrolysates produced by porcine pepsin, cod pepsin, or the aspartic protease from *P. duponti* significantly reduced the viability of the

test strain. Reverse-phase HPLC fractionation of the peptides generated by porcine pepsin hydrolysis of bovine lactoferrin showed that a single peptide peak contained all the antibacterial activity. The active peptide present in this peak was sequenced and was shown to be a 25 amino acid long peptide, which was identical to a sequence in the N-lobe of bovine lactoferrin from residues 17 to 41. This peptide was named lactoferricin. Evidence for the production of antibacterial peptides *in vivo* was also provided (Tomita *et al.*, 1994; Tomita *et al.*, 1994a). Bovine lactoferricin (LFcin-B) could be isolated from the gastrointestinal contents of rats fed a diet containing bovine lactoferrin. This lactoferricin B peptide has also been purified by affinity chromatography using immobilised heparin (Shimazaki *et al.*, 1998). The peptide displayed an essentially unordered β-sheet structure as assessed by circular dichroism. When this peptide was mixed with heparin, a distinct spectral change was observed indicating a change in the conformation of the peptide, which was reversible by the removal of heparin.

The physical and biological properties of this lactoferricin peptide are discussed in the following section.

1.4 Lactoferricin

1.4.1 Identification, isolation and activity of the antimicrobial domain of lactoferrin

Bellamy *et al.*, (1992) reported the presence of an antimicrobial domain present near the N-terminus of both human and bovine lactoferrins in a region distant from the iron-binding sites. There was very little or no similarity with the sequence in the corresponding region of the C-terminal lobe (Bellamy *et al.*, 1992). A single active peptide representing this domain was isolated from the low molecular weight peptides generated by gastric pepsin cleavage of lactoferrin using reverse-phase HPLC and testing for antimicrobial activity against *E. coli* 0111. They named these active peptides as Lactoferricin H (LFcin-H) and Lactoferricin B (LFcin-B) for the peptide from human and bovine respectively (Bellamy *et al.*, 1992a). The antimicrobial peptide sequence was found to consist of a loop of 18 amino acid residues formed by a disulphide bond

between cysteine residues 20 and 37 of human lactoferrin and 19 and 36 of bovine lactoferrin. This antimicrobial peptide was devoid of Tyr and His residues, which are essential for the metal-chelating functions of lactoferrin. While Bellamy *et al.*, (1992) reported the isolation of a single active peptide from pepsin-digested bovine lactoferrin, Dionysius and Milne (1997) reported the isolation of three peptides from pepsin-digested bovine lactoferrin that have antibacterial activity against enterotoxigenic *E. coli* (Dionysius and Milne, 1997). All three peptides were cationic and originated from the N-terminus of lactoferrin. Peptide I was almost identical to LFcin-B comprising residues from 17 to 42. Peptide II, a less active peptide, consisted of two peptides (derived from residues 1 to 16 and 43 to 48) linked by a single disulphide bond and peptide III, also a disulphide linked heterodimer, which corresponded to residues 1 to 48 and had been cleaved between residues 42 and 43. This confirms that the bactericidal domain of lactoferrin is localised in the N-terminus and does not involve the iron-binding sites. Hoek *et al.*, (1997) reported the generation of several peptides sharing high sequence homology with LFcin B when bovine lactoferrin was digested with recombinant chymosin, which is an aspartyl proteinase with specificity similar to pepsin. Recombinant chymosin was used to digest bovine lactoferrin under acidic conditions to generate these LFcin-B like peptides. Native lactoferrin is not hydrolysed by this enzyme at pH 6.3 to 6.5. Lactoferricin has a relatively high proportion of and asymmetric clustering of basic amino acid residues which are essential for its activity (Hoek *et al.*, 1997).

Since then a lot of work has been done on the antimicrobial properties of lactoferricin and their synthetic analogues.

Lactoferricin B was shown to be more potent than Lactoferricin H. However, both peptides were bactericidal, causing a rapid loss of colony-forming capability against a physiologically diverse range of Gram-negative and Gram-positive bacteria. Bellamy *et al.*, (1992a) showed that lactoferricin H and B were about 2-fold and 12-fold more effective than the corresponding undigested lactoferrin. Lactoferricin B was 9-fold more effective than lactoferricin H (Bellamy *et al.*, 1992a).

Table 1.4 summarises the findings of Bellamy *et al.*, (1992a).

In the presence of 0.1 mM ferrous sulphate bovine lactoferrin was inactive. No inhibition of *E. coli* 0111 growth was observed even with 100 μM of bovine lactoferrin whereas, lactoferricin B under the same conditions retained 40% of its activity. Lactoferricin B also has a diminished antimicrobial effectiveness in the presence of Na^+, K^+, Ca^{2+} and Mg^{2+} ions at concentrations above 1.0 mM.

Table 1.4 ***Effectiveness of antimicrobial activity of lactoferrin and its derivatives against Escherichia coli 0111*** [adapted from (Bellamy *et al.*, 1992a)]

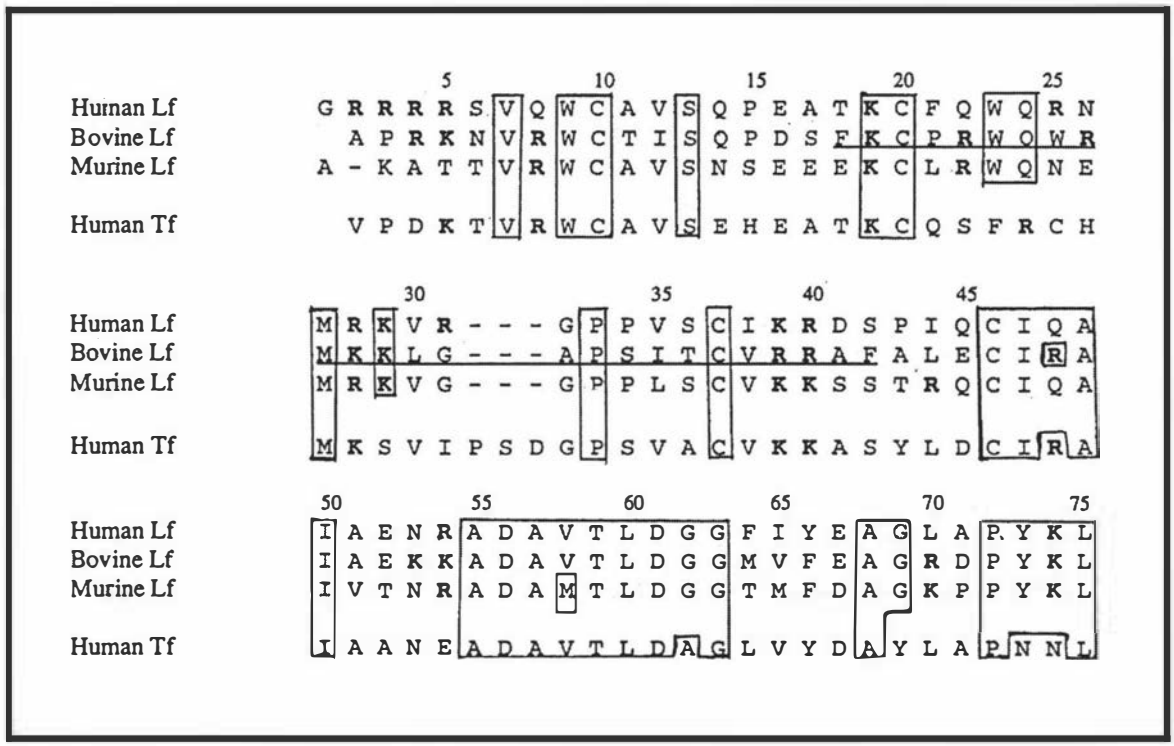
Lactoferrin or derivative	**MIC**	
	μg/ml	**(μM)**
Human lactoferrin	3000	(37)
Human lactoferrin, pepsin hydrolysed	500	
Lactoferricin H	100	(18)
Bovine lactoferrin	2000	(25)
Bovine lactoferrin, pepsin hydrolysed	100	
Lactoferricin B	6	(2)
Bovine lactoferrin + 0.1 mM $FeSO_4$	>8000	(>100)
Lactoferricin B + 0.1 mM $FeSO_4$	15	(5)
Lactoferricin B, pyridylethylated	6	(2)

Similar effects have been observed with apo-lactoferrin in the presence of the same ions. This effect could be due to the neutralising effect of these cations on the negatively charged target sites on the surface of the membrane thereby reducing the affinity of polycationic membrane-active agents for these target sites. If this is true, it suggests that binding of peptide to the cell surface is necessary for its antibacterial effect

and that ionic interactions have an important role in the cell-binding event. These negatively charged elements in the membranes of susceptible bacteria to which cationic antibacterial peptides bind are lipopolysaccharide (LPS) in Gram-negative bacteria and lipotechoic acid in Gram-positive bacteria.

Fig.1.2 gives a comparison between the N-terminal sequences of human lactoferrin, bovine lactoferrin, murine lactoferrin and human transferrin.

Fig. 1.2 ***Comparison of N-terminus sequences of human, bovine and murine lactoferrin and human transferrin*** [adapted from (Ellison III, 1994)]



The boxed sequence indicates regions of identity. The underlined sequence is the antimicrobial bovine lactoferricin peptide. Amino acid residues indicated in bold are positively charged.

By the use of radiolabelled substrate binding studies, lactoferricin B was shown to have a direct interaction with the cell surface. The extent of binding was greater than 10^6 peptide molecules per cell at optimal pH, which is higher than expected for binding to specific receptors (Bellamy *et al.*, 1993). The optimal pH for binding was shown to be strain dependent. The killing effect was maximal near the optimal pH for cell binding, with individual test strains (Bellamy *et al.*, 1993).

Bellamy and co-workers also showed that the disulphide bond responsible for the looped structure of the isolated peptides was not essential for activity. This was shown by modifying lactoferricin B by pyridylethylation of its cysteine residues and assaying for activity. There was no difference in the activity of the unmodified and modified lactoferricin-B against *E. coli* 0111. A synthetic 23-mer corresponding to residues 18 to 40 of human lactoferrin has been shown to have similar antibacterial effectiveness as native lactoferricin H and similarly, a synthetic 25-mer corresponding to residues 17 to 41 of bovine lactoferrin has also shown similar antibacterial effectiveness as lactoferricin B (Bellamy *et al.*, 1992a). Eight of the 25 residues in lactoferricin B and seven of the corresponding 25 residues in lactoferricin H are basic residues and these are important for activity (Bellamy *et al.*, 1992a). Similar asymmetric clustering of basic residues has also been observed in the other antimicrobial peptides such as magainins, cecropins and defensins though lactoferricins do not share any sequence similarity with them.

Cationic peptides have been shown to kill sensitive microorganisms by inducing an increase in cell permeability (Yamauchi *et al.*, 1993). This leads to a disruption of energy metabolism or other essential functions in the organism. The lethal effect of lactoferricins appears to be due to a similar mechanism. Similar to the effects of apo-lactoferrin, lactoferricin has been shown to cause substantial release of lipopoly-saccharide (LPS) from the outer membrane of Gram-negative bacteria. This is followed by the immediate development of electron-dense 'membrane blisters' and /or outer membrane dislocation and subsequent affect to cytoplasmic contents [(Yamauchi *et al.*, 1993) and results of this investigation].

Yamauchi *et.al.*, (1993) reported that under most conditions, more LPS was released by the peptide fragment than by whole bovine lactoferrin and that bovine lactoferrin was at most bacteriostatic whereas, lactoferricin demonstrated consistent bactericidal activity against Gram-negative bacteria. This confirms the fact that the lactoferricin domain is the structural region responsible for the membrane disruptive effects of lactoferrin. Lactoferricin B has been shown to have a broad antibacterial spectrum (Bellamy *et al.*, 1992; Jones *et al.*, 1994). The antimicrobial effectiveness of lactoferricin B was studied under varying conditions of growth of the microorganisms including growth phase and size of the inoculum, pH and ionic strength of the medium. Jones *et al.*, (1994), have shown that the bactericidal activity of LFcin-B was inhibited by increasing the ionic strength, size and age of bacterial inoculum and at acidic pH. The activity of LFcin-B was completely inhibited by the addition of 5% whole cow's milk and was reduced in the presence of increasing concentrations of BSA and mucin.

These results raise doubts about LFcin-B's role *in vivo* because of its sensitivity to changes in physical variables although it can reduce the viability of sensitive organisms in simple medium (Jones *et al.*, 1994).

1.4.2 Structure vs biological activity relationships of the amino acid residues in lactoferricin

In vitro studies on a wide range of Gram-negative microorganisms suggested that lactoferrin can bind to lipopolysachharide (LPS) in the bacterial cell membrane. Applemelk *et al.*, (1994) found high-affinity binding of human lactoferrin to the lipidA region of *E. coli* LPS (Appelmelk *et al.*, 1994). With this knowledge, a lot of work was done on the N-terminal region and also by changing the amino acid residues of lactoferrin in the region of the molecule that is thought to be responsible for binding the LPS of *E. coli.* Elass-Rochard *et al.*, (1995) have performed many experiments to identify the domain of the hLF molecule implicated in the endotoxin (LPS) recognition (Elass-Rochard *et al.*, 1995). They studied both direct binding and competitive binding assays performed with different hLF fragments. Their findings are summarised in Table 1.5. These authors have shown that the 28-34 amino acid sequence present in a

synthetic octadecapeptide corresponding to the cationic antimicrobial peptide of hLF (20-37 residues) produced 62% inhibition of the hLF-LPS interaction. Similarly, lactoferricin B (residues 17-41), a proteolytic fragment from bLF, revealed the importance of the region of bLF homologous to residues 28-34 for LPS binding. They have concluded that the residues 28-34 of the loop region of hLF and the homologous region in bLF, is involved in the high-affinity binding to *E. coli* 055B5 LPS (Elass-Rochard *et al.*, 1995).

Table 1.5 ***Binding parameters of hLF, bLF, rhLF, EGS-loop rhLF and proteolytic fragments derived from hLF to E. coli 055B5 LPS*** (Elass-Rochard *et al.*, 1995)

Proteins	K_d (nM)	
	High-affinity Binding site	**Low-affinity binding site**
hLF	3.6 ± 1	390 ± 20
bLF	4.5 ± 2	576 ± 30
hLF N-tryptic fragment	1 3 ± 3	Not measurable
hLF C-tryptic fragment	Not present	580 ± 30
rhLF	7.6 ± 1	650 ± 20
EGS-loop rhLF	Not present	220 ± 14

(Results are means ± S.E.M)

The EGS-loop hLF mutated protein corresponds to rhLF in which the sequence ^{28}RKVRGPP^{34} was replaced by the sequence ^{365}EGS^{367}, located in the C-terminal lobe counterpart.

Odell *et al.*, (1996) also reported that synthetic peptides homologous to the loop region in human lactoferrin had antibacterial activity (Odell *et al.*, 1996). They showed that synthetic peptides corresponding to residues 20-35 (whole loop) and 20-30

(positively charged portion of loop) in hLF had significant antibacterial effects against *E. coli* serotype 0111 strains NCTC 8007 and ML35. They reconfirmed that antibacterial activity is independent of the disulphide bridge as a much smaller 11-residue peptide homologous to just over half the loop region of Lactoferricin H has potent antibacterial activity. These authors compared the sequences of lactoferricin H & B with other cationic antimicrobial peptides. This comparison is shown in Table 1.6.

Table 1.6 ***Sequence alignment of antimicrobial peptides with lactoferrins including regions beyond the antimicrobial segments*** [adapted from (Odell *et al.*, 1996)].

Peptide	Sequence	Reference
Dermaseptin I	A L **W** K T M L **K K L** - **G** T M A **L** H A **G** K A A L G **A** . . .	(1)
Magainin I	**E V** R G I G **K F** L H S A G **K F** - **G** K A F **V** G E **I** M K S **K R D** . . .	(2)
Magainin II	**E V** R G I G **K F** L H S A **K K F** - **G** K A F **V** G E **I** M N S **K R D** . . .	(2)
Bovine Lf	**E** W F K C R **R W** Q W R M **K K L** - **G** A P S **I** T C V - - - **R R A** F . .	(3)
Mouse Lf	**E** E E K C L **R W** Q N E M **R K V** - **G** - P P **V** S C I - - - **K K** S . . .	(4)
Human Lf	**E A** T K C F Q **W** Q R N M **R K V** R **G** - P P **V** S C L - - - **K R D**	(5)
LFcin-B	F K C R **R W** Q W R M **K K L** - **G** A P S **I** T C V - - - **R R A** F A	This
LFcin-H	T K C F Q **W** Q R N M **R K V** R **G** - P P **V** S C I - - - **K R**	Study

(1) (Mor and Nicolas, 1994) (2) (Zasloff, 1987) (3) (Pierce *et al.*, 1991)
(4) (Pentecost and Teng, 1987) (5) (Norris *et al.*, 1991)

Residues in bold are identical/similar between one or more lactoferrin sequences and one or more antibacterial sequences. Identities or similarities within each group are not highlighted. Underlined sequences represent bovine lactoferricin and the longer peptide chain of human lactoferricin, which contains the loop region.

A similar study was done with chemically synthesised lactoferricin B and its substituted peptides to investigate the structure-antimicrobial activity relationships (Kang *et al.*, 1996). They selected the 11-residue highly basic peptide segment of bovine lactoferricin B (RRWQWRMKKLG) (residues 20-30) for their detailed antimicrobial activity study. The antimicrobial activities of the peptides were tested by determining the minimal inhibitory concentrations (MIC) for *E. coli* and *B. subtilis* and the disruption of the outer cell membrane of *E. coli* by measuring the β-lactamase activity from *E. coli* 0111 harbouring pUC19 plasmid. The toxicity of the peptide was assayed by haemolysis of red blood cells (RBC). They reported that a short basic peptide composed of only 11 residues (residues 20-30 of bLF or LFcin-B) had similar antimicrobial activity to the 25-residue long LFcin-B but the haemolytic activity was significantly reduced.

Short peptides with two arginine residues at the N-terminus had more potent antimicrobial activity than those with two lysine residues. When all basic amino acids in the peptide were substituted with glutamic acid and when all hydrophobic amino acid residues were substituted with alanine, both antimicrobial activity and haemolytic activity were abolished.

A three-dimensional solution structure of lactoferricin B was determined using 2D ^{1}H NMR spectroscopy (Hwang *et al.*, 1998). The structure of Lactoferricin B in solution was shown to be a somewhat distorted antiparallel β-sheet. This is in contrast with the X-ray crystallographic-determined structure of bovine lactoferrin in which residues 1-13 of LFcin-B (ie. residues 17-29 in bovine lactoferrin) forman α-helix (Moore *et al.*, 1997). Hence, this region of lactoferricin B appears to be able to adopt either a helical or sheet-like conformation, similar to that has been proposed for the amyloidogenic prion proteins and Alzheimer's β-peptides. Lactoferricin B bears numerous similarities to a number of cationic peptides, which exert their antimicrobial activities through membrane disruption. Since the structure of these peptides is quite well defined, it might be suggested that the NMR solution structure of LFcin-B is more relevant to membrane interaction than that suggested by the X-ray structure in intact lactoferrin. This α- to β-secondary structure transition may be beneficial for lactoferrin

in exerting its antimicrobial activity. This transition could be induced by the release of LFcin-B from bovine lactoferrin by pepsin cleavage. In the absence of proteolysis, it is possible that membrane interactions alone are capable of inducing a similar α-to β-secondary structure transition in the N-terminal region of intact lactoferrin. LFcin-B could bind membranes either by forming oligomeric pores by inserting its multimeric strands in the membrane vertically like defensins or by interacting with a single leaflet of the lipid bilayer, so that its backbone lies parallel to the membrane surface like magainin 2. Based on its NMR solution structure it is likely that LFcin-B prefers an orientation parallel to the membrane bilayer. The β-sheet structure of LFcin-B would appear to be better suited for making initial contacts with membranes than the helical structure found in intact lactoferrin. This conclusion could be challenged by the results of Chapple *et al.*, (1998). These authors synthesised an analogue of the lactoferricin H peptide with a proline substituted for methionine in its loop region. This change, which is predicted to disrupt the helical region, also abolished antibacterial activity against *E. coli* 0111 and substantially reduced antibacterial activity against *Staph. aureus* and *Acinetobacter* strains. This confirms the idea that structural considerations are important for activity (Chapple *et al.*, 1998a). They checked the mode of action of this synthetic peptide against *E. coli* serotype 0111 (NCTC 8007) by using flow cytometry, surface plasmon resonance and transmission electron microscopy. LFcin-H was found to act at the cell membrane, causing complete loss of membrane potential after 10 min and of membrane integrity within 30 min, with irreversible damage to the cell as shown by rapid loss of viability. The number of particles measured by light scattering on the flow-cytometer dropped significantly, showing that bacterial lysis resulted. The peptide was shown to bind to *E. coli* 0111 lipopolysaccharide by using surface plasmon resonance. Transmission electron microscopy revealed bacterial distortion with the outer membrane becoming detached from the inner cytoplasmic membrane. Similar observations were made by using enterohaemorrhagic *E. coli* 0157:H7 [(Shin *et al.*, 1998) and also by us as described in this thesis].

1.4.3 Antimicrobial activity of lactoferricin B

Lactoferricin has been shown to have a potential anti-microbial activity against a wide range of Gram-negative and Gram-positive bacteria and against yeasts, similar to that shown by lactoferrin but with a higher potential at very low concentrations. Table 1.7 gives a summary of the susceptible microorganisms with their MICs.

Table 1.7 ***List of various microorganisms tested for antimicrobial activity with lactoferricin B*** [adapted from (Bellamy *et al.*, 1992)].

Strain	MIC (μg/ml) of Lfcin B
Gram-negative bacteria	
Escherichia	
coli 0111	6
coli IID-861	6
Klebsiella pneumoniae JCM-1662T	9
Salmonella enteritidis IID-604	12
Proteus vulgaris JCM-1668T	12
Yersinia enterolitica IID-981	6
Psedomonas	
aeruginosa MMI-603	12
aeruginosa IFO-3445	6
aeruginosa IFO-3446	9
aeruginosa IFO-3448	9
aeruginosa IFO-3452	6
fluorescens IFO-14160	>60
Gram-positive bacteria	
Staphylococcus	
aureus JCM-2413	6
aureus JCM-2179	3
aureus JCM-2151	3
epidermidis JCM-2414T	3
haemolyticus JCM-2416T	0.6
hominu JCM-2419T	2

Strain	MIC (μg/ml) of Lfcin B
Enterococcus faecalis ATCC-E19433	>60
Streptococcus	
mutans JCM-5705T	2
bovis JCM-5672	3
Corynebacterium	
ammoniagenes JCM-1306	0.3
renale JCM-1322	0.6
diptheriae JCM-1310	6
Listeria	
monocytogenes JCM-7673	0.3
monocytogenes JCM-7674	0.6
Bacillus	
subtilis ATCC-6633	0.6
natto IFO-3009	1
circulans JCM-2504T	0.3
cereus MMI-272	9
Clostridium	
perfringens ATCC-6013	12
Yeasts	
Candida albicans	11.7
Candida parapsilosis	7.8

A standard logarithmic-phase inoculum of a size of 1 x 10^6 cfu/ml grown on 1% peptone media was taken in all these experiments. The minimal inhibitory concentration (MIC) was taken as the lowest concentration of lactoferricin B that caused complete inhibition of bacterial growth.

These authors also showed that the MIC values were higher for the same bacteria when they were grown on PYG (Peptone-yeast extract-glucose) media instead of 1% peptone.

Lactoferricin B had been shown to have parasiticidal activity (Tanaka *et al.*, 1995). Lactoferricin B has been found to inhibit tumour growth, but whether it is more related to its antimicrobial activity or to a stimulatory effect on the immune system has yet to be established (Yoo *et al.*, 1997).

1.4.4 Affect of lactoferrin and lactoferricin on Gram-negative cell membranes

Ellison *et al.*, (1988) have shown from their *in vitro* studies that human apo-lactoferrin has direct bactericidal activity. This is brought about by binding to sensitive Gram-negative bacteria, damaging their outer membrane by the release of lipopolysaccharide (LPS) and altering the permeability properties of the outer membrane (Ellison III *et al.*, 1988). This release of LPS from the outer membrane of Gram-negative bacteria was shown to increase with increasing concentrations of lactoferrin. Transferrin also could cause LPS release from susceptible strains in Hank's salt solution lacking calcium and magnesium. Damage to the outer membrane of Gram-negative bacteria by lactoferrin and transferrin was modulated by the concentration of Ca^{+2} and Mg^{+2} and the release of LPS could be blocked by concurrent addition of Ca^{+2} and Mg^{+2} (Ellison III *et al.*, 1990). Transmission electron microscopy studies on bacterial cells exposed to the N-terminal antibacterial peptide of bovine lactoferrin (Lactoferricin B) showed immediate development of electron dense 'membrane blisters' and under most conditions, more LPS was released by Lactoferricin B than by the whole bovine lactoferrin molecule (Yamauchi *et al.*, 1993). These authors also reported that bovine lactoferrin was mostly bacteriostatic, whereas lactoferricin B consistently showed bactericidal activity against Gram-negative bacteria and that this bactericidal activity was modulated by the concentration of the cations, Ca^{+2}, Mg^{+2} and Fe^{+2} but was independent of osmolarity of the medium.

Recently, Chapple *et al.*, (1998) used a synthetic peptide of an 11-amino-acid amphipathic alpha-helical region of lactoferricin H (HLP 2) and showed that it exhibited potent antibacterial activity against *E. coli* serotype O111 (NCTC 8007). There was a complete loss of bacterial membrane potential and membrane integrity. They also showed that this peptide bound *E. coli* O111 lipopolysaccharide and caused bacterial

distortion, with the outer membrane becoming detached from the inner cytoplasmic membrane as revealed by transmission electron microscopy (Chapple *et al.*, 1998b). They concluded that the membrane disruption capability of the HLP 2 peptide was dependent upon its helical structure (Chapple *et al.*, 1998a).

Shin *et al.*, (1998) reported that lactoferricin B exerted its antibacterial activity against enterohaemorrhagic *E. coli* 0157:H7, initially on the bacterial cell surface and then subsequently affecting the cytoplasmic contents. With their transmission electron microscopy experiments, they showed that LFcin-B initially caused some membrane blisters and subsequently affected the cytoplasmic contents with the formation of electron-dense aggregates (Shin *et al.*, 1998).

1.4.5 Bovine lactoferricin (LFcin-B)

Phe-Lys-Cys-Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys-Leu-Gly-Ala-Pro-Ser-Ile-Thr-Cys-Val-Arg-Arg-Ala-Phe

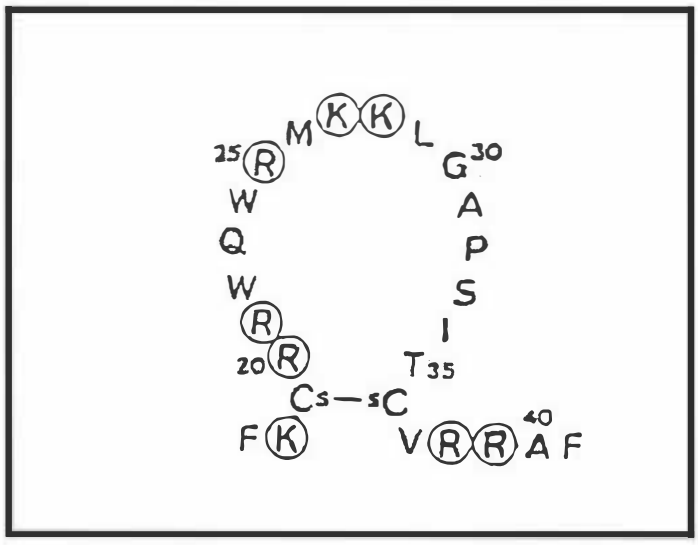


Fig 1.3 ***Primary loop structure of Lactoferricin B. Basic residues are circled.***

1.4.6 Human Lactoferricin (LFcin-H)

Thr-Lys-Cys-Phe-Gln-Trp-Gln-Arg-Asn-Met-Arg-Lys-Val-Arg-Gly-Pro-Pro-Val-Ser-Cys-Ile-Lys-Arg

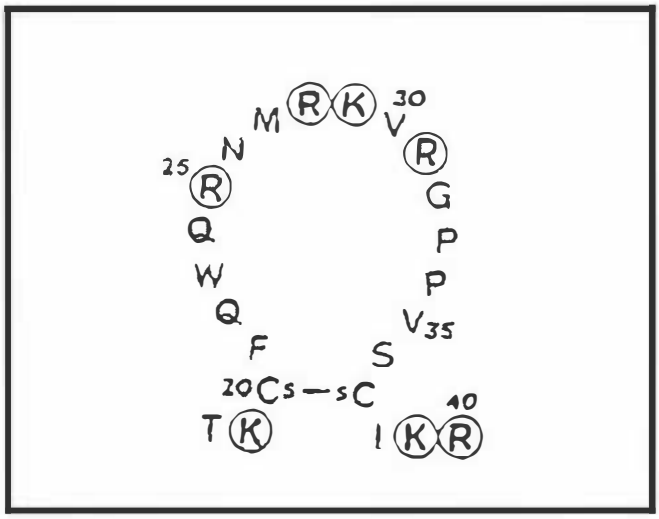


Fig 1.4 ***Primary loop structure of Lactoferricin H. Basic residues are circled.***

1.5 Expression of Lactoferrin *in vivo*

The similarities between lactoferrin and transferrin in their synthesis and secretion suggest that they may have related transcriptional and translational regulation patterns. The differential expression of lactoferrin in both bovine and human mammary glands throughout the lactational cycle suggests a possible hormonal regulation of the lactoferrin gene expression and also regulation by iron status as in the case of transferrin.

Lactoferrin has been shown to be the major oestrogen-inducible protein in mouse uterine secretions (Pentecost and Teng, 1987). Human lactoferrin secretion has also been shown to be regulated by oestrogen (Teng *et al.*, 1992). Rado *et al.*, (1984) suggested that lactoferrin synthesis in developing neutrophils is highly controlled as it occurs only during granulocyte differentiation (Rado *et al.*, 1984). Siebert and Huang

(1997) identified an alternative form of human lactoferrin mRNA that is expressed differentially in normal tissues and tumour-derived cell lines (Siebert and Huang, 1997). They have shown that there is a novel form of lactoferrin mRNA called ΔLF mRNA, at various levels in all the normal adult and foetal tissues they tested. However, they reported that they could not find ΔLF mRNA in tumour-derived cell lines. The ΔLF mRNA was nearly identical to that of LF mRNA except that it had a novel sequence that would change the N-terminal signal peptide sequence of lactoferrin. They concluded that ΔLF mRNA was the product of alternative splicing of the LF gene and that it might be using an alternative promoter (Siebert and Huang, 1997).

Recently, it has been shown *in vitro* that the expression of ferritin and lactoferrin by respiratory epithelial cells is metal dependent, especially catalytically active iron which causes oxidative injury to the cells (Ghio *et al.*, 1998). They have shown that lactoferrin and ferritin protein levels are up regulated whereas, transferrin is down regulated upon exposure to residual oil fly ash containing catalytically active metals. The metal chelator, deferoxamine inhibited these responses.

1.6 Heterologous expression of transferrin and lactoferrin and its variants

There have been many studies on the heterologous expression of both transferrin and lactoferrin proteins, which are quite large in size and in the case of lactoferrin is very basic. Successful expression of lactoferrin and transferrin has only been achieved in eukaryotic expression hosts using vector constructs, which cause the protein to be secreted during expression. Transferrin has been expressed in BHK cells and in yeast. Lactoferrin has been expressed in BHK cells and in a number of strains of A*spergillus.* Expression levels of 20-40 mg/L of BHK cell culture medium have been reported for full-length recombinant hLF. Recombinant full-length human lactoferrin was shown to have identical spectroscopic properties and identical pH-dependent iron release profile as native human milk lactoferrin (Stowell *et al.*, 1991). *E. coli* expression systems were found to be unsuccessful in all cases with the exception of human serum transferrin expressed in *E. coli* as inclusion bodies. The recombinant product was not well characterised (Ikeda *et al.*, 1992). Recently, Maheswari *et al.*, (1998) have expressed a

series of the bLF N-terminal deletion mutants in *E. coli* that bind to the Ca^{+2}- dependent lactoferrin receptor on isolated rat hapatocytes. Attempts to heterologously express the C-terminal lobe of human lactoferrin were not successful (Tweedie, J.W., personal communication).

Table 1.8 summarises previous reports of heterologous expression of both proteins.

Table 1.8 ***Heterologous expression of transferrin and lactoferrin proteins and their variants***

Protein	Expression System	Reference
hTf N-terminal half	BHK cell line	(Funk *et al.*, 1990)
hTf N-terminal half	*Pichia pastoris*	(Mason *et al.*, 1996)
hTF full length	BHK cell line	(Mason *et al.*, 1993)
hLF full length	BHK cell line	(Stowell *et al.*, 1991)
hLF N-terminal half and its mutants	BHK cell line	(Day *et al.*, 1992)
hLF full length	*Aspergillus awamori*	(Ward *et al.*, 1995)
hLF full length	*Aspergillus nidulans*	(Ward *et al.*, 1992)
hLF full length	*Aspergillus oryzae*	(Ward *et al.*, 1992a)
hLF full length	Mammary gland of transgenic mice	(Nuijens *et al.*, 1997)
hLF full length	*Saccharomyces cerevisiae*	(Liang and Richardson, 1993)
hLF protein fragments	*Nicotiana tabacum L.* (Tobacco cell line)	(Mitra and Zhang, 1994)
bLF deletion mutants	*Escherichia coli*	(Maheswari *et al.*, 1998)

1.7 Structure of lactoferrin

All members of the transferrin family of proteins are glycoproteins consisting of a single polypeptide chain of 670-700 amino acid residues. Approximately 60 to 70% sequence identity is seen between lactoferrins of different species and approximately 50-60% between the lactoferrins and the mammalian serum transferrins. Insect transferrins

have a lower sequence identity to the transferrins in higher animals. Analysis of sequences of the transferrin family of proteins shows that each polypeptide can be divided into two homologous halves. This internal amino acid similarity suggests that the transferrins have evolved by means of a gene duplication event. The structures of both human lactoferrin and bovine lactoferrin in both apo- and iron-loaded forms have been determined. The details of the structure of these individual lactoferrins are discussed in the following sections with special reference to the putative antimicrobial regions.

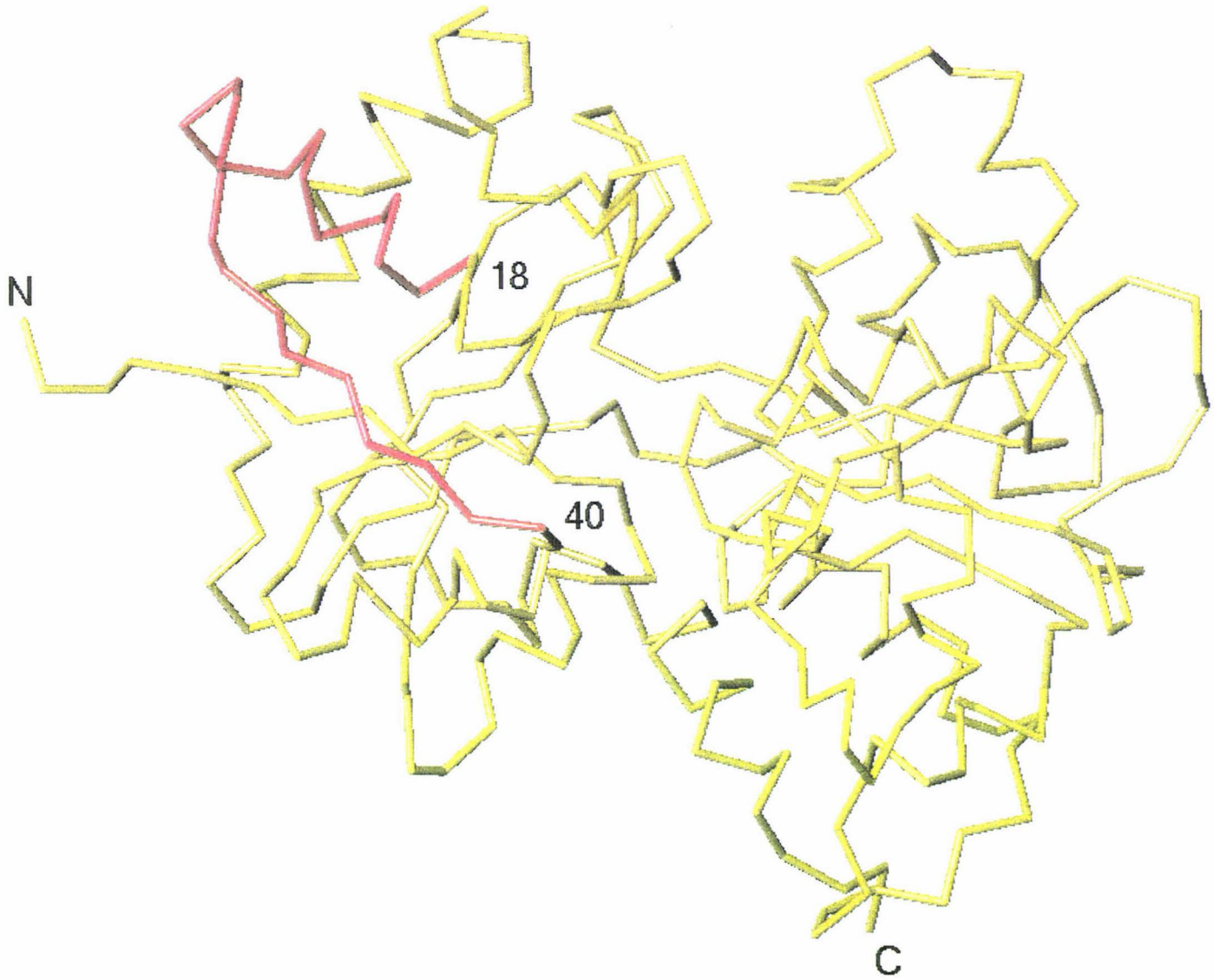
1.7.1 Human Lactoferrin

Metz-Boutigue *et al.*, (1984) have determined the complete amino acid sequence of lactoferrin isolated from human milk (Metz-Boutigue *et al.*, 1984). Later, the complete cDNA sequence of human neutrophil lactoferrin was determined (Rado *et al.*, 1987). As a consequence, human lactoferrin is currently accepted to have 691 amino acid residues (Anderson *et al.*, 1987). The tertiary structure of iron-loaded lactoferrin has been determined at 3.2 Å resolution (Anderson *et al.*, 1987) and later refined at 2.8 Å resolution (Anderson *et al.*, 1989). The structure of diferric human lactoferrin was further refined at 2.2 Å resolution (Haridas *et al.*, 1994). The structure of human apolactoferrin has also been reported by the same group at 2.8 Å resolution (Anderson *et al.*, 1990).

The structural determination of diferric lactoferrin (Anderson *et al.*, 1989) shows that human lactoferrin is a globular protein consisting of two structurally similar globular lobes, with differences mainly in loops and turns (Figure 4). The N-lobe is comprised of amino acid residues 1 to 333 while the C-lobe is comprised of residues 345 to 691 with 40% identity in their primary amino acid sequences. These two lobes are connected by a three-turn α-helix, which is formed by residues 334 to 344. Both these lobes are further subdivided into two domains of approximately 160 residues each. The four structural domains of human lactoferrin are labelled as follows: N1 (residues 1 to 90 and 252 to 320), N2 (residues 91 to 251), C1 (residues 345 to 433 and 596 to 663) and C2 (residues 434 to 595). The structural similarity between the domains is significantly

higher than that of the complete lobes. That is, the structures of N1 and C1 domains superimpose with much higher fidelity than the complete lobes.

Fig. 1.5 *Tertiary structure of Human Lactoferrin*



Similarly, the N2 and C2 domains have significantly higher structural similarity than the N- and C-terminal lobes (Anderson *et al.*, 1989). Each lobe of lactoferrin contains a single iron-binding site, which is located at the inner end of each inter-domain cleft. The iron atoms are located individually in a deep cleft and are coordinated to ligand from both domains and from two inter domain crossover strands. The ligands involved in iron binding are one carboxylate oxygen, two phenolate oxygens and one imidazole nitrogen. In the N-lobe these are provided by the side chains of Asp 60, Tyr 92, Tyr 192 and His 253. The corresponding amino acid residues in the C-lobe are Asp 395, Tyr 435, Tyr 528 and His 597. The widely spaced arrangement of these residues along the polypeptide chain has been suggested to be advantageous in terms of protein function when iron binding and release is accompanied by large-scale conformational changes. Each of these lobes contains one iron-binding site, which binds one Fe (III) ion concomitantly with one CO_3^{2-} anion. The relationship between the metal ion and the anion is such that neither is bound strongly in the absence of other (Anderson *et al.*, 1989). The ligands involved in binding the anion are Arg 121 (Arg 465 in the C-lobe) and the associated N-terminus of a α-helix formed by residues 121-136 (465-481 in the C-lobe). Despite the similarity in sequence and in the three-dimensional structure between the N- and C-terminal halves of lactoferrin, the overall environment of the two lobes is not equivalent. The 'head to tail' joining of the two lobes gives the lactoferrin molecule an asymmetry, which may have implications for interactions with possible receptors. Due to this asymmetric orientation of the lobes, equivalent parts of the two lobes have different environments. The inter-domain cleft of the N-lobe is more exposed to the solvent than the C-lobe cleft. It has also been shown that one iron binding site has a higher affinity for Fe^{3+}, which may be the result of this asymmetry (Ainscough *et al.*, 1980).

The X-ray crystallographic structure of copper (II)- and oxalate-substituted human lactoferrin was determined (Smith *et al.*, 1991) and it was shown that the overall structure of the molecule is not changed. They proved that lactoferrin can accommodate different sized ionic species by making minor changes to the protein structure to accommodate these bulkier ions and hence allow lactoferrin to bind a variety

of metals and synergistic anions.

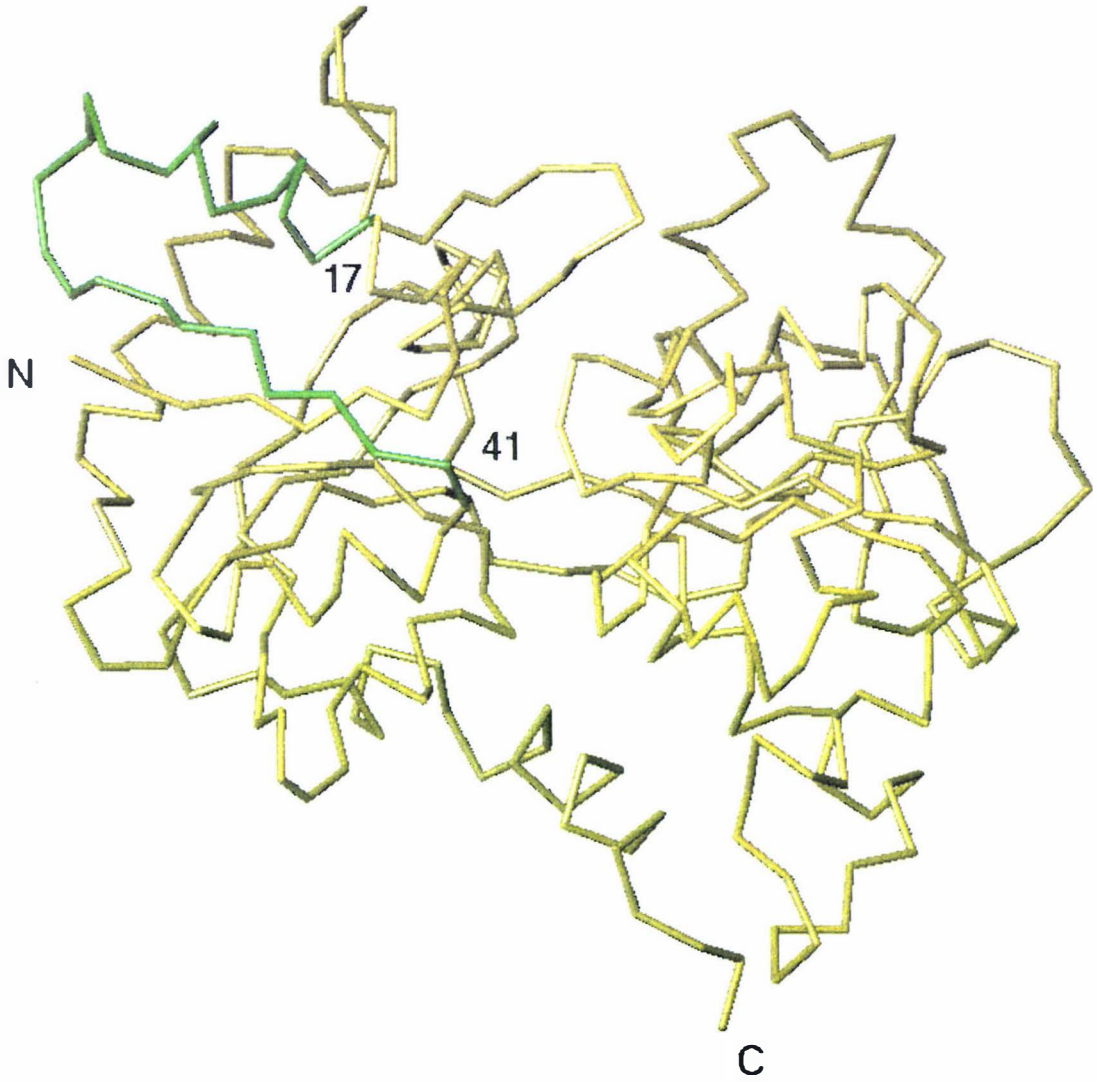
The differences in the structures between diferric lactoferrin and apo-lactoferrin show that the protein undergoes substantial conformational changes during iron binding and release (Anderson *et al.*, 1990). The deglycosylated apo-lactoferrin had been shown to have identical properties of iron binding and release and identical spectroscopic properties and formed crystals, which diffracted to 2 Å resolution. The X-ray analysis showed that the N-lobe had undergone a very large conformational change, where as the C-lobe remained essentially unchanged even though no metal was bound (Anderson *et al.*, 1990). The conformational change in the N-lobe involves an opening of the binding cleft through relative movement of the two domains; the N2 domain rotates 54° relative to N1, about an axis passing through the two backbone strands that run behind the iron site, connecting the two domains. The 54° domain movement is one of the largest seen in any protein. It has the effect of opening the binding cleft wide, thus exposing a number of residues previously buried, including several basic side chains. This might explain why the apo-lactoferrin has potent antimicrobial property compared to the diferric lactoferrin. The loop region of lactoferricin, the antimicrobial domain of lactoferrin comprises of an amphipathic alpha helical region exposed on the outer surface of the amino-terminal lobe of lactoferrin.

1.7.2 Bovine lactoferrin

The three-dimensional structure of diferric bovine lactoferrin (bLF) has been determined by X-ray crystallography in order to investigate the factors that influence iron binding and release by the transferrin family of proteins (Moore *et al.*, 1997). The structure was solved by molecular replacement, using the coordinates of diferric human lactoferrin (hLF) as a search model, and then was refined to 2.8 Å resolution (Fig 5). The folding of the bLF molecule was essentially the same as that of hLF, but bLF differed in the extent of closure of the two domains of each lobe, and in the relative orientations of the two lobes. Differences in domain closure are attributed to amino acid changes in the interface, and differences in lobe orientations to slightly altered packing of two hydrophobic patches between the lobes. This changed inter domain interactions

might explain the lesser iron affinity of bLF, compared with hLF and the two lysine residues behind the N-lobe iron site of bLF may offer new insights into the `dilysine trigger' mechanism proposed for iron release by transferrins. The bLF structure is also notable for several well-defined oligosaccharide units, which demonstrate the structural factors that stabilise carbohydrate structure. One glycan chain, attached to Asn545, appears to contribute to inter domain interactions and this might modulate iron release from the C-lobe.

Fig. 1.6 *Tertiary structure of Bovine Lactoferrin*



1.8 Heterologous gene expression systems

Many biologically interesting and important proteins are present naturally in very small quantities. Hence they are not amenable to structural and biochemical characterisation. With the advent of recombinant DNA technology, it is now possible to heterologously produce these proteins in large quantities in both prokaryotic and eukaryotic host systems. Since each protein has its own 'personality', the maximum yield and biological property of individual recombinant proteins varies greatly and depends upon a large number of factors such as protein stability, solubility, size and post-translational modifications. These factors often differ between expression systems.

The most important aspects to be considered in heterologous protein production are the host-vector system, the choice of protein to be produced, the genetic stability of the vector and the gene coding for the protein of interest.

1.8.1 Yeast expression systems

Yeasts have a specific advantage in being hosts for heterologous expression of eukaryotic proteins because they are eukaryotic microorganisms. Yeast genetics is more advanced than that of any other eukaryote, so the host can be genetically manipulated almost as easily and rapidly as *Escherichia coli.* As a eukaryote, it is more likely to provide a suitable environment for the folding of foreign eukaryotic proteins than does *E. coli*, and can be more readily used for secretion of proteins. Yeast can be grown on simple media, and to high cell density. Many yeast species are highly accepted for the production of pharmaceutical proteins because of their generally regarded as safe (GRAS) status. The review by Rallabhandi & Yu (1996) discusses the advantages of various yeast host systems and different protein products produced heterologously by using them (Rallabhandi and Yu, 1996a).

1.8.2 *E. coli* expression vector systems

Escherichia coli has been and continues to be the "workhorse" for the heterologous expression of a large number of known gene products at levels sufficient for detailed biochemical analysis and product development. The *E. coli* expression

system has been successfully used to express chemically synthesised genes like somatostatin (Itakura *et al.*, 1977) and human insulin (Goeddel *et al.*, 1979). In both cases the cloned synthetic genes were fused to an *E. coli* β-galactosidase gene to provide efficient transcription and translation and a stable precursor protein. The peptides were later cleaved from β-galactosidase.

A vast number of *E. coli* expression vectors and host strains are commercially available to express the required gene product as a fusion protein for an easy purification using affinity purification methods.

1.9 Justification of this project

Perspectives

Unlike bovine lactoferrin, the availability of human lactoferrin is limited because of the scarcity in the availability of the raw material, the human milk from which hLF can be isolated. Because of the species specificity of the receptors of lactoferrin, human lactoferrin is the most relevant protein to use in our food supplements. As described earlier, human lactoferrin has been heterologously expressed in different expression systems. While animal cell line and *Aspergillus* systems proved to be very good heterologous systems for the expression of hLF cDNA, tobacco cell line and *Saccharomyces cerevisiae* have shown a very poor expression of the whole protein. The yeast *Kluyveromyces lactis* expression system has several advantages in comparison to other yeast species. Because *K. lactis* has better secretion capability in addition to its ability to grow on whey-based media, we decided to over-express hLF and its variants in this system. As the work proceeded, we had to make a lot of changes to the project based on the progress at each stage. Finally the heterologous expression, purification and characterisation of antimicrobial peptides of both bovine lactoferrin and human lactoferrin were carried out in *E. coli*. A substantial amount of work was done on the membrane ultrastructural studies of both sensitive and relatively resistant *E. coli* strains to native lactoferricin B peptide using transmission electron microscopy.

Aims

We were compelled to make some changes to the actual aims with which the project was started, based on the progress at each stage. The consolidated aims of this project are as follows.

- To express the whole molecule of human lactoferrin in both yeast *Kluyveromyces lactis* and *Saccharomyces cerevisiae* expression systems.
- To express the amino terminal half of human lactoferrin (Lfn) in these yeast expression systems.
- To express antibacterial peptides of both human and bovine lactoferrins using an *E. coli* expression system.
- To isolate, purify and characterise these recombinant peptides and compare their efficacy with the native peptides.
- To conduct transmission electron microscopy studies on lactoferricin treated sensitive and relatively resistant *E. coli* strains.

Various expression systems, both prokaryotic and eukaryotic were used with different human and bovine lactoferrin cDNA sequences and their variants. Chapters 3 and 4, each describes one of the heterologous expression systems used and the results obtained with it.

Chapter 6 includes a general discussion of the different expression systems used and the reasons for making all the changes in the project as it progressed. Finally, the prospects for future studies will be discussed.

2.1 Materials

2.1.1 Enzymes and chemicals

All restriction endonucleases, RNAseA, RNAse free DNAse, T4 DNA polymerase, T4 DNA ligase, *E. coli* DNA polymerase I (Klenow fragment) and calf intestinal alkaline phosphatase (CIAP and Ts-CIAP) were obtained from the following companies: Amersham Life Science (Buckinghamshire, UK), Boehringer Mannheim (West Germany), Promega Corporation (WI, USA), New England Biolabs (MA, USA) and Pharmacia AB (Uppsala,Sweden).

All antibiotics, IPTG, low EEO type I-A agarose, SDS, ethidium bromide, TEMED, ammonium persulphate, bovine serum albumin (fraction V), imidazole, pure human lactoferrin, EGTA and lysozyme were supplied by Sigma Chemical Company (St. Louis, USA) and Progen Industries Inc., USA.

Yeast extract, tryptone, NaCl, bactopeptone, bacteriological agar, D-amino acids, LB broth base, Tris, tricine and yeast nitrogen base (YNB) (without amino acids) were obtained from GibcoBRL Ltd., (Paisley, Scotland, UK), Oxoid Ltd., (Basingtonstoke, England), Difco Laboratories (Detroit, Michigan, USA), Serva (West Germany), Ajax Chemicals (Sydney, Australia) and BDH Limited (Poole, England).

All common chemicals used were of Analar^{R} grade obtained from BDH Limited and GibcoBRL. Pure water (>10 M Ω/cm, milli-Q H_2O) was produced using a Sybron/Barnstead (MA, USA) NANOpure filtration apparatus.

Microspin S-400 HR columns, microspin G-50 columns, GST-purification module, SDS-LMW marker kit, peptide molecular weight marker kit, pGEX 5' and 3'-sequencing primers, dATP, dGTP, dTTP and dCTP and Hitrap HR columns (1 ml) were obtained from Pharmacia-LKB Biotechnology (Uppsala, Sweden). Radio labelled [^{32}P] dCTP nucleotide was bought from New England Research products (MA, USA)

(3000 ci/mmole). TRIzol™ LS Reagent kits for total RNA isolation from liquid samples were obtained from Life-Technologies Inc., (MD, USA).

Wizard DNA Miniprep kits were obtained from Promega Corporation (WI, USA) and Prep-A-Gene DNA Purification Kits were from Bio-Rad Laboratories (CA, USA). Sterile filters of 0.2 μm pore size were from Gelmann sciences (MI, USA) or Millipore Corporation (MA, USA). Filtron Technology Corporation (MA, USA) supplied 10 K cut-off Filtrons. Spectra/por molecular porous dialysis membranes were obtained from Spectrum (CA, USA).

96-well plates for ELISA (# 442404) were obtained from Nunc, Denmark. Blotting paper (3 MM) was supplied by Whatman, England. X-ray film, photographic developer and fixer were obtained from Eastman Kodak (NY, USA) or Fuji Photo Film Company Ltd., Japan and polaroid film was bought from Polaroid Corporation (Cambridge, MA, USA).

ECL western blotting analysis kits were obtained from Amersham Life Science (Buckinghamshire, UK). DIG-DNA non-radioactive labelling and detection kits were supplied by Boehringer Mannheim (West Germany). Vectastain ABC kit for lactoferrin ELISA work was obtained from Vector Laboratories Inc., (CA, USA).

All synthetic oligonucleotides, 1 Kb and 1 Kb plus DNA ladders and rTEV protease were obtained from Bethedsa Research laboratories (GibcoBRL) (MD, USA). Phosphorylated *SacI* and *BamHI* linkers were obtained from Promega Corporation (WI, USA).

Bovine lactoferrin was supplied by Tatua Biologicals (Morrinsville, NZ) and native lactoferricin B cationic peptide was a gift from Prof. E.N. Baker.

For transmission electron microscopy experiments, gluteraldehyde, para-formaldehyde, osmium tetroxide and lead acetate were obtained from Agar Scientific Ltd., UK. Phosphate buffers and alcohol were from BDH chemicals and L.R.White was obtained from London Resin Company, UK.

2.1.2 Bacterial strains, yeast strains and plasmids used in this study

The bacterial and yeast strains and plasmids used in this study are listed in the following Table 2.1.

Table 2.1 Bacterial and yeast strains and plasmids used in this study

Strains and plasmids	Source or Reference
Bacterial Strains	
E. coli 0111:K58(B4):H2 (NCTC 8179)	New Zealand Reference Culture Collection, ESR, Porirua, NZ
E. coli 0111:K58(B4):H12 (NCTC 8008)	Massey University collection #254
E. coli DH5α™ (Lab strain)	GibcoBRL
E. coli TG1 (Lab strain)	PET Department collection Massey University
E. coli BL21 (DE3) (Lab strain)	PET Department collection
E. coli 0157 (Food pathogen, New Zealand isolate)	PET Department collection
Yeast strains	
Kluyveromyces lactis MW98-8c	PET Department collection
Saccharomyces cerevisiae DY150	Clonetech Labs (CA, USA)
Plasmids	
pET-15b	Novagen Inc (WI, USA)
pPROEXHTa,b,c	GibcoBRL
pGEX-4T 1,2,3	Pharmacia-LKB, Sweden
pEPS1	Obtained from Dr. Ian Macreadie Biomolecular Research Institute, Australia
pYEXS1	Clonetech Labs (CA, USA)
pSPHO4	Obtained from Hiroshi Fukuhara Institute Curie, France

2.1.3 Genotypes of bacterial and yeast strains used in this study

The genotypes of the bacterial and yeast strains used in this study are listed in the following Table 2.2.

Table 2.2 Genotypes of bacterial and yeast strains used in this study

Bacterial and yeast strains	Genotype
***E. coli* strains**	
E. coli DH5α	*SupE44 Δlac U169(ϕlacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*
E. coli BL21(DE3)	*hsd gal (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene1)*
Yeast strains	
Kluyveromyces lactis MW98-8c	*α UraA arg A lys A* K^+
Saccharomyces cerevisiae DY150	*MATa Ura 3-52 Leu 2-3 trp 1-1 ade 2-1 his 3-11 can 1-100*

2.1.4 Bacterial and yeast growth media

LB broth

Bacto-tryptone 10 g/l, bacto-yeast extract 5 g/l, NaCl 10 g/l, (pH 7.0). Pre-mixed LB Broth base supplied by GibcoBRL was also used.

SOB broth

Bacto-tryptone 20 g/l, bacto-yeast extract 5 g/l, NaCl 0.5 g/l, 2.5 mM KCl, 10 mM $MgCl_2$, (pH 7.0).

SOC broth

SOB broth containing 20 mM glucose.

Rich glucose medium (YPD broth)

Bacto-yeast extract 10 g/l, bacto-peptone 20 g/l, glucose 20 g/l.

***K. lactis* minimal medium**

Yeast nitrogen base (without amino acids) 6.7 g/l, Glucose 20 g/l, arginine 0.02 g/l, lysine 0.02 g/l.

***S. cerevisiae* minimal medium**

Yeast nitrogen base (without amino acids) 6.7 g/l, Glucose 20 g/l, histidine 0.02 g/l, tryptophan 0.02 g/l, adenine sulphate, 0.02 g/l, leucine, 0.01 g/l.

All media were sterilised by autoclaving for 20 minutes at 121°C and 15 lb/in^2. All liquid media were solidified by the addition of 15 g/l agar for hard agar media and 7.5 g/l for soft agar media prior to autoclaving. All antibiotics were added after autoclaving, when the temperature of the medium was less than 50°C.

2.1.5 Commonly used solutions and buffers

TE

10 mM Tris.Cl, 1 mM EDTA, (pH 8).

Transformation buffer (TB) for *E. coli*

10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, (pH 6.7).

HEPES buffer was also used instead of PIPES buffer. Similar transformation efficiencies were obtained.

Solutions for plasmid DNA minipreps

Solution I 25 mM Tris.HCl (pH 8), 10 mM EDTA, 50 mM glucose, 0.8 mg/ml lysozyme, 50 µg/ml RNase A.

Solution II 200 mM NaOH, 1% (w/v) SDS. This was always prepared fresh from 10x stocks.

Solution III 5 M or 7.5 M ammonium acetate.

Phenol : chloroform

50% (v/v) phenol, 48% (v/v) chloroform, 2% (v/v) isoamyl alcohol, 0.1% (w/v) hydroxyquinoline, (pH 8.0).

TAE electrophoresis buffer

40 mM Tris-acetate, 1 mM EDTA, (pH 8).

TBE electrophoresis buffer

45 mM Tris-borate, 1 mM EDTA, (pH 8).

MOPS electrophoresis buffer (1X)

20 mM MOPS, 0.1 mM EDTA, 1 mM sodium acetate, (pH 7).

1X SSC

150 mM NaCl, 15 mM sodium citrate, (pH 7).

1x Denhardt's reagent

0.2 mg/ml Ficoll (Type 400), 0.2 mg/ml polyvinylpyrrolidone, 0.2 mg/ml BSA.

50x Denhardt's reagent

10 mg/ml Ficoll (Type 400), 10 mg/ml polyvinylpyrrolidone, 10 mg/ml BSA.

HEPES-buffered saline

600 mM NaCl, 5 mM HEPES (pH 7.4), 5 mM EDTA, 1 mM EGTA.

SDS-PAGE solutions

Stacking gel 5% (w/v) (37.5 : 1) acrylamide : bisacrylamide, 125 mM Tris.HCl (pH 6.8), 0.1% (w/v) SDS.

Resolving gel	10-15% (w/v) (37.5 : 1) acrylamide : bisacrylamide, 375 mM Tris.HCl (pH 8.8), 0.1% (w/v) SDS.
Running buffer	25 mM Tris, 200 mM glycine, 0.1% (w/v) SDS, (pH 8.5).
Sample buffer (2x)	62.5 mM Tris.HCl (pH 6.8), 3% (w/v) SDS, 700 mM mercaptoethanol, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue.
	OR
Sample buffer (6x)	0.35 M Tris-HCl (pH 6.8), 10.28% SDS (w/v), 36%(v/v) Glycerol, 0.6 M dithiothreitol (or 5% 2-marcaptoethanol), 0.012% (w/v) bromophenol blue (Pharmacia GST-purification module manual).

Tris-tricine buffer system

Gel buffer	3 M Tris-HCl (pH 8.5), 0.3% (w/v) SDS.
Anode buffer	0.2 M Tris-HCl, (pH 8.9).
Cathode buffer	0.1 M Tris base, 0.1 M Tricine, 0.1% (w/v) SDS.

Phosphate-buffered saline (PBS)

0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.115% (w/v) Na_2HPO_4, 0.02% (w/v) KH_2PO_4, (pH 7.5).

PBS-Tween.

0.05% (v/v) Tween-20 in PBS, (pH 7.5).

2.1.6 Maintenance, storage and propagation of bacterial cultures

All *E. coli* strains used in this study were streaked from frozen glycerol stocks [30% (v/v) glycerol in LB broth, stored at -70°C], onto LB-agar plates [1.5% (w/v) agar in LB broth], supplemented if necessary with antibiotic and incubated at 37°C for 12-16 hrs. Liquid cultures of *E. coli* were grown in LB media with antibiotic (if necessary) at 37°C for 12-16 hrs with shaking (200 rpm). Plasmids were selected and maintained in *E.*

coli with appropriate antibiotic pressure, which in most cases was ampicillin (50 - 100 µg/ml).

2.1.7 Growth and storage of M13 phage stocks

A bacteriophage M13 infected culture was prepared by the inoculation of a single plaque into a 5 ml culture of *E. coli* XL1 strain (containing F′ plasmid- tetracycline resistant) having an OD_{600} of ~0.6, followed by incubation at 37°C for a further 5 to 6 hrs. Cells and cell debris were removed from the media by centrifugation in a microfuge at 12,000 rpm for 5 min. The resulting supernatant containing approximately 10^{10}-10^{12} pfu/ml of phage suspension could be stored indefinitely at 4°C.

2.1.8 Growth and maintenance of yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*

Saccharomyces cerevisiae strain DY150 and *Kluyveromyces lactis* strain MW98-8c were stored as frozen glycerol stocks [30% (v/v) in YPD broth] at -70°C, on agar stabs [2% (w/v) agar in YPD broth] at 4°C or as dried cells on sterile filter paper at room temperature in vials. Cells were grown by streaking onto agar plates [1.5% (w/v) agar in YPD broth] supplemented with tetracycline (12.5 µg/ml) and incubated at 30°C for 36-48 hours.

Liquid cultures of *Saccharomyces cerevisiae* and *Kluyveromyces lactis* were grown in YPD broth at 30°C for 36-48 hrs with shaking (200 rpm). Plasmids were selected and maintained in transformed *S. cerevisiae* and *K. lactis* by growing for several days at 30°C on minimal medium lacking the appropriate amino acids. Tetracycline (12.5 µg/ml) was included in all cultures in order to prevent growth of contaminating microorganisms.

2.2 Methods

All procedures for recombinant DNA techniques were carried out essentially as described in Sambrook *et al.*, (1989), except where noted below.

2.2.1 General precautions in DNA handling

All glassware, plastic-ware and solutions in direct contact with DNA were sterilised by autoclaving at 15 psi for 15 to 20 min. Temperature sensitive compounds such as antibiotics, some salt solutions and volatiles like DTT and acetate were filter sterilised through 0.2 μm membrane filters. Gloves were always worn while handling DNA to minimise nuclease contamination.

2.2.2 DNA and RNA quantitation

The concentration of purified DNA in solution was generally determined spectrophotometrically. The quantitation of small quantities of DNA was done by comparing with DNA quantitation standards or a plasmid of known concentration following agarose gel electrophoresis. Pure preparations of DNA had an A_{260}/A_{280} ratio of approximately 1.8 (Sambrook *et al.*, 1989). Contamination with phenol or protein would decrease this ratio. The concentration of DNA and RNA solutions was calculated using the following relationships.

1 A_{260} unit of double stranded DNA = 50 μg/ml

1 A_{260} unit of single stranded DNA = 33 μg/ml

1 A_{260} unit of single stranded RNA = 40 μg/ml

2.2.3 Protein quantitation

Protein concentration was determined by the dye-binding method using Coomassie blue G-250. The dye-binding reagent contained 300 mg of Coomassie blue G-250 dissolved in 14.28 ml of perchloric acid and diluted to 500 ml with milli-Q water. This reagent was filtered before use. Protein samples (0.1 ml) were mixed with 2.5 ml of dye reagent and left to stand for 20-30 minutes. The blue colour was measured at 595 nm. This method has a very high sensitivity and protein amounts as low as 10-50 μg could be measured with moderate accuracy.

2.2.4 Preparation of M13 replicative form

M13 phage suspension (5 ml) (prepared as described in section 2.1.7) was added to 300 ml of XL1 culture (grown on LB^{tet} media) at OD_{600} ~ 0.1-0.2. The suspension was incubated at 37°C with shaking for exactly 15 min to enable phage to attach to *E.*

coli XL1 cells. Chloramphenicol was added to a final concentration of 15 μg/ml and the incubation was continued for a further 2 hrs with shaking at 37°C. The accumulated replicative form of M13 in *E. coli* XL1 cells was isolated by using standard plasmid isolation methods.

2.2.5 Plasmid DNA and ds-M13 Replicative Form DNA Preparations from *E. coli*

Plasmid DNA Minipreps

Plasmid DNA minipreps were made using the alkaline lysis method (Sambrook *et al.*, 1989).

Single *E. coli* colonies were inoculated into LB broth (5 ml) containing the appropriate antibiotic to maintain the plasmid (typically ampicillin 100 μg/ml), vortexed and incubated for 12-16 hours at 37°C with shaking (200 rpm).

Cells from 1.5 ml of this overnight culture were pelleted by centrifugation in a microfuge (6,000 rpm, 1 min), and resuspended in Solution I (200 μl). After standing for 5 minutes at room temperature, Solution II (400 μl) was added and mixed by inversion. After another 5 minutes of incubation at room temperature, Solution III (300 μl) was added, and the solutions were thoroughly mixed by inversion. After standing on ice for 10 minutes, the resultant flocculant mixture was centrifuged (13,000 rpm, 10 min), the supernatant transferred to a fresh tube, and the plasmid DNA precipitated by the addition of 2 volumes of isopropanol followed by centrifugation (13,000 rpm, 10 min). The final DNA pellet was washed with 70% ethanol (-20°C), dried and finally dissolved in TE, pH 8.0 (50 μl).

For making pure plasmid DNA for the purpose of DNA sequencing, the Wizard miniprep kit from Promega Corporation or the DNA isolation and purification kit from Boehringer Mannheim or the Quantum-prep plasmid miniprep kit from BioRad was used. The pure minipreps were made as per the instructions of the suppliers. Double-stranded M13 replicative form DNA was also isolated and purified using the same methods.

Large-scale preparation of plasmid DNA

Large scale preparations of plasmid DNA were carried out either by using the Wizard Maxiprep DNA purification system (Promega) or by using the alkaline lysis procedure described in Sambrook, *et al.*, (1989), followed by equilibrium density gradient centrifugation using a CsCl-ethidium bromide gradient. The band corresponding to the plasmid was recovered and then the ethidium bromide was removed by extraction with water saturated butanol. Caesium chloride was removed by dialysis against several changes of 1x TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer.

2.2.6 Isolation of plasmids from *K. lactis*

Recombinant strains of *K. lactis* were grown in selective medium (10 ml) for 24 hrs. The culture was centrifuged (4,000 rpm, 5 min) and the pellet was washed in 0.9% NaCl and recentrifuged. The supernatant was discarded and the pellet was resuspended in Lyticase buffer (0.5 ml: 0.8 M sorbitol, 10 mM β-mercaptoethanol, 100 mM sodium phosphate, pH 6.5). A 10 µl aliquot of the suspension was diluted (1:100) in 1% SDS and the absorbance was measured at 600nm. The suspension was transferred to a microfuge tube and Lyticase enzyme (20 mg/ml) was added. The absorbance at 600 nm was monitored using 10 µl aliquots as described above until it reached 10% of the original value. After centrifugation (13,000 rpm, 30 sec) the pellet was gently resuspended in suspension solution (Wizard miniprep kit) and the manufacturer's instructions were followed to isolate the plasmid DNA. The eluted plasmid DNA was mixed with isopropanol (0.5 vol) and was held at RT for 20 min. After centrifugation, the supernatant was carefully removed and the pellet was washed in 70% ethanol (-20°C) and recentrifuged. The pellet was dried and resuspended in minimum volume (~ 20 µl).

2.2.7 Restriction digestion of DNA with endonucleases

Minipreps of high copy number plasmids yield between 100 and 150 ng/µl DNA in the final solution. Routinely, 5 µl (~0.5 µg plasmid) of this final solution was digested using 1-5 units of restriction enzyme in a total volume of 25 µl of 1x enzyme buffer, as recommended by the manufacturer, for 1-2 hours usually at 37°C unless a different temperature was recommended for an enzyme. Digestion of larger amounts of DNA was

scaled up accordingly and incubated for longer periods of time to ensure complete digestion.

2.2.8 Agarose gel electrophoresis of DNA

After digestion by restriction endonucleases, DNA fragments were analysed using agarose (low EEO agarose) gel electrophoresis in 1x TAE buffer at 3-5 V/cm, essentially as described in Sambrook *et al.*, (1989), using 0.2% bromo-phenol blue in 50% glycerol as 6x loading buffer. Agarose gels of between 0.7 and 1.0% were routinely used for most separations. Agarose gels of 2% were used to resolve DNA fragments smaller than 500 bp. The inclusion of ethidium bromide in the gel (0.5 μg/ml) allowed the DNA to be visualised by illumination with ultraviolet light (302 nm). Permanent record of gels was obtained by photography using either a Polaroid Land camera or a Gel Doc system (Alpha Innotech Corporation, USA).

2.2.9 Size determination of DNA fragments

Sizes of DNA fragments were estimated by comparing with standards. The usual sizing standard used for DNA fragments larger than 500 bps was 1 Kb or 1 Kb plus DNA ladders (GibcoBRL) or *Hind*III-digested λ-phage DNA. Standards were loaded at a concentration to give a total of 1.5 μg of DNA per lane. Semi-quantitative estimation of DNA samples was possible by comparison of the relative intensities of bands with the quantification standards. Size estimation for smaller DNA fragments was carried out by comparison with 1 Kb or 1 Kb plus DNA ladder markers (GibcoBRL) on a 2% agarose gel.

2.2.10 Precipitation of DNA and purification of DNA fragments from agarose gels

Plasmid DNA was routinely precipitated from aqueous solutions by the addition of sodium acetate (0.1 vol., 3 M, pH 5.2) and 100% ethanol (2 vol., -20°C) as described in Sambrook, *et al.*, (1989). Precipitated DNA was recovered by centrifugation (13,000 rpm, 10 min., 4°C) and washed with 70% ethanol (-20°C) to remove excess salt and dried in a vacuum concentrator (SpeedVac) (Savant, NY,USA). Plasmid DNA was also concentrated by using Nanospin columns supplied by BioRad.

Restriction-digested DNA fragments were purified from agarose gels by using a Prep-A-Gene kit supplied by Bio-Rad, following the manufacturer's instructions. Plasmid DNA in solution was purified from protein and other contaminants either by extraction with phenol : chloroform (Sambrook *et al.*, 1989) or using a Prep-A-Gene kit, following the manufacturer's instructions.

2.2.11 Modification of DNA fragments

When required plasmid DNA fragments were blunt-ended using Klenow fragment of *E. coli* DNA polymerase I or T4 DNA polymerase as appropriate (Sambrook *et al.*, 1989) and/or dephosphorylated using ordinary CIAP or thermo-sensitive CIAP (Ts-CIAP) before being ligated. The CIAP was inactivated by heat treatment and later removed by using Prep-A-Gene DNA purification kit.

2.2.12 Ligation of DNA fragments

Ligations were routinely carried out using bacteriophage T4 DNA ligase (either at 10 U/µl or 1 U/µl concentration) in as small a volume as possible (typically 10 µl). Ligation reactions were usually incubated at 12-16°C overnight. Incubation at room temperature for several hours or at 37°C for 30 minutes was also used. A portion of the ligation reaction mix (usually 1-2 µl) was then used to transform competent *E. coli* cells as described in the following section.

2.2.13 Preparation of *E. coli* competent cells

Plasmid vectors were introduced into *E. coli* cells made competent using a chemical procedure based on that of Inoue *et al.*, (Inoue *et al.*, 1990).

Several large colonies of the required strain (usually DH5α) were inoculated into SOB medium (300 ml) in a 2 litre conical flask. The culture was then incubated at 18°C or 22°C with shaking (200 rpm) until the culture reached an optical density of A_{600} ~ 0.6. The cells were pelleted by centrifugation (4,000 rpm, 10 min, 4°C) and gently resuspended in ice-cold transformation buffer (TB). All subsequent steps were carried out at 4°C or on ice. The cells were pelleted and gently resuspended a second time in TB (total volume, 24 ml). DMSO (1.8 ml) was added drop-wise to the cell suspension

with gentle mixing. The cells were allowed to stand on ice for 10 minutes before being divided into small aliquots, flash frozen in liquid nitrogen and stored at -70°C.

2.2.14 Transformation of *E. coli* competent cells

Competent cells (100 µl) were incubated with plasmid DNA (1-10 µl) on ice for 30 minutes before being heat-shocked at 42°C for 30 - 45 seconds. After being returned to ice, SOC or LB medium (0.9 ml) was added to the cells, which were then incubated at 37°C with shaking (200 rpm) for 60 minutes. The transformed cells were then plated out onto LB-agar plates [1.5% (w/v) agar in LB broth] with appropriate antibiotic selection [typically ampicillin (100 µg/ml)] and incubated at 37°C for 12-16 hours. If appropriate, β-galactosidase activity of transformed cells was assessed by plating onto agar containing IPTG (20 mM) and the chromogenic substrate X-Gal (80 µg/ml).

2.2.15 Preparation of *K. lactis* competent cells

K. lactis cells were grown in YPD broth (100 ml) aerated at 30°C until mid-log phase (A_{600} ~0.6-1.0), harvested by centrifugation (4°C, 8,000 rpm, 5 min) and washed with ice-cold electroporation buffer [EB: 10 mM Tris-HCl (pH 7.5), 270 mM sucrose, 1 mM $MgCl_2$). The cells were resuspended in YPD broth (10 ml) containing dithiothreitol (DTT, 25 mM) and HEPES buffer (20 mM, pH 8), and incubated at 30°C for 30 min. The cells were recentrifuged, washed in EB and resuspended in EB (1 ml). The competent cells were stored at -70°C.

2.2.16 Electroporation of *K. lactis* competent cells

Ligation reactions were dialysed to remove salts. A 100 µl aliquot of competent cells was mixed with 10 µl of ligation mix (~ 0.2 µg of DNA) and placed on ice for 15 min. The mixture was then transferred to a sterile Gene Pulsar cuvette (Bio-Rad, 2 mm electrode gap). For Gene Pulsar, one pulse at 900 V (4,500 V cm^{-1}) and 25 µF was applied. The cells were immediately cooled in ice and resuspended in YPD broth (1 ml) and incubated at 30°C for one hour before diluting and plating on selection minimal media agar. The plates were incubated at 30°C for 2-3 days before counting colonies and examination of transformants.

2.2.17 Transformation of *S. cerevisiae*

Transformation of *S. cerevisiae* DY150 was performed as described below. A 5 ml liquid culture of *S. cerevisiae* DY150 was grown at 30°C for 12-16 hours in YPD broth before being diluted into 100 ml of the same medium. Incubation continued until the culture had an optical density of A_{600} ~1.3 to 1.5. The cells were harvested by centrifugation (3,000 rpm, 10 min, 4°C), washed once with 100 ml of ice-cold sterile milli-Q H_2O. The cells were kept cold during the entire process. Washing was repeated with 50 ml of ice-cold sterile milli-Q H_2O. The cells were resuspended in 4 ml of ice-cold 1 M sorbitol and centrifuged. The pellet was resuspended in 100 µl of ice-cold 1 M sorbital. The cells could be stored at 4°C for up to a week without a significant drop in transformation efficiency.

Electro-competent yeast cell suspension (65 µl) was mixed with dialysed ligation mix (~ 1-3 µg), mixed and incubated on ice for 5 min. The cell/DNA mixture was then transferred to a prechilled sterile Gene Pulsar cuvette (2 mm) (BioRad) and subjected to a pulse of 1,500 V (7.5 kV/cm) and 1 ml of cold sorbitol was added immediately. Aliquots of the cells were plated onto selective media containing 1 M sorbitol. The plates were incubated at 30°C for 2 to 3 days till the colonies appeared.

2.2.18 Sequence analysis of DNA

DNA sequencing was carried out using the dideoxy chain-termination method (Sanger *et al.*, 1977) or dye-termination method using double-stranded plasmid as template on an ABI Prism 377-36 automated DNA Sequencer.

2.3 Working with RNA

2.3.1 Precautions to be taken in RNA work

For all the work involving the manipulation of RNA, glass and plastic ware were rendered RNase-free by soaking in a 0.1% (v/v) DEPC solution for 24 hours before being autoclaved at 15 lb/in^2 for at least 1 hour (or 4 to 5 times for 15 min each) to remove any residual DEPC. All solutions were made using DEPC-treated milli-Q pure water and were treated with 0.1% (v/v) DEPC prior to autoclaving, if applicable.

2.3.2 RNA isolation from *E. coli*

Total RNA was isolated from *E. coli* using TRIzol™ LS Reagent from Life Technologies Inc following manufacturer's instructions.

2.3.3 RNA gel electrophoresis

Total RNA was analysed using denaturing agarose gel electrophoresis. RNA was dissolved in loading buffer [50% (w/v) formamide, 0.7% (v/v) formaldehyde, 1x MOPS buffer, 0.03 mg/ml ethidium bromide], heated to 65°C for 10 minutes, quenched on ice and loaded onto a denaturing gel [1.2 - 2% (w/v) low EEO agarose, 0.7% (v/v) formaldehyde, 1x MOPS buffer], which had been pre-run at 5 V/cm for 5 min in running buffer (1x MOPS buffer, 0.7% (v/v) formaldehyde). Electrophoresis was carried-on at the same voltage for 2-4 hours, and the RNA visualised as described previously for DNA gels (section 2.2.8). The sizes of RNA bands were estimated by comparison with an RNA standard ladder (GibcoBRL).

2.3.4 Southern and northern blotting

After electrophoresis of DNA or RNA, transfer of nucleic acids to nitrocellulose or nylon membrane (Hybond-N^+) was performed by capillary transfer, as described in Sambrook *et al.*, (1989). The nucleic acids were fixed to the membrane by baking at 80°C for 2 hours or by exposing to UV light for 2 min on each side.

Radio-labelled cDNA probes of high specific activity were produced using random priming with small oligonucleotides, essentially as described in Sambrook *et al.*, (1989), or by end-filling the lactoferricin primers using [α-^{32}P] dCTP as the radiolabel. Unincorporated radiolabel was removed from labelled DNA by chromatography through pre-packed microspin Sephadex G-50 supplied by Pharmacia.

Hybridisation of radio-labelled probes to nucleic acids immobilised on nylon or nitrocellulose filters was carried out essentially as described in Sambrook *et al.*, (1989). Membrane prehybridisation (2 hrs) and hybridisation (14-16 hrs) was performed at 65°C in the following solution (solutions were freshly made for both steps).

6xSSC, 0.5% SDS, 5x Denhardt's reagent

175 μg/ml sheared salmon sperm DNA

The filters were subsequently washed in 5x SSC/0.1% SDS followed by 2x SSC/0.1% SDS and then followed by 0.1x SSC/0.1% SDS at 65°C. The stringency of washing was also changed by varying the temperature of the wash solutions. The hybridisation of the probe to the filter was checked by Geiger-Mullar counter for radioactivity at each step and then visualised by autoradiography.

Generally the blots were stripped of the probe by placing the membrane in hot (100°C) 0.1% SDS solution and allowing it to cool slowly to RT. This step was repeated at least twice and autoradiography was used to check for any residual probe.

2.4 Expression and analysis of recombinant proteins

2.4.1 Expression studies of recombinant proteins in *E. coli*

Routinely, cultures of OD_{600} ~0.6 were induced with 1 mM IPTG and grown for 3-4 hrs at 37°C. IPTG induction was also done at different concentrations of IPTG (0.1 mM, 1 mM and 2 mM) and different temperatures (16°C, 25°C and 30°C).

2.4.2 Disruption of *E. coli* cells

The cell pellet from 1 ml of IPTG induced culture was resuspended in 200 μl of 50 mM Tris.HCl (pH 7.0), 10 mM $MgCl_2$ buffer or other suitable buffer and sonicated for 20 to 30 seconds using a Virsonic sonicator (Virtis, USA) with an optimum power setting for maximum cell lysis. The sonicated suspension was spun in a microcentrifuge at 12,000 rpm to separate the supernatant fraction from the pellet.

2.4.3 SDS-polyacrylamide gel electrophoresis using minigels

Proteins were separated both analytically and preparatively using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using a Hoefer Mighty Small II vertical slab minigel apparatus. Routinely, 15% or 20% (w/v) (37.5 : 1 acrylamide: bisacrylamide) resolving gels and 5% (w/v) (19:1 acrylamide : bisacrylamide) stacking

gels were used. Both SDS-PAGE gels and Tris-tricine gels were used depending upon the size of the protein/polypeptide to be separated. Normalised volumes of the samples were loaded on the SDS-PAGE gels with respect to the elution volumes of the fusion proteins.

2.4.4 Fixing, staining and destaining of polyacrylamide gels

After electrophoresis, gels were stained using Coomassie blue. Gels were routinely fixed and stained by soaking in 2% (w/v) Coomassie Brilliant Blue R-250 dissolved in 10% (v/v) acetic acid, 30% (v/v) methanol, and destained by soaking in several changes of 10% (v/v) acetic acid, 30% (v/v) methanol.

Molecular masses of proteins were estimated by comparison with known standards. Peptide molecular weight (PMW) and Low molecular weight (LMW) SDS-Markers were routinely used as molecular weight markers. Stained gels were air-dried between cellulose acetate membranes (Bio-Rad) or vacuum dried (80°C, 2 hours) by sandwiching the gels between cellulose acetate membranes or onto 3 MM paper (Whatman) after soaking in 2% (v/v) glycerol in 30% (w/v) methanol. The inclusion of glycerol reduces gel shatter on drying. Gels were dried using a Bio-Rad gel drier.

2.4.5 Electroblotting of proteins from acrylamide gels

After electrophoresis, proteins were electro-blotted by soaking the gel in transfer buffer (10 mM CAPS, pH 11.0, 10% (v/v) methanol) for 5 minutes, before being assembled in an electroblotter (BioRad) between sheets of 3 MM paper soaked in transfer buffer, sandwiched next to a sheet of PVDF membrane that had been wetted in methanol and then equilibrated in transfer buffer. Proteins were transferred at a constant current of 60 mA for 90-120 minutes. Protein transfer was assessed by staining a sample strip of blotted membrane in 0.005% (w/v) Poncue S dissolved in 30% (v/v) methanol, 0.2% (v/v) acetic acid.

2.4.6 Western blotting

Proteins were separated by SDS-PAGE as described in section 2.4.3. and transferred to PVDF membrane as described in section 2.4.5. Detection of transferred protein using antisera was carried out as described below.

All incubations and washes were carried out at 30°C. To reduce non-specific antibody binding, the membrane was incubated (30 min) with 3% (w/v) casein in PBS-Tween 20, before being washed (10 min) three times with PBS-Tween 20. The membrane was then incubated (30-60 min) with antibody, diluted according to estimated titre in PBS-Tween 20, before being washed as before.

Antibody binding was visualised by incubation (45 min) with HRP-conjugated goat anti-rabbit antiserum (1:1000 in PBS-Tween) followed by washing, immersing in 2 ml mixture of ECL reagents and then exposing to X-ray film by following the manufacturer's instructions. This system utilises the horse readdish peroxidase (HRP) catalysed luminol based chemiluminescence reaction enabling HRP-labelled secondary antibody to be detected by light emission, with the signal being captured on X-ray film.

2.4.7 Enzyme-linked immunosorbant assay (ELISA) of lactoferrin

Lactoferrin secreted into the media by *K. lactis* was estimated using the ELISA method developed for lactoferrin (Vilja *et al.*, 1985). All procedures were carried out in a 96 well flat-bottomed micro-titre plate. The wells were coated with anti-lactoferrin antibodies (100 µl of 0.1 µg/ml) in 0.1 M sodium carbonate/bicarbonate buffer (pH 9.6). The antibodies were attached to the plate by incubation at 4°C for 12-16 hours. The wells were washed 3 times with wash buffer (1x PBS, pH 7.4 and 0.5% Tween 20), non-specific binding of lactoferrin was reduced by pre-incubation (37°C, 30 min.) with diluent buffer [1% (w/v) BSA in PBS-Tween 20 (250 µl/well)], the diluent buffer was discarded and lactoferrin standards (0-80 ng) or samples were added to the wells with 100 µl of diluent buffer in each well and incubated for 1 hr at 37°C. The wells were washed 3 times with washing buffer. Biotinylated anti-lactoferrin antibodies diluted in diluent buffer were added to the wells (100 µl) and incubated for 1 hr at 37°C. The wells were washed again 3 times with wash buffer. To each well 100 µl of pre-incubated Vectastain ABC-reagent (A, 20 µl, B, 20 µl in 10 ml diluent buffer) was added and incubated for 1 hr at 30°C. The wells were washed again for 5 times with wash buffer. Then 100 µl of OPD substrate solution was added to the plate, incubated at room temperature in the dark for 20 min. Reaction was stopped by adding 100 µl of 2 M H_2SO_4. The plate was read at 492 nm using an ELISA reader (SLT Laboratory Instruments). The limit of detection with this method is 1 µg/ml concentration of LF.

2.5 Purification and characterisation of recombinant proteins

2.5.1 Poly-His affinity tagged protein purification

The pET-15b and pPROEXHTa vectors used in this study have a stretch of DNA coding for 6 consecutive histidine residues that can be expressed at the N-terminal end of a target protein. This His-tag sequence can bind to divalent cations (eg. Ni^{2+}) immobilised in a metal chelation resin. After the unbound proteins have been washed away, the target protein can be recovered by elution with imidazole. Hitrap HR metal chelation columns (1 ml size) supplied by Pharmacia were used for this purpose. Procedures supplied by Pharmacia (Sweden) and Novagen (WI, USA) were followed for the purification of both soluble proteins and solubilised inclusion bodies.

2.5.1.1 Buffers for the purification of inclusion bodies on Ni-NTA column

All buffers contained urea at a final concentration of 8 M urea. Urea was used in preference to guanidine-HCl as urea-containing samples can be loaded directly to SDS-polyacrylamide gels whereas, guanidine-HCl samples must be diluted 1:5 in water or dialysed before electrophoresis.

1x binding buffer	5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9 at RT
1x wash buffer	60 mM imidazole, 0.5 M NaCl, 20 mM Tris. HCl, pH 7.9 at RT
1x elution buffers	80, 100, 120, 160, 200 and 300 mM of imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9 at RT.

The elution buffers were prepared with increasing imidazole concentrations to enable the elution of the recombinant protein in a step-wise fashion.

1x strip buffer	100 mM EDTA, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9 at RT
1x charge buffer	50 mM $NiSO_4$ (for recharging the stripped NTA-resin with Nickel)

2.5.2 GST-tagged protein purification

The glutathione S-transferase (GST) fusion proteins expressed in the pGEX4T-1 vector were purified directly from bacterial lysates using the affinity matrix glutathione-Sepharose 4B. Fusion proteins were eluted from the resin under mild, non-denaturing

conditions using 10 mM reduced glutathione, which preserves protein antigenicity and functionality. Procedures supplied by Pharmacia (Sweden) were used to purify GST fusion proteins.

2.5.2.1 Buffers for the purification of GST-tagged proteins expressed as inclusion bodies

1x PBS buffer	0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.3 at RT)

For the solubilisation of inclusion bodies, 1x PBS buffer included 8 M urea. After solubilising the inclusion bodies, the supernatant was diluted with 1x PBS to a final urea concentration of 3 M before binding to the glutathione-Sepharose 4B column. After binding the GST-fused recombinant protein to glutathione-Sepharose 4B resin, it was washed extensively with 1x PBS before elution with elution buffer.

1x elution buffer	10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0) (1x elution buffer did not contain urea)

2.5.3 N-terminal protein sequencing of recombinant lactoferricins

Proteins were electro-blotted onto PVDF membrane as described in section 2.4.5. The blot was stained with Coomassie brilliant blue. A single band corresponding to the protein to be sequenced was cut out from the Coomassie blue stained blot. N-terminal sequence was determined by automatic protein sequencing using an Applied Biosystems 477A Protein Sequencer.

2.6 Antibacterial assays of native Lactoferricin B (LFcin-B)

2.6.1 Preparation of bacterial cultures for antibacterial assay

Five *E. coli* strains selected for this study have different growth rates when grown on 1% peptone liquid media at 37°C with aeration. To assist in obtaining the cultures of different *E. coli* strains at the same initial cell density, calibration curves (OD_{600} vs Log_{10} CFU) were constructed for all these strains during growth on 1% peptone at 37°C.

Overnight inoculum of these *E. coli* strains was diluted in fresh 1% peptone in a 1:100 ratio. The freshly inoculated culture was grown at 37°C with shaking. Samples were collected initially and after every 20 min up to 120 min. Absorbance at 600 nm was measured at each sampling point. Then a serial 10-fold dilution of these cultures in 1% peptone at each sampling point was plated in triplicate on LB agar media and incubated overnight at 37°C. The colony forming units (CFU) were counted. The limit of detection was 100 CFU/ml. A correlation was drawn between OD_{600} vs Log_{10} CFU (Fig. 5.1). This assisted in obtaining the cultures at desired OD_{600} and hence at desired cell number. For all assays, typically standard inoculum of logarithmic-phase cells (~$1x10^5$ to $1x10^7$ CFU/ml) was taken for all strains.

2.6.2 Determination of minimum inhibitory concentration (MIC) of LFcin-B

Stock solutions of native lactoferricin B (1 mg/ml) were prepared in distilled water and filter-sterilised. Protein estimation was done by dye-binding assay as described in section 2.2.3. For assay of antibacterial activity, a standard inoculum of logarithmic-phase cells (~$1x10^6$ CFU/ml and $1x10^7$ CFU/ml for all strains) was incubated for 24 hrs in a series of tubes containing 1% peptone medium supplemented with various concentrations of native lactoferricin B (0, 1, 2, 4, 8, 10, 12, 16, 33, 50 and 100 µg/ml of culture). Each series of tubes was prepared in triplicate. After 24 hrs of incubation at 37°C in a shaker-incubator water bath, serial 10-fold dilutions were prepared in 1% peptone medium in triplicate and plated 100 µl of diluted samples on LB agar media. The plates were incubated at 37°C for 12-14 hrs for the determination of colony-forming units (CFU). Then the CFU were calculated back per millilitre. The limit of detection was 100 CFU/ml. No loss of viability occurred in the absence of added lactoferricin B.

The minimum inhibitory concentration (MIC) was taken as the lowest concentration of lactoferricin B that caused complete inhibition of growth in all three series.

2.6.3 Assay of the bactericidal activity of LFcin-B

For the assay of bactericidal activity, a standard inoculum of logarithmic-phase cells ($1x10^6$ CFU/ml and $1x10^7$ CFU/ml for all strains) was cultured for specified periods of time in a series of tubes containing 1% peptone medium supplemented with various

concentrations of native lactoferricin B (0, 1, 10, 33, 50 and 100 µg/ml of culture). Each series of tubes was prepared in triplicate. Samples were collected initially and after 1 hr, 2 hrs, 3 hrs and 24 hrs time periods of incubation at 37°C in a shaker-incubator water bath and serial 10-fold dilutions of them were prepared in 1% peptone medium in triplicate and plated on LB-agar media. The plates were incubated at 37°C for 12-14 hrs for determination of colony-forming units (CFU). The limit of detection was 100 CFU/ml. No loss of viability occurred in the absence of added lactoferricin B. For data analysis, if no viable bacteria were observed at the lowest dilution, the bacterial count was recorded as 1 CFU at that dilution. For example, if the lowest dilution without bacterial growth for a given experiment was $1:10^2$, the bacterial CFU was considered to be 100 CFU/ml.

The minimum bactericidal concentration (MBC) was defined as the lowest concentration of lactoferricin B that resulted in at least 99.9% killing in all three series after 24 hrs of incubation at 37°C when compared to the series where no lactoferricin B was added.

2.7 Transmission Electron Microscopy (T.E.M.)

2.7.1 Sample Preparation

Primary fixation

The purpose of fixation is to preserve the structure of a biological specimen with minimal alteration during dehydration, embedding, cutting, staining and viewing in the electron microscope. Primary fixation of the *E. coli* cell suspension was done by mixing equal volumes of cell suspension and primary fix solution (25% gluteraldehyde, 8% paraformaldehyde in 0.2 M phosphate buffer, pH 7.2). The mixture was incubated at RT for 1 hr. The cells were pelleted and washed 3 times in 0.2 M phosphate buffer (each wash for 10-15 min) and were left over night at 4°C.

Post-fixation

The cell pellet was resuspended in 1% Osmium tetroxide in 0.2 M phosphate buffer, pH 7.2 and incubated at RT in a fume cupboard for 30 min. The pellet turned

black. The pellet was washed 3 times with water (each wash for10-15 min.). The post-fixed cell pellet was then embedded in 1% agar for further dehydration with a series of alcohol (from 30% to 100%) (10 minutes each) solutions.

Embedding

The dehydrated agar blocks were embedded into L.R.White resin in closed gelatine capsules. The samples were placed into the capsules using toothpicks and small labels were put into each block before pouring the resin mix. The polymerisation was carried out in an oven, vented into a fume hood, at 60°C for up to 24 hrs, till the resin was polymerised.

2.7.2 Cutting semi-thin and ultra-thin sections and their observation

Preparation of glass knife and observation of sections

Glass knives were made on a LKB Knifemaker II using standard methods. Semi-thin sections were cut using a microtome (Reichert-Jung) and viewed under a dissection microscope (Olympus) after staining with 1% Toluidine blue. Once the semi-thin sections showed the presence of sample,thin sections (70 mm)were cut using a fresh knife to obtain sections suitable for T.E.M. The thin sections were collected on 400 mesh grids (supplied by Agar Co., Ltd.,), air-dried and stained using saturated uranyl acetate in 50% ethanol for 10 min. (by floating the thin sections upside down on the stain) and then washed thoroughly in water. Then the sections were stained with lead citrate (Reynold's lead stain) in the same way, washed in water and air-dried prior to viewing.

The sections were viewed and studied using a Philip's 201C transmission electron microscope at 60 kV.

3.1 Introduction

In this study, an attempt was made to express full-length human lactoferrin (hLF) in the yeasts *Kluyveromyces lactis* and *Saccharomyces cerevisiae.* Human lactoferrin has been successfully expressed in BHK cells (Stowell *et al.*, 1991) and in a number of strains of *Aspergillus* (Ward *et al.*, 1992a; Ward *et al.*, 1992; Ward *et al.*, 1995) but, with limited success in the yeast *Saccharomyces cerevisiae* (Liang and Richardson, 1993).

The yeast *Kluyveromyces lactis* can grow on whey-based media. Whey is abundantly available in New Zealand as a by-product of the dairy industry. The production of ethanol through fermentation of whey by the yeast *Kluyveromyces marxianus* has proven successful in New Zealand (Mawson, 1987). This project was initially started with the aim to produce large quantities of human lactoferrin in *K. lactis* by utilising the whey-based media.

At the outset of this project, the *Kluyveromyces lactis* vectors, pEPS1 (Macreadie *et al.*, 1993) and pSPHO4 (from Fukuhara, H.) and the *Saccharomyces cerevisiae* vector pYEXS1 (Castelli *et al.*, 1994) were available. The cDNA encoding intact human lactoferrin (hLF) in the construct pGEM:hLF (Stowell, 1990) and M13 phage containing the coding sequences for the N-lobe of hLF cDNA with a *SacI* site present at the immediate upstream position of the first codon of mature hLF cDNA designated as M13:Lfn (1-296) (*SacI*) (Peterson, 1999) were also available.

The *K. lactis* vector, pEPS1 and the *S. cerevisiae* vector, pYEXS1 comprise the constitutive *pPGK* promoter and *tPGK* terminator for the expression of the cloned DNA. They also contain sequences encoding the leader of the α-subunit of *K. lactis* killer toxin for the transport of mature protein across the cell wall and secretion into the medium.

The construct pGEM:hLF had been made by ligating a blunt-ended 2.3 kb RT-PCR product into the unique *Hinc II* site in the multiple cloning site (MCS) of pGEM1 to make a construct of ~5.1 kb (Stowell, 1990). A partial restriction map of this construct is shown in

Fig. 3.1. The nucleotide and deduced amino acid sequence of hLF cDNA is available in the Ph.D. thesis of Stowell (1990). The pGEM:hLF construct contained the complete human lactoferrin cDNA including the sequences for the native secretion signal peptide and also contained additional restriction sites from pGEM1 flanking the hLF cDNA. This construct needed modification before transferring hLF cDNA to the pEPS1 and pYEXS1 yeast vectors. Both the yeast vectors have a unique *SacI* cloning site and also have a yeast signal for the transport of mature protein across the cell wall and secretion into the medium. So, the sequences for the native secretion signal peptide of the hLF cDNA have to be replaced with the yeast secretion signal sequences by creating a *SacI* site downstream of hLF native secretion signal sequence. It was also necessary to introduce a *SacI* restriction site into the 3′-end of hLF cDNA so that, the entire 2.1 kb hLF cDNA with the complete coding sequence could be directly sub-cloned into the pEPS1 and pYEXS1 yeast vectors.

There are many approaches available to introduce a *SacI* site at the start and the end of the coding sequence of mature hLF. One approach is to use PCR with appropriate primers to create the desired restriction sites. The other commonly used approach is to digest the required insert with appropriate restriction endonucleases and ligate it into the respective sites of the digested vectors. The latter sub-cloning method was followed throughout this study. Although this is laborious, it has the advantage of not introducing any errors in the sequence as is possible in the PCR method. All ligation junctions were checked for correctness by DNA sequencing.

A double stranded (ds) cDNA containing the N-lobe sequences of hLF cDNA (*SacI/SmaI* fragment) obtained from the replicative form of M13:Lfn (1-296) (*SacI*) was used to bring about the required changes at the 5′-end of the hLF cDNA. This *SacI/SmaI* fragment was sub-cloned into the *SacI* and *SmaI* digested pGEM:hLF to obtain hLF cDNA without the native human lactoferrin secretion signal sequence.

The construct M13:Lfn (1-296) (*SacI*) had been made using the *in vitro* site-specific mutagenesis method to introduce a *SacI* site at the immediate upstream position of the first

codon of the mature hLF cDNA in the construct M13:Lfn (1-296) (Peterson, 1999). A partial restriction map of the construct M13:Lfn (1-296) (*SacI*) is shown in Fig. 3.2.

Fig. 3.1 Partial restriction map of pGEM:hLF

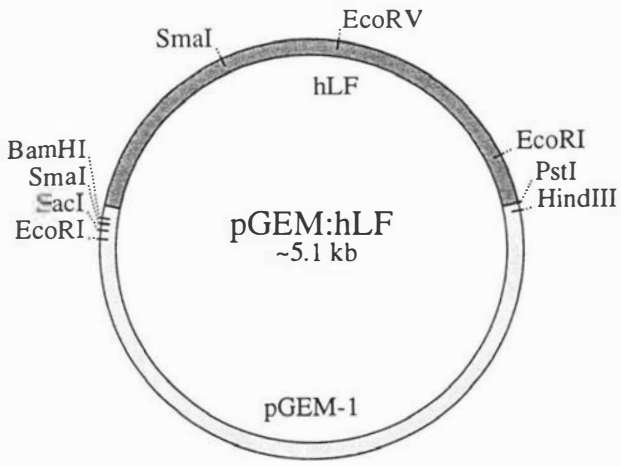
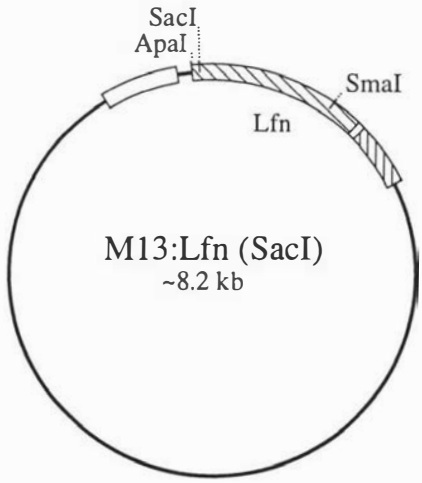


Fig. 3.2 Partial restriction map of M13: Lfn (1-296) (*SacI*)



3.2 Results

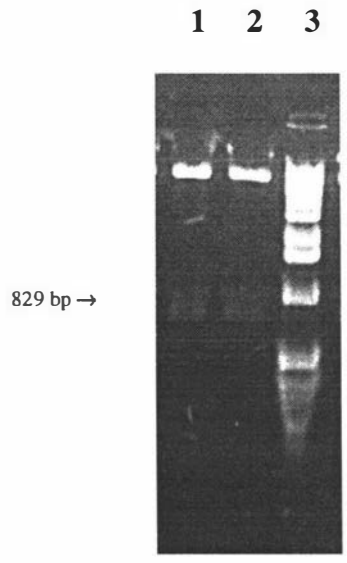
3.2.1 Preparation of replicative form of M13 containing the N-lobe of hLF cDNA with a *SacI* restriction site

The M13 phage containing single stranded M13:Lfn with a *SacI* site [M13:Lfn (1-296) (*SacI*) construct] (Peterson, 1999) was used to infect *E. coli* XL1 cells using the method described in section 2.2.4. The amplified replicative form of M13:Lfn was isolated from the *E. coli* XL1 cells using a Promega miniprep DNA isolation kit.

3.2.2 Sub-cloning of *SacI/SmaI* fragment from replicative form of M13:Lfn (1-296) (*SacI*) into pGEM:hLF construct

The sub-cloning strategy is depicted in the Figure 3.4. The *SacI/SmaI* double-stranded fragment from the M13 replicative form of N-lobe hLF cDNA (~ 0.8 kb) containing the N-terminal lobe (Fig. 3.3) was ligated into the *SacI* and *SmaI* digested pGEM:hLF. This construct was designated as pGEM:hLF (-1) (*SacI*).

Fig 3.3 Analysis of M13:Lfn (*SacI*) by *SacI* and *SmaI* digestion

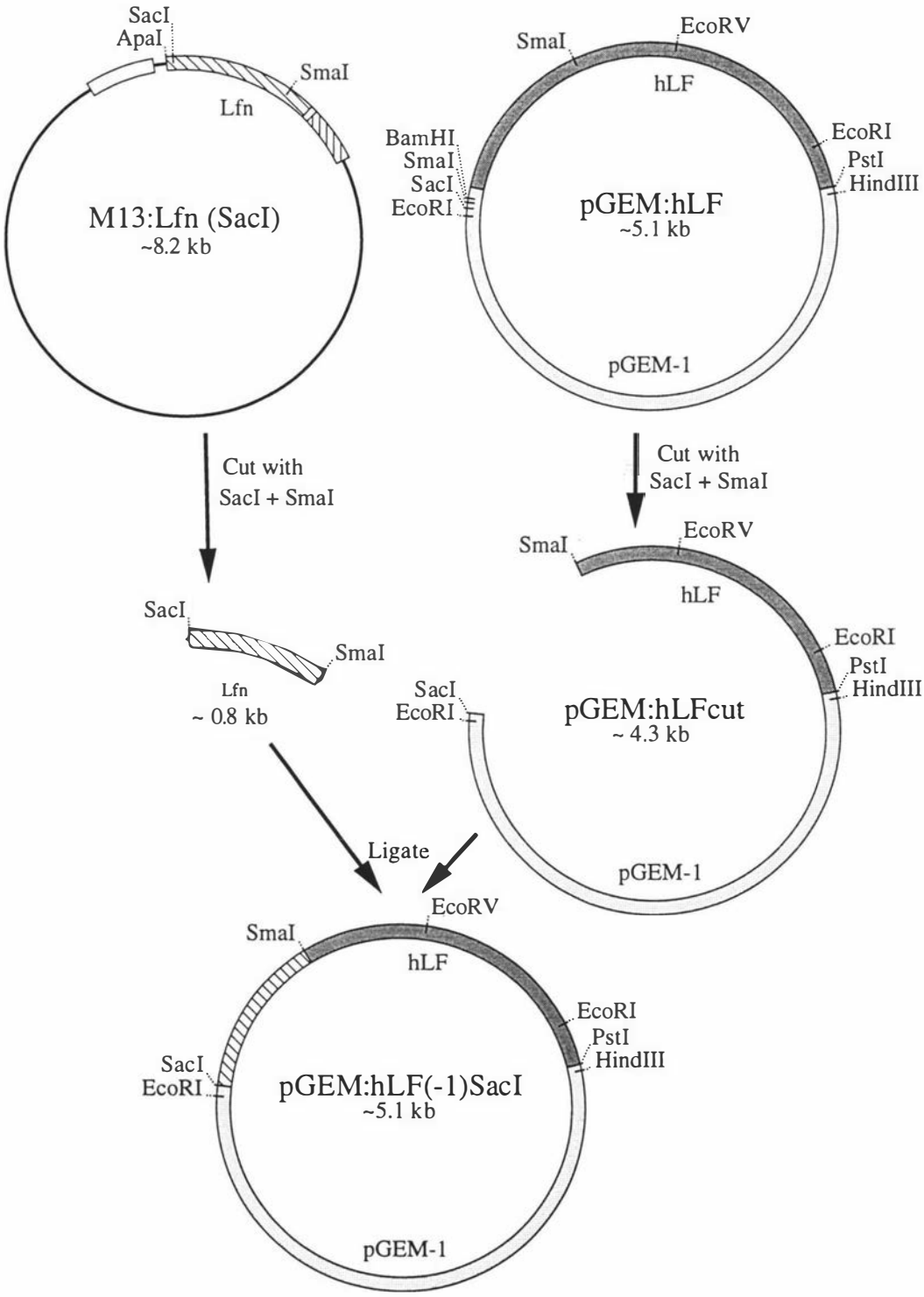


Lanes

(1 and 2) M13:Lfn digested with *SacI* and *SmaI*,

(3) BRL 1 kb ladder

Fig. 3.4 Schematic diagram of the preparation of pGEM:hLF (-1) (*SacI*) construct



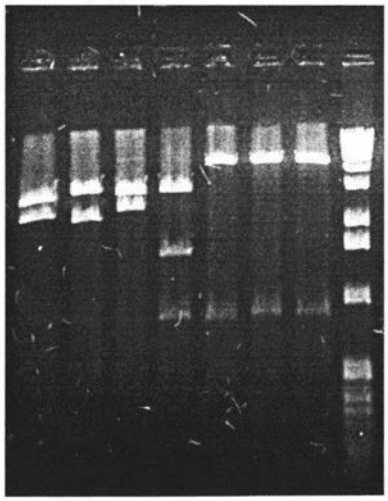
The ligation mix was transformed into *E. coli* DH5α. Recombinant clones were selected on LB^{amp} agar media. The presence of the expected insert was confirmed by restriction digestion with appropriate restriction enzymes (Fig. 3.6 & Fig 3.7, lane 1, *SacI* and *Hind III* double digestion). Double digestion of the plasmid DNA isolated from these transformants with *BamHI* and *SmaI* confirmed loss of the *BamHI* site and the second *SmaI* site present in the MCS of pGEM:hLF (Fig. 3.6, lane 6) when compared to *BamHI* and *SmaI* double digested original pGEM:hLF construct as shown in Fig. 3.5, lane 6. This clone, which was designated as pGEM:hLF (-1) (*SacI*) and contained a ~0.8 kb *SacI/SmaI* fragment with the *SacI* site at the immediate upstream position of the first codon of mature hLF cDNA.

3.2.3 Changing of *HindIII* at the 3'-end of hLF cDNA in pGEM:hLF (-1) (*SacI*) to *SacI*

It was also necessary to create a *SacI* site at the 3′-end of full-length hLF cDNA to produce an approximately 2.1 kb *SacI* fragment which could be introduced into the unique *SacI* site of the yeast expression vectors. To obtain this fragment pGEM:hLF (-1) (*SacI*) was digested with *HindIII* and end-filled using the Klenow fragment of *E. coli* DNA polymerase I. The blunt-ended DNA was ligated to a 50-fold molar excess of phosphorylated *SacI* linker [5′ d(pCGAGCTCG) 3′]. The ligation mix was transformed into *E. coli* DH5α. Transformants were selected on LB^{amp} agar media. The presence of the expected insert and the introduced *SacI* site were confirmed by digesting the construct with *SacI* restriction enzyme. A fragment of ~2.1 kb was excised by *SacI* enzyme (Fig. 3.7, lane 2). Digestion of the plasmid DNA with *HindIII* restriction enzyme showed loss of the *HindIII* site in the construct (Fig. 3.6, lane 1). This clone was designated as pGEM:hLF (2) (*SacI*).

Fig. 3.5 Restriction digestion analysis of pGEM:hLF

1 2 3 4 5 6 7 8

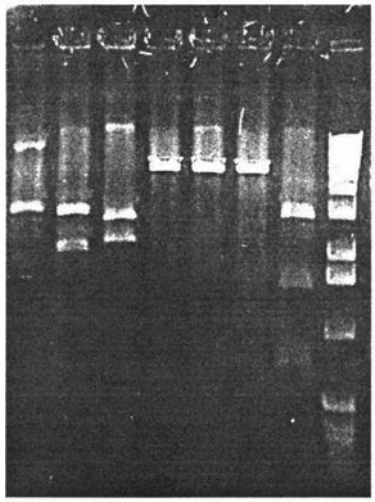


Lane

(1) With *BamHI* and *HindIII*
(2) With *EcoRI*
(3) With *SacI* and *HindIII*
(4) With *SmaI* and *HindIII*
(5) With *SmaI*
(6) With *SmaI* and *BamHI*
(7) With *SmaI* and *SacI*
(8) BRL 1 kb ladder

Fig. 3.6 Restriction digestion analysis of pGEM:hLF (2) (*SacI*)

1 2 3 4 5 6 7 8

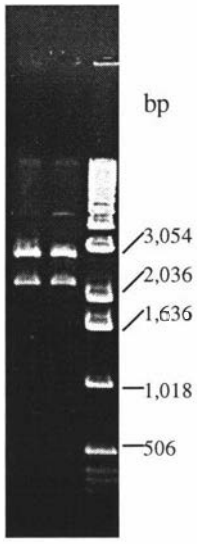


Lane

(1) With *BamHI* and *HindIII* (no digestion)
(2) With *EcoRI*
(3) With *SacI* and *HindIII*
(4) With *SmaI* and *HindIII*
(5) With *SmaI*
(6) With *SmaI* and *BamHI*
(7) With *SmaI* and *SacI*
(8) BRL 1 kb ladder

Fig. 3.7 Restriction digestion analysis of pGEM:hLF (-1) *SacI* and pGEM:hLF (2) (*SacI*) constructs

1 2 3



Lane

(1) pGEM:hLF (-1) *SacI* digested with *SacI* and *HindIII*
(2) pGEM:hLF (2) (*SacI*) digested with *SacI*
(3) BRL 1 kb ladder

Explanation for figures 3.5, 3.6 and 3.7

Fig. 3.5 Restriction fragment analysis of the original pGEM:hLF construct (Stowell, 1990) with different restriction enzymes and their combinations.

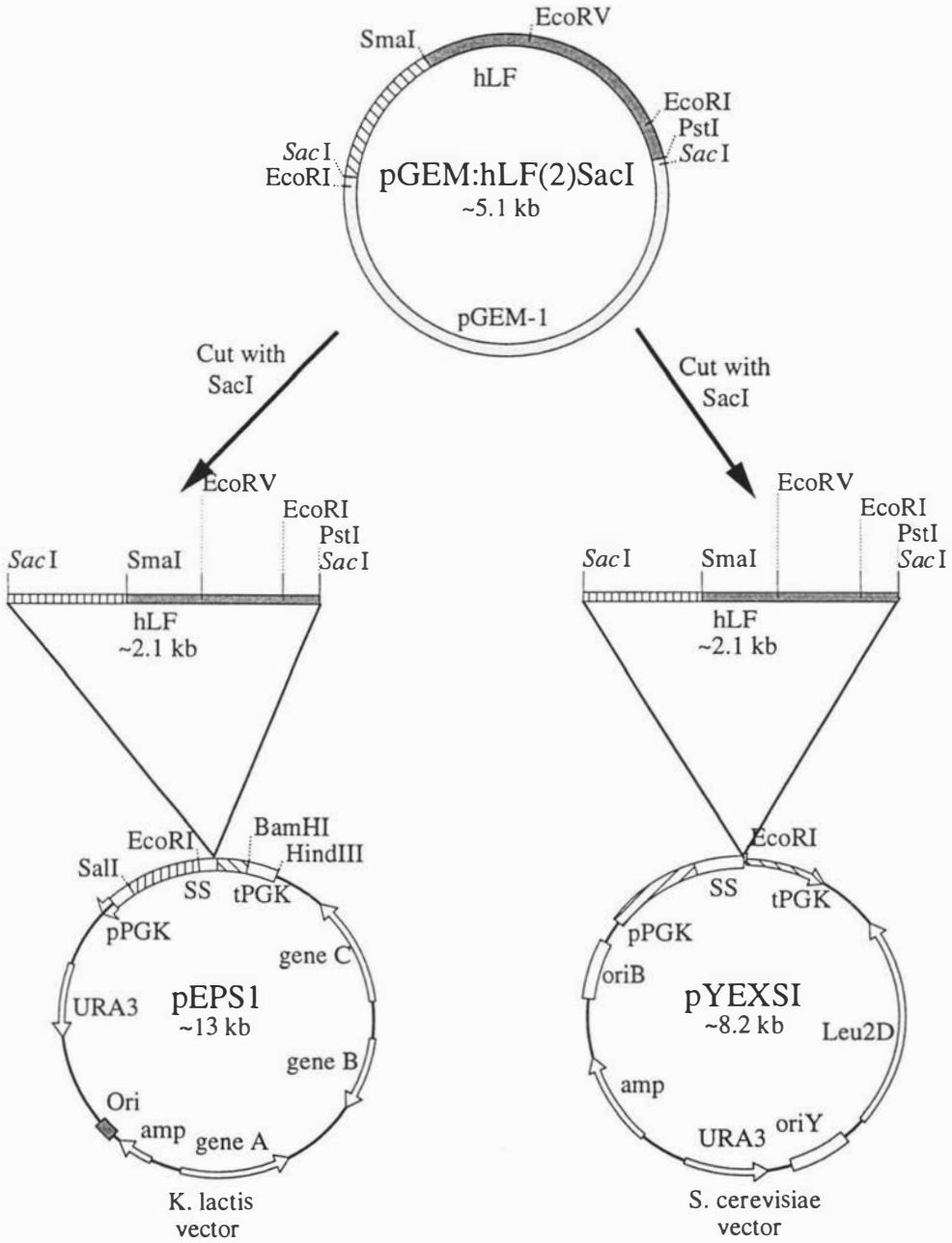
Fig 3.6 Restriction fragment analysis of the construct pGEM:hLF (2) (*SacI*) (the construct with two *SacI* sites flanking the hLF cDNA) digested with the same set of restriction enzymes as in Fig. 3.5.

Fig. 3.7 This gives a comparison between the constructs pGEM:hLF (-1) (*SacI*) and pGEM:hLF (2) (*SacI*) digested with suitable enzymes.

3.2.4 Sub-cloning of ~2.1 kb hLF *SacI* fragment into pEPS1 and pYEXS1 expression vectors

Ligation reactions were assembled with the 2.1 kb *SacI* fragment of hLF cDNA and *SacI* cut and calf-intestinal alkaline phosphatase (CIAP) treated pEPS1 (*K. lactis* expression vector) or pYEXS1 (*S. cerevisiae* expression vector) at a 3:1 molar ratio of insert to vector. The cloning strategy is shown in Fig. 3.8.

Fig. 3.8 Schematic diagram of the preparation of the constructs of hLF cDNA in the yeast expression vectors



Ligation reactions were incubated with T4 DNA ligase at 4°C for 16-18 hrs. The ligation mixes were transformed into *E. coli* DH5α and transformants were obtained on LB^{amp} agar media. Digestion of the plasmid DNA isolated from the transformants with suitable restriction enzymes showed no inserts and all turned out to be only vector. In control ligation reactions, the self-ligation of the *SacI* digested and CIAP treated vector did not yield any transformants.

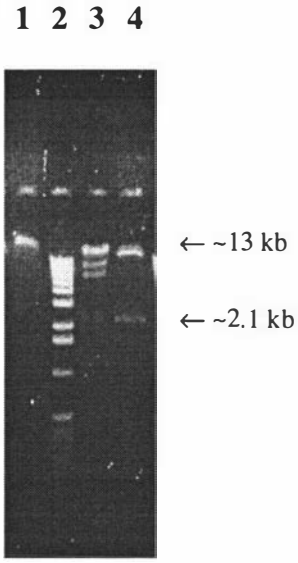
The ligation reactions were repeated many times and transformed into different *E. coli* strains. Also *E. coli* strains of higher transformation efficiency [including Max.Efficiency DH5α (GibcoBRL) (greater than $1x10^9$ cfu/μg of DNA)] were used. The same observations were made with the transformants containing only vector devoid of insert. We then contacted Dr. Ian G. Macredie from whom these yeast vectors were obtained. He also confirmed that his group had never managed to establish constructs with the β-lactamase gene in either of these yeast vectors in *E. coli.* This led us to believe that it was not possible to establish the construct in *E. coli.*

Possible reasons for the non-establishment of the constructs in *E. coli* are as follows.

Ligation of the fragment into vectors

There is always a possibility that a problem with ligation of insert into vectors can lead to non-establishment of constructs. Ligation mix of the *SacI* fragment of hLF cDNA and *SacI* digested and CIAP treated yeast vectors was assembled as described in section 2.2.12. A part of the ligation mix was checked by running it on a 0.75% agarose gel. The fragments were ligated as shown by the disappearance of the vector and insert fragments and the appearance of higher molecular weight species as shown in Fig. 3.9. This suggested that the ligation reaction was successful.

Fig. 3.9 Ligation test between the *SacI* digested and CIAP treated linear pEPS1 and ~2.1 kb hLF fragment with *SacI* ends



Lane

(1) Ligation mix of *SacI* digested and CIAP treated pEPS1 *K. lactis* vector and ~2.1 kb hLF fragment with *SacI* ends

(2) BRL 1 kb marker

(3) λ/*HindIII* marker and

(4) Ligation mix as in lane (1) without T4 DNA ligase added

Size of the construct

The total size of the *K. lactis* vector construct pEPS1/hLF would be ~15 kb and the *S. cerevisiae* vector construct would be ~10.3 kb. It is known that large plasmid constructs generally have low transformation efficiency and a low copy number. This might have influenced the establishment of these constructs in *E. coli.* In spite of using maximum efficiency DH5α competent cells (GibcoBRL) (>1x10 ^{9} cfu/μg of DNA), these constructs could not be established. However, the pEPS1 vector is ~13 kb and the pYEXS1 vector is ~8.2 kb and they could both be established (without insert) in *E. coli* in spite of their large size. These yeast vectors have been reported to be of high-copy-number (Castelli *et al.*, 1994; Macreadie *et al.*, 1993).

Nature of hLF cDNA and the vectors

Previous attempts in our laboratory to sub-clone and express full-length hLF cDNA in *E. coli* were unsuccessful. This may have to do with the mammalian codon bias of the hLF cDNA and the vectors used. In this study, the constructs carrying hLF cDNA in the yeast expression vectors, pEPS1 and pYEXS1 could not be established in *E. coli.* The same hLF cDNA could be sub-cloned into pGEM1 vector (~2.8 kb) and also into pNUT vector (~7.4 kb) and the constructs were established in *E. coli.* Recombinant human lactoferrin could successfully be expressed in BHK-cells using the pNUT:hLF construct (Stowell *et al.*, 1991).

3.2.5 Direct transformation of competent yeast cells with the ligation mix

The constructs of pEPS1 and pYEXS1 vectors with full-length hLF cDNA could not be established in *E. coli* (section 3.2.4). For this reason, electro-competent *K. lactis* and *S. cerevisiae* cells were directly transformed separately, with the dialysed ligation mixes. The cells of both *K. lactis* and *S. cerevisiae* were then plated on minimal media. After 2 to 3 days of incubation at 30°C the plates developed recombinant colonies. *K. lactis* gave the required transformants. No colonies appeared on the negative control plate where *K. lactis* cells were transformed with milli-Q H_2O. However, *S. cerevisiae* DY150 showed a lot of background with the negative control (transformation with milli-Q H_2O) on selective media. This might be because of its leaky selection markers as observed with this yeast host by me.

3.2.6 Expression studies of full-length hLF or its fragments in recombinant *K. lactis*

The lactoferrin ELISA method was used to check for the expression of full-length human lactoferrin or its fragments by the recombinant yeast as described in section 2.4.7. Ten transformants selected from the *K. lactis* selective agar media were grown on 10 ml of liquid selective media for two to five days. The media was tested for the presence of hLF protein products on days 2 and 5. Of the ten transformants tested, two expressed hLF products initially at 1 to 2 mg/L. Changes were made in the growth media in an attempt to enhance the production human lactoferrin products. The media tested were minimal media + 1% casamino acids, YEPD and YEPD + 1% casamino acids. The expression levels of

hLF protein products increased to approximately 5 mg/L on YEPD + 1% casamino acids media (Rallabhandi and Yu, 1996b).

Expression of hLF protein products from these clones continued for several transfers, but gradually fell to levels that were not detectable by lactoferrin ELISA. Similar results were seen with both the working liquid stocks (4°C) and the glycerol stocks stored at -70°C. This could be because of the rapid loss of plasmid construct and/or the selective removal of the hLF cDNA by the yeast host during sub-culturing and storage. This drop in the expression levels happened in a gradual manner as expression of hLF products could be seen with the initial transfers of culture. The ligation reaction and electro-transformation of *K. lactis* with dialysed ligation mix were repeated for a further two times. No lactoferrin-expressing clones were obtained from these two transformations.

3.2.7 Southern blot analysis of plasmid DNA isolated from recombinant *K. lactis*

Plasmid DNA was isolated from the recombinant *K. lactis* cultures that were expressing hLF products, but had subsequently stopped as assessed by using the method described in section 2.2.6. This analysis was attempted after observing the loss of expression of hLF protein products by *K. lactis* as assayed by lactoferrin ELISA. The plasmid DNA preparations were run on a 0.8 % agarose gel along with standard hLF cDNA. Southern blot analysis was carried out using the method described in section 2.3.4. The probing was done with hLF cDNA labelled with DIG-DNA labelling and detection kit. The hLF cDNA standard showed hybridisation with the probe on the blot, but no hybridisation was seen with the lanes having plasmid minipreps from recombinant *K. lactis* (results not shown). Since the cultures were able to grow on selection media, only the vector (pEPS1) might be being maintained after a selective removal of the hLF cDNA from the construct in *K. lactis* host. Attempts were not made to probe the plasmid DNA preparations from the recombinant *K. lactis* with pEPS1 vector probe. This was further evidence that the construct with the desired insert could not be stably maintained in *K. lactis*.

Because attempts to establish stable insert-carrying constructs of pEPS1 and pYEXS1 vectors, which have constitutive promoters, were not reproducible, we turned our

attention to an another *K. lactis* expression vector, pSPHO4, which has an inducible promoter.

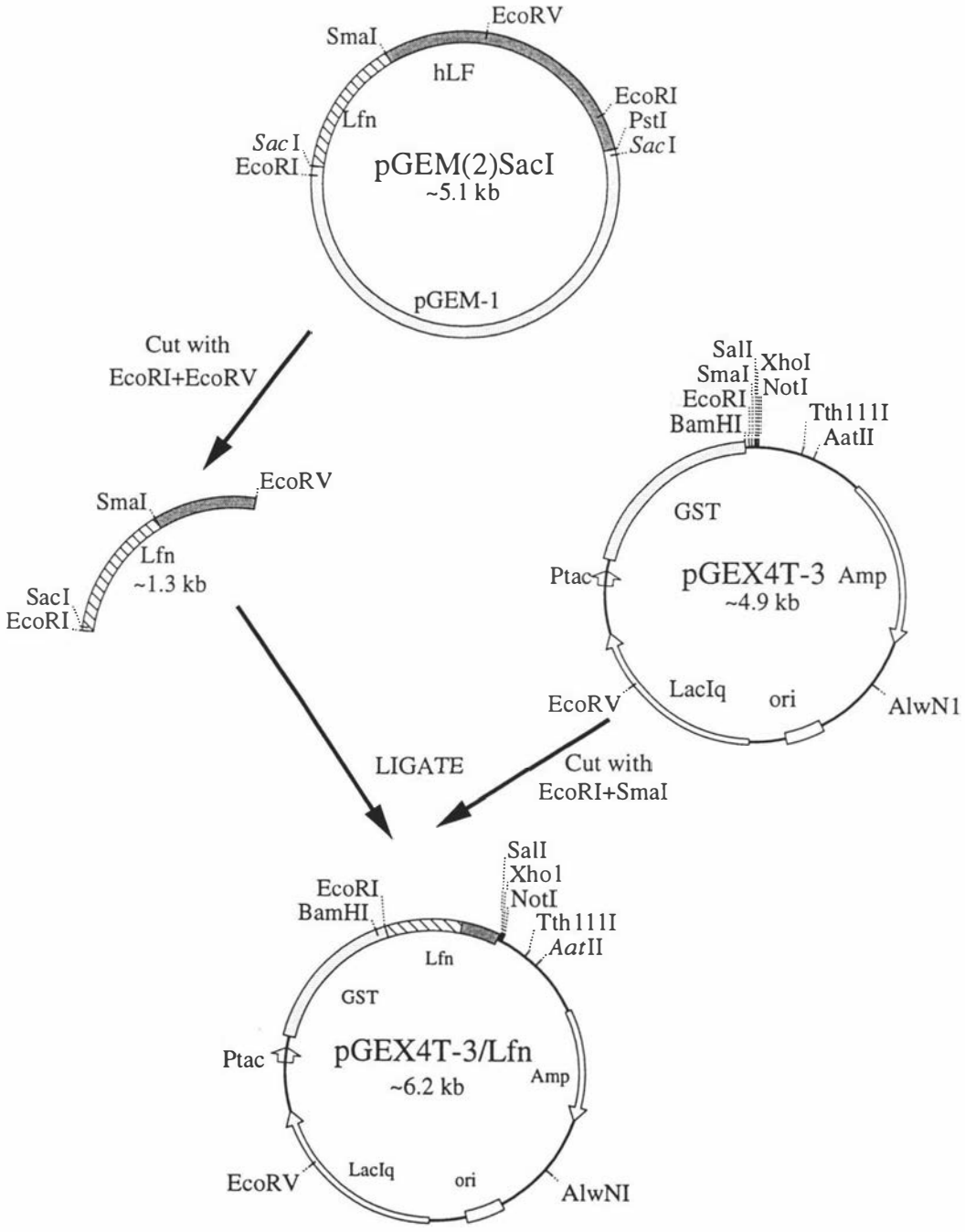
3.2.8 Sub-cloning of hLF cDNA into pSPHO4, *K. lactis* vector with an inducible promoter

The *K. lactis* expression vector, pSPHO4 contains the PHO5 promoter that can be induced by inorganic phosphate. The *EcoRI* fragment (~ 2 kb) from the pGEM:hLF (2) (*SacI*) construct was ligated into the unique *EcoRI* site of pSPHO4 vector after dephosphorylating the linear vector with CIAP. The ligation mix was transformed into *E. coli* DH5α and transformants were selected on LB^{amp} agar media. Thirty transformants were screened by digesting isolated plasmid DNA with appropriate restriction enzymes. No ampicillin resistant clones had full-size hLF cDNA inserts and some clones had only vector. The process of ligation and transformation was repeated for a total of five times. The desired construct could not be obtained. This problem was never resolved. Though *E. coli* DH5α, a recombination minus (rec^-) mutant was used, recombination seemed to be occurring. Transformation of the ligation mix straight into *K. lactis* by electroporation was not tried.

3.3 Expression of the N-terminal lobe of hLF cDNA in yeast host-vector systems

Since constructs of yeast vectors carrying the full-length hLF cDNA could not be obtained (sections 3.2.4 and 3.2.8), an attempt was made to express the N-lobe half-molecule of hLF in yeast. The following approach was taken to sub-clone sequences of the N-terminal lobe of hLF cDNA into yeast vectors. The *EcoRI/EcoRV* fragment (N-terminal lobe sequence) of hLF cDNA does not have a stop-codon at its 3′- end. So, the *EcoRI/EcoRV* fragment of hLF cDNA was ligated into *EcoRI* and *SmaI* digested pGEX4T-3 (an *E. coli* expression vector) to add a stop-codon that is present in the down-stream 3′-end sequence of the pGEX-4T3 MCS. Fig. 3.10 gives a schematic representation of the sub-cloning strategy to introduce the hLF cDNA N-lobe into the pGEX4T-3 vector.

Fig. 3.10 Schematic representation of sub-cloning of N-terminal lobe of hLF cDNA into *E. coli* expression vector pGEX4T-3



3.3.1 Sub-cloning of the N-terminal lobe of hLF cDNA into *E. coli* vector pGEX4T-3

Ligation reaction was assembled with the *EcoRI/EcoRV* fragment of hLF cDNA and *EcoRI* and *SmaI* digested pGEX4T-3 vector. The ligation mix was transformed into *E. coli* DH5α and transformants were selected on LB^{amp} agar media.

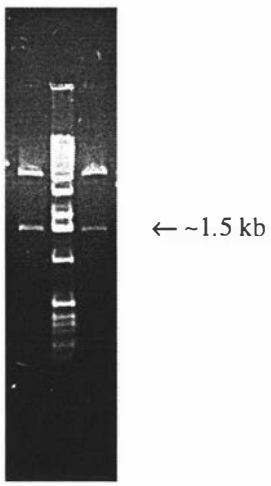
Restriction digestion analysis of plasmid DNA prepared from a number of transformants with suitable enzymes showed the presence of the expected insert. This construct was designated as pGEX4T-3/Lfn.

3.3.2 Conversion of *AatII* restriction site to either *SacI* or *BamHI* in pGEX4T-3/Lfn construct

The yeast vectors, pEPS1 and pYEXS1 have a unique *SacI* cloning site. To sub-clone the hLF cDNA N-terminal lobe into these vectors, it was necessary to introduce a *SacI* site after the stop-codon. A restriction site (*AatII*) present down-stream to the stop-codon in pGEX-4T3 vector was changed to a *SacI* site. The pGEX4T-3/Lfn construct was cut with *AatII* enzyme and the linear fragment was gel purified. The fragment was blunt-ended by using T4 DNA polymerase. The blunt-ended linear fragment was dephosphorylated using CIAP. The dephosphorylated linear fragment of pGEX4T-3/Lfn was ligated to a 50-fold molar excess of phosphorylated *SacI* linker. The ligation mix was transformed into *E. coli* DH5α and transformants were selected on LB^{amp} agar media. Plasmid DNA preparations from these transformants digested with *SacI* gave a fragment of ~1.5 kb, which could be sub-cloned into the yeast vectors (Fig. 3.11, lane 1). This construct was designated as pGEX4T-3/Lfn (*SacI*).

Similarly, the *AatII* digested, blunt-ended and dephosphorylated linear fragment of pGEX4T-3/Lfn construct was ligated to 50-fold molar excess of phosphorylated *BamHI* linker. The ligation mix was transformed into *E. coli* DH5α and transformants were selected on LB^{amp} agar media. Plasmid DNA preparations from these transformants digested with *BamHI* excised a fragment of ~1.5 kb, which could be sub-cloned into pET-15b, an *E. coli* expression vector (Fig. 3.11, lane 3). This construct was designated as pGEX4T-3/Lfn (*BamHI*).

Fig. 3.11 Restriction digestion analysis of pGEX4T-3/Lfn (*SacI*) and pGEX4T-3/Lfn (*BamHI*)



Lane

(1) pGEX3/Lfn (*SacI*) construct digested with *SacI*

(2) BRL 1 kb ladder

(3) pGEX3/Lfn (*BamHI*) construct digested with *BamHI*

3.3.3 Sub-cloning of *SacI* fragment of hLF cDNA N-lobe into yeast vectors

The same procedure described in section 3.2.4 was followed to sub-clone the *SacI* fragment of N-terminal lobe of hLF cDNA into *SacI* digested and CIAP treated pEPS1 and pYEXS1. Many transformants were obtained on LB^{amp} agar media in *E. coli*. Upon digesting the plasmid DNA isolated from these transformants with suitable restriction enzymes, none of the clones showed the presence of the desired insert but showed the presence of only vector. Self-ligated CIAP treated vector did not yield any transformants. This result demonstrated the difficulty in establishing constructs having the N-terminal lobe of hLF cDNA in the yeast expression vectors, pEPS1 and pYEXS1 in *E. coli*.

Direct transformation of the ligation mix into *K. lactis* by electroporation was carried out as described in section 3.2.5. Transformants were obtained on *K. lactis* selective media. Ten transformants were checked for the secretion of the hLF N-lobe

protein products using lactoferrin ELISA. None of them showed the secretion of protein products in the medium. This study was not pursued further.

3.3.4 Sub-cloning of *BamHI* fragment of hLF cDNA N-lobe from pGEX4T-3/Lfn (*BamHI*) into pET-15b vector

The *BamHI* fragment corresponding to the N-lobe of hLF cDNA from pGEX4T-3/Lfn (*BamHI*) was sub-cloned into *BamHI* digested and CIAP treated pET-15b vector. Transformants were selected on LB^{amp} media. Digestion of plasmid DNA with suitable restriction enzymes showed the presence of the expected insert both in the correct and reverse orientations in roughly equal proportion. The construct having the insert in the correct orientation was designated pET-15b/Lfn (*BamHI*).

3.3.5 Expression studies of pGEX4T-3/Lfn (*SacI*) and pET-15b/Lfn (*BamHI*) constructs in *E. coli*

Induction studies of these two constructs in *E. coli* BL21 (DE3) were carried out with 1 mM of IPTG at 37°C. Both these constructs expressed the recombinant proteins as GST and poly-His fusions respectively, in inclusion bodies. The inclusion bodies were isolated, solubilised in 8 M urea and analysed on 10% SDS-PAGE gels (data not shown). Further purification and characterisation of the recombinant protein was not carried out with these constructs in *E. coli.*

Since the expression of the full-length and N-terminal lobe of hLF cDNA in yeast has proven to be unsuccessful (sections 3.2.4, 3.2.5 and 3.3.1), a decision was made at this stage to attempt to heterologously express the synthetic DNAs corresponding to the sequences of the antimicrobial domains of both human and bovine lactoferrin in ***E. coli.*** This part of the work is described in Chapter 4.

3.4 Discussion and conclusions

(a) Establishment of the constructs of yeast vectors with inserts of hLF cDNA and its N-terminal lobe

Many attempts were made to express hLF cDNA and its N-terminal lobe sequence in the yeasts *Kluyveromyces lactis* and *Saccharomyces cerevisiae*, but none were successful. A major problem was the failure to establish stable constructs of the yeast vectors pEPS1 and pYEXS1 carrying hLF cDNA inserts in *E. coli.* Several *E. coli* hosts and *E. coli* strains of higher transformation efficiency were used without success. Transformation was also tried with the addition of exogenous glucose from 0 to 5% to the selection media and with changes in the reaction conditions for ligation. Dr. Ian Macredie's group, which developed the pEPS1 and pYEXS1 yeast expression vectors, also encountered the same problem with the establishment of yeast vector/β-lactamase gene constructs in *E. coli* (Macredie, I.G., personal communication). There are no known reports on the use of these vectors for the expression of mammalian proteins. Failure to establish the constructs in *E. coli* meant that we did not have a fully characterised construct for yeast transformation experiments.

(b) Direct transformation of competent yeast cells with the ligation mix by electroporation

The alternative approach is to directly electroporate the ligation mix into yeast. Dr. Ian Macreadie's group directly transformed *K. lactis* electro-competent cells with a ligation mix and selected for transformants. They obtained transformants and screened them for β-lactamase reporter gene expression. Similarly, we also electroporated *K. lactis* cells with dialysed ligation mix and selected for transformants. The transformants were screened for the secretion of recombinant hLF protein products using the lactoferrin ELISA method. This method was fairly laborious for screening a large number of transformants. Two transformants constitutively expressing and secreting recombinant hLF products were identified. Growth of these recombinant *K. lactis* clones on the selective minimal media was very slow. The expression of lactoferrin was sustained for only a few transfers of culture and was gradually lost from both working liquid cultures (4°C) and with glycerol stocks stored at -70°C. *S. cerevisiae* transformants carrying hLF cDNA constructs could

not be obtained because of a very high background on the negative control plate (cells only) with the *S. cerevisiae* strain DY150 used in this study.

Dr. Ian Macreadie's group has also reported that plasmid loss was very high with a pEPS1/β-lactamase construct when grown on rich YEPD media. This group reported that only 15% of the plasmid retention could be seen on YEPD media and that this level of plasmid retention was maintained for up to 8 days at which time the culture was in stationary phase (Macreadie *et al.*, 1993). The selective removal of the hLF cDNA insert from the yeast vector constructs could be the reason for the loss of expression of human lactoferrin protein products in this study. This has been evidenced by the Southern blot analysis as described in section 3.2.7.

Direct electroporation of dialysed ligation mix into *K. lactis* on a routine basis is very difficult when an easy screening method for the selection of transformants is not available. This was the case in this study. Difficulty in establishing the constructs in *E. coli* meant that it was necessary to go back to the ligation step every time transformants were not obtained. The same approach was taken for the expression of the N-lobe of hLF cDNA in both *K. lactis* and *S. cerevisiae*. Similar problems occurred with the establishment of these constructs in yeast.

In conclusion, the *K. lactis* expression vector, pEPS1 and the *S. cerevisiae* expression vector, pYEXS1 were unsuitable vectors for the expression and secretion of recombinant human lactoferrin.

Non-*Saccharomyces* yeasts like *K. lactis* have recently become popular for the production of foreign proteins because of their higher secretion and better post-translational processing abilities compared to *Saccharomyces* (Rallabhandi and Yu, 1996a). In spite of this, the developments in the host genetics and the plasmid vector systems for these non-*Saccharomyces* systems have been very slow. There are only few reports on the use of these yeast vectors for the production of foreign proteins (refer to review by Rallabhandi and Yu, 1996a).

4.1 Introduction

Natural polycationic peptides have been found in many different species of animals, plants and insects and shown to have a broad range of antimicrobial activities. These antimicrobial peptides serve as one of the primary defence agents of innate immunity in animals. These peptide antibiotics are gene-encoded, usually range between 15 and 34 amino acids in length and contain a minimum of four Lys and/or Arg residues (Piers *et al.*, 1993). They are present abundantly in the organisms or cells in which they are found. They can be divided into subsets of molecules such as defensins, cecropins, magainins, mellitin and others, all with specific characteristics that distinguish the groups. These peptides are interesting antimicrobial agents that are able to bind to the endotoxin and permeabilise the outer membrane of a variety of important medical pathogens to ultimately kill them.

The wide-spread clinical use and misuse of conventional antibiotics has led to the development of plasmid-borne resistance genes, which pose an increasing threat to the future medical use of these agents. In this context, there has been considerable interest in the use of natural antimicrobial peptides as therapeutic agents. These peptides have an ability to kill target cells rapidly and have an unusually broad activity spectrum. They also show activity against some of the more serious antibiotic-resistant pathogens in clinics (Hancock and Lehrer, 1998). The cationic peptides initially exhibit an electrostatic interaction with the cell membrane due to their charged nature and subsequently result in pore formation in the membranes by a barrel-stave type mechanism, allowing leakage of the cytoplasm to the exterior (Dykes *et al.*, 1998). It is relatively difficult to select for bacterial mutants, which are resistant to cationic peptides *in vitro* (Yamauchi *et al.*, 1993). Because of their broad antimicrobial activity, they could potentially be used to improve animal and plant husbandry.

Both human and bovine lactoferrin have been shown to have antimicrobial peptide domains resident in their N-lobe. The antimicrobial peptide derived from the acid pepsin hydrolysate of lactoferrin is called lactoferricin and has been shown to have antimicrobial

activity against a wide range of Gram-negative and Gram-positive bacteria and yeasts (Bellamy *et al.*, 1992). A detailed discussion of the structure and antimicrobial activity of native lactoferricins from both human and bovine lactoferrin has been given in section 1.4.

Currently, the only way to obtain native lactoferricins is to isolate the peptides from the acid pepsin hydrolysate of lactoferrin, which gives very low yields, or to synthesise them by protein chemical methods, which is very expensive on a large scale. All previous investigators working with lactoferricin have taken one of these routes. To further study these antimicrobial peptides from lactoferrin, we identified a need to develop a bacterial expression system to produce lactoferricins in large quantities. Since lactoferricin is a short peptide, the complementary DNA that encodes it can be synthesised as overlapping oligonucleotides, enabling us to introduce the restriction sites of our choice for sub-cloning into various commercially available bacterial expression vectors.

There are a number of advantages to producing these peptides in a heterologous system. If the peptide is expressed heterologously, it is relatively easy to scale-up the system at low cost. Site-directed mutagenesis can be used to introduce changes into the peptide and study the physiological and structural consequences of these changes.

4.2 Results

4.2.1 Design of overlapping synthetic oligonucleotides to construct full-length DNA for lactoferricin

Several forward and reverse oligonucleotides, each of approximately 70 bases in length were designed to give an overlap of at least 30 bases in the middle portion of the DNA and to produce the required restriction sites at both the ends. The codons chosen in these synthetic oligonucleotides were based on those found in proteins expressed at high levels in *E. coli* (refer to Appendix 1). The resulting lactoferricin-encoding DNA had *EcoRI* and *NdeI* sites on the 5′ (N-terminal) end and *BamHI* and *XbaI* sites on the 3′ (C-terminal) end.

A set of reverse oligonucleotides for both bovine lactoferricin (LFcin-B) and human lactoferricin (LFcin-H) were designed in such a way that with the same forward oligonucleotides, three different types of 3′- end in the insert could be obtained. They are,

1. Without a stop-codon in the DNA insert
2. With codons for four hydrophilic amino acids before a stop-codon at the 3′- end of the insert
3. With a stop-codon at the 3′- end of the insert.

All constructs of LFcin-H & B would be expressed as fusion proteins with an N-terminal fusion partner (either poly-His or GST) and variable length C-terminal extensions: a hydrophobic C-terminal extension added to the peptide from the vector sequences, a hydrophilic C-terminal extension (Gly-Ser_3) or no C-terminal extension.

The same restriction sites were used for both bovine and human lactoferricin coding sequences to enable sub-cloning into the same set of *E. coli* expression vectors pPROEXHTa, pGEX-4T1 and pET-15b. The *EcoRI* and *XbaI* sites were designed for the pPROEXHTa vector. Once the construct was established in the pPROEXHTa vector and checked for the correct sequence, it was moved into the other vectors, pGEX-4T1 and pET-15b. The lactoferricin peptides would be expressed as poly-His fusion proteins in pPROEXHTa and pET-15b vectors and as GST-fusion proteins in pGEX-4T1 vector.

Table 4.1 gives a summary of the forward and reverse primers used to produce LFcin-B DNA and its variants and similarly, Table 4.2 gives a summary of primers used for LFcin-H DNA and its variants.

Table 4.1

Synthetic oligonucleotides for bovine lactoferricin (LFcin-B)

(1) **EnlacBbx 70** (or Forblb 70) (5'→3') (common to all constructs)

```
          EcoRI       NdeI
1     GGA ATT CCA TAT GTT CAA ATG CCG TCG TTG GCA GTG GCG TAT
43    GAA AAA ACT GGG TGC TCC GTC CAT CAC C
```

(2) **Revblb 70** (5'-→3') (without a stop-codon in the DNA insert)

```
          XbaI        BamHI
1     GCT CTA GAG GAT CCA GCG AAA GCA CGA CGA ACG CAG GTG ATG
43    GAC GGA GCA CCC AGT TTT TTC ATA CGC C
```

(3) **Mrevmblb#71** (5'→3') (with codons for four hydrophilic amino acids before a stop-codon at the 3′- end of the insert)

```
          XbaI                              BamHI
1     GC TCT AGA ATTA ACT GCT GGA TCC AGC GAA AGC ACG ACG AAC
43    GCA GGT GAT GGA CGG AGC ACC CAG TTT TT
```

(4) **Revblb#65** (5'→3') (with a stop-codon at the 3′- end of the insert)

```
          XbaI        BamHI
1     GC TCT AGA GGA TCC ATTA AGC GAA AGC ACG ACG AAC GCA GGT
43    GAT GGA CGG AGC ACC CAG TTT TT
```

The sequences underlined are the restriction sites and those shown in bold are stop-codons.

Table 4.2

Synthetic oligonucleotides for human lactoferricin (LFcin-H)

(1) Forhlh 65 (5'→3') (common to all constructs)

EcoRI *NdeI*

```
1    GGA ATT CCA TAT GAC CAA ATG CTT CCA GTG GCA GCG TAA CAT
43   GCG TAA AGT TCG TGG TCC GCC GG
```

(2) Revhlh 63 (5'→3') (without a stop-codon in the DNA insert)

XbaI *BamHI*

```
1    GCT CTA GAG GAT CCA CGT TTG ATG CAG GAA ACC GGC GGA CCA
43   CGA ACT TTA CGC ATG TTA CGC
```

(3) Mrevmhlh#73 (5'→3') (with codons for four hydrophilic amino acids before a stop-codon at the 3′- end of the insert)

XbaI *BamHI*

```
1    GC TCT AGA ATTA ACT GCT GGA TCC ACG TTT GAT GCA GGA AAC
43   CGG CGG ACC ACG AAC TTT ACG CAT GTT ACG C
```

(4) Revhlh#67 (5'→3') (with a stop-codon at the 3′- end of the insert)

XbaI *BamHI*

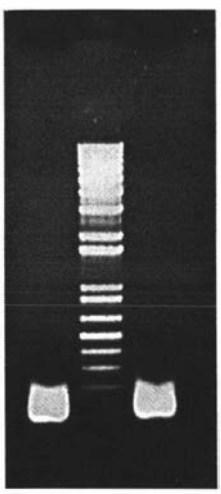
```
1    GC TCT AGA GGA TCC ATTA ACG TTT GAT GCA GGA AAC CGG CGG
43   ACC ACG AAC TTT ACG CAT GTT ACG C
```

The sequences underlined are the restriction sites and those shown in bold are stop-codons.

4.2.2 Preparation of full-length lactoferricin H & B DNA from overlapping oligonucleotides

The forward and reverse oligonucleotides were resuspended in either distilled water or TE buffer at pH 8.0. The respective oligonucleotides were mixed at an equimolar ratio and about 2 μM final concentration of each, heated to 100°C and cooled slowly to room temperature (RT) to allow them to anneal. The ends were filled-in by using either the Klenow fragment of *E. coli* DNA polymerase I or T4 DNA polymerase. DNA products of expected size were obtained as observed by running on a 1.2% agarose gel (Fig. 4.1). This gel is an example of one of the DNA fragments for each lactoferricin H & B synthetic DNAs that have different 3′-ends.

Fig. 4.1 End-filled fragments of LFcin-H & B DNAs



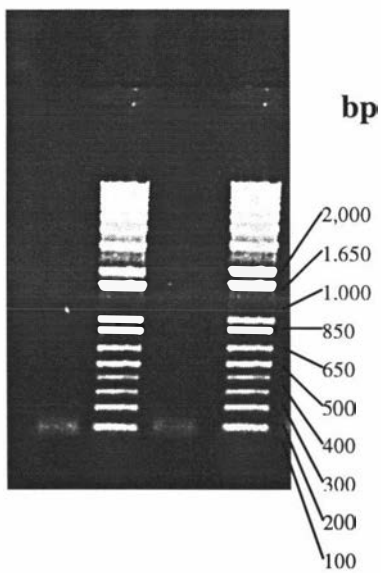
Lane

(1) Lactoferricin H DNA

(2) BRL 1 kb ladder

(3) Lactoferricin B DNA

Fig. 4.2 Restriction enzyme digested and purified LFcin-H & B DNAs



Lane

(1) Lactoferricin H DNA

(2) BRL 1 kb ladder

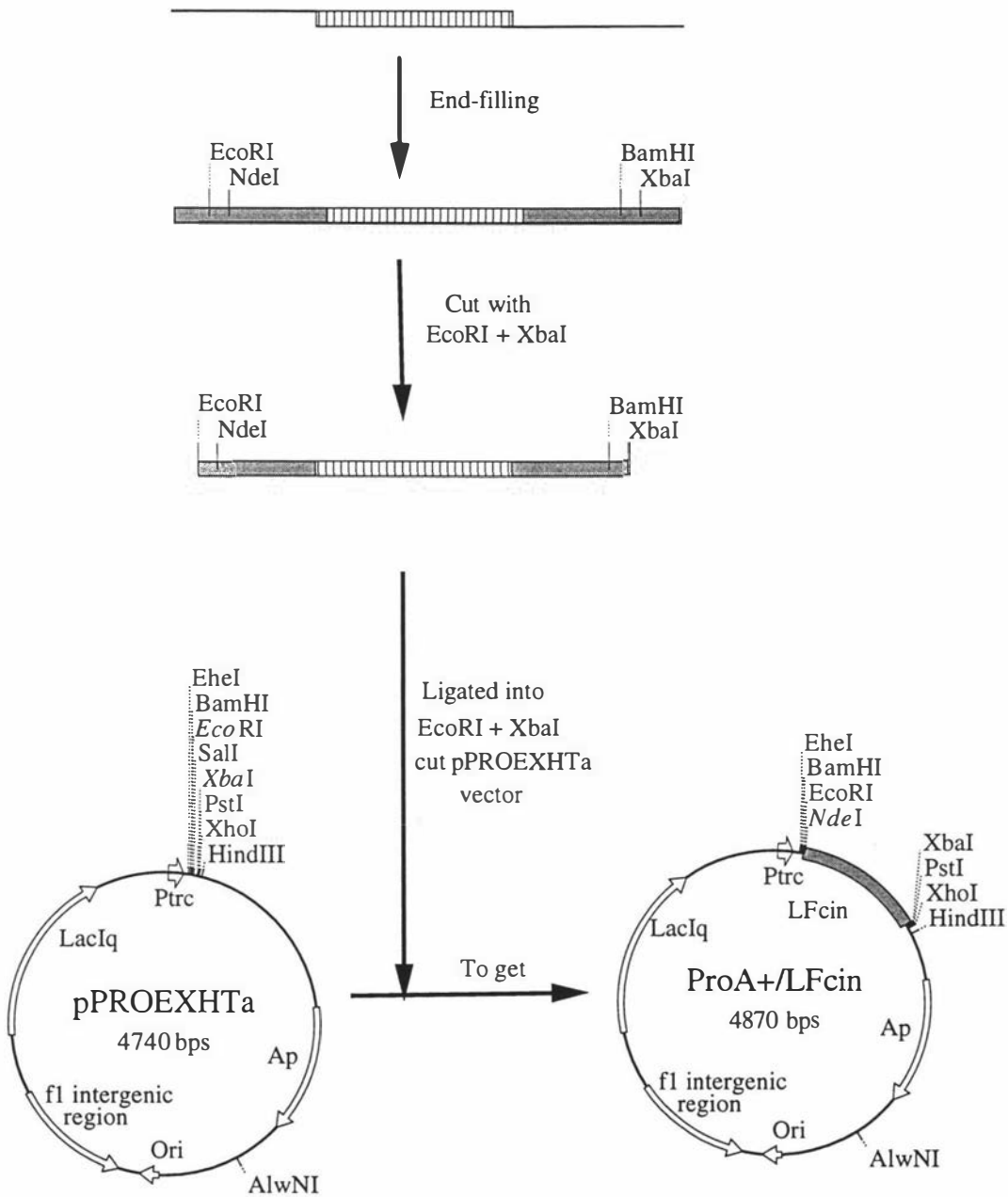
(3) Lactoferricin B DNA

4.2.3 Cloning of the end-filled DNA fragments into *E. coli* expression vectors

The end-filled lactoferricin DNA fragments were digested with *EcoRI* and *XbaI* restriction endonucleases to give the right ends for sub-cloning into the pPROEXHTa vector digested with the same restriction enzymes. The restriction-digested DNA fragments were purified using Microspin S-400 HR columns to remove the restriction enzymes and short fragments produced by restriction digestion. The digested fragments were run on a 1.2% gel and lactoferricin fragments of expected size were seen, which could be sub-cloned into the pPROEXHTa vector. Lactoferricin B fragment was 105 bp long and the lactoferricin H fragment was 96 bp long (Fig. 4.2).

Fig. 4.3 describes the cloning strategy that was used to sub-clone these fragments into the pPROEXHTa *E. coli* expression vector.

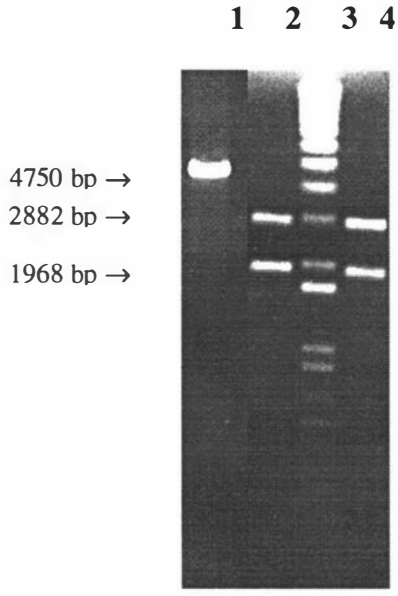
Fig. 4.3 Schematic diagram of sub-cloning strategy of lactoferricin DNA into *E. coli* expression vector pPROEXHTa



The ligation reaction was assembled using a 3:1 molar ratio of insert to linear vector (pPROEXHTa) in a total volume of 10 µl. Following the ligation, half of the ligation mix was used to transform *E. coli* DH5α. Cells were plated on LB^{arnp} agar media to select ampicillin-resistant transformants. Plasmid DNA isolated from several transformant colonies was digested with *NdeI* and *AlwNI* restriction enzymes and electrophoresed on a 0.75% agarose gel. Some of the transformants showed the required insert of approximately 2 kb in size (Fig. 4.4) (the pPROEXHTa vector does not contain a *NdeI* site).

Similar restriction analysis was carried out on the plasmid DNA isolated from the transformants obtained with LFcin-H & B DNA with different C-terminal modifications that had been sub-cloned into the pPROEXHTa vector. The presence of required inserts of expected size was confirmed.

Fig. 4.4 Restriction digestion analysis of LFcin-B & H constructs in pPROEXHTa vector



Lane

(1) pPROEXHTa vector cut with *NdeI* and *AlwNI*

(2) ProA+/LFcin-B construct cut with *NdeI* and *AlwNI*

(3) BRL 1 kb plus ladder

(4) ProA+/LFcin-H construct cut with *NdeI* and *AlwNI*

The DNA sequence of the LFcin-B and LFcin-H fragments in the pPROEXHTa vector was confirmed by DNA sequencing using the M13-reverse primer on an ABI Prism 377-36 automatic DNA sequencer (dye-terminator method). The DNA and deduced amino acid sequences of the LFcin-H & B constructs in pPROEXHTa *E. coli* expression vector are shown in sections 4.2.4 and 4.2.5. The lactoferricin sequences are shown in bold and their N-terminal and C-terminal extensions in normal type face.

4.2.4 DNA and amino acid sequences of the LFcin-B constructs in pPROEXHTa *E. coli* expression vector

a. LFcin-B expression in pPROEXHTa (without a stop-codon in the DNA insert)

```
1    ATG TCG TAC TAC CAT CAC CAT CAC CAT CAC GAT TAC GAT ATC
     Met Ser Tyr Tyr His His His His His His Asp Tyr Asp Ile

43   CCA ACG ACC GAA AAC CTG TAT TTT CAG GGC GCC ATG GAT CCG
     Pro Thr Thr Glu Asn Leu Tyr Phe Gln Gly Ala Met Asp Pro

95   GAA TTC CAT ATG TTC AAA TGC CGT CGT TGG CAG TGG CGT ATG
     Glu Phe His Met Phe Lys Cys Arg Arg Trp Gln Trp Arg Met

137  AAA AAA CTG GGT GCT CCG TCC ATC ACC TGC GTT CGT CGT GCT
     Lys Lys Leu Gly Ala Pro Ser Ile Thr Cys Val Arg Arg Ala

179  TTC GCT GGA TCC TCT AGA GCC TGC AGT CTC GAG GCA TGC GGT
     Phe Ala Gly Ser Ser Arg Ala Cys Ser Leu Glu Ala Cys Gly

211  ACC AAG CTT GGC TGT TTT GGC GGA TGA
     Thr Lys Leu Gly Cys Phe Gly Gly ★★★
```

b. LFcin-B expression in pPROEXHTa (With codons for four hydrophilic amino acids before a stop-codon at the 3′- end of the insert)

```
1    ATG TCG TAC TAC CAT CAC CAT CAC CAT CAC GAT TAC GAT ATC
     Met Ser Tyr Tyr His His His His His His Asp Tyr Asp Ile

43   CCA ACG ACC GAA AAC CTG TAT TTT CAG GGC GCC ATG GAT CCG
     Pro Thr Thr Glu Asn Leu Tyr Phe Gln Gly Ala Met Asp Pro

95   GAA TTC CAT ATG TTC AAA TGC CGT CGT TGG CAG TGG CGT ATG
     Glu Phe His Met Phe Lys Cys Arg Arg Trp Gln Trp Arg Met

137  AAA AAA CTG GGT GCT CCG TCC ATC ACC TGC GTT CGT CGT GCT
     Lys Lys Leu Gly Ala Pro Ser Ile Thr Cys Val Arg Arg Ala

179  TTC GCT GGA TCC AGC AGT TAA
     Phe Ala Gly Ser Ser Ser ★★★
```

c. LFcin-B expression in pPROEXHTa (with a stop-codon at the 3′- end of the insert)

```
1    ATG TCG TAC TAC CAT CAC CAT CAC CAT CAC GAT TAC GAT ATC
     Met Ser Tyr Tyr His His His His His His Asp Tyr Asp Ile

43   CCA ACG ACC GAA AAC CTG TAT TTT CAG GGC GCC ATG GAT CCG
     Pro Thr Thr Glu Asn Leu Tyr Phe Gln Gly Ala Met Asp Pro

95   GAA TTC CAT ATG TTC AAA TGC CGT CGT TGG CAG TGG CGT ATG
     Glu Phe His Met Phe Lys Cys Arg Arg Trp Gln Trp Arg Met

137  AAA AAA CTG GGT GCT CCG TCC ATC ACC TGC GTT CGT CGT GCT
     Lys Lys Leu Gly Ala Pro Ser Ile Thr Cys Val Arg Arg Ala

179  TTC GCT TAA
     Phe Ala ★★★
```

4.2.5 DNA and amino acid sequences of the LFcin-H constructs in pPROEXHTa *E. coli* expression vector

a. LFcin-H expression in pPROEXHTa (without a stop-codon in the DNA insert)

```
1    ATG TCG TAC TAC CAT CAC CAT CAC CAT CAC GAT TAC GAT ATC
     Met Ser Tyr Tyr His His His His His His Asp Tyr Asp Ile

43   CCA ACG ACC GAA AAC CTG TAT TTT CAG GGC GCC ATG GAT CCG
     Pro Thr Thr Glu Asn Leu Tyr Phe Gln Gly Ala Met Asp Pro

95   GAA TTC CAT ATG ACC AAA TGC TTC CAG TGG CAG CGT AAC ATG
     Glu Phe His Met Thr Lys Cys Phe Gln Trp Gln Arg Asn Met

137  CGT AAA GTT CGT GGT CCG CCG GTT TCC TGC ATC AAA CGT GGA
     Arg Lys Val Arg Gly Pro Pro Val Ser Cys Ile Lys Arg Gly

179  TCC TCT AGA GCC TGC AGT CTC GAG GCA TGC GGT ACC AAG CTT
     Ser Ser Arg Ala Cys Ser Leu Glu Ala Cys Gly Thr Lys Leu
```

```
211  GGC TGT TTT GGC GGA TGA
     Gly Cys Phe Gly Gly ★★★
```

b. LFcin-H expression in pPROEXHTa (With codons for four hydrophilic amino acids before a stop-codon at the 3′- end of the insert)

```
1    ATG TCG TAC TAC CAT CAC CAT CAC CAT CAC GAT TAC GAT ATC
     Met Ser Tyr Tyr His His His His His His Asp Tyr Asp Ile

43   CCA ACG ACC GAA AAC CTG TAT TTT CAG GGC GCC ATG GAT CCG
     Pro Thr Thr Glu Asn Leu Tyr Phe Gln Gly Ala Met Asp Pro

96   GAA TTC CAT ATG ACC AAA TGC TTC CAG TGG CAG CGT AAC ATG
     Glu Phe His Met Thr Lys Cys Phe Gln Trp Gln Arg Asn Met

137  CGT AAA GTT CGT GGT CCG CCG GTT TCC TGC ATC AAA CGT GGA
     Arg Lys Val Arg Gly Pro Pro Val Ser Cys Ile Lys Arg Gly

179  TCC AGC AGT TAA
     Ser Ser Ser ★★★
```

c. LFcin-H expression in pPROEXHTa (with a stop-codon at the 3′- end of the insert)

```
1    ATG TCG TAC TAC CAT CAC CAT CAC CAT CAC GAT TAC GAT ATC
     Met Ser Tyr Tyr His His His His His His Asp Tyr Asp Ile

43   CCA ACG ACC GAA AAC CTG TAT TTT CAG GGC GCC ATG GAT CCG
     Pro Thr Thr Glu Asn Leu Tyr Phe Gln Gly Ala Met Asp Pro

97   GAA TTC CAT ATG ACC AAA TGC TTC CAG TGG CAG CGT AAC ATG
     Glu Phe His Met Thr Lys Cys Phe Gln Trp Gln Arg Asn Met

137  CGT AAA GTT CGT GGT CCG CCG GTT TCC TGC ATC AAA CGT TAA
     Arg Lys Val Arg Gly Pro Pro Val Ser Cys Ile Lys Arg ★★★
```

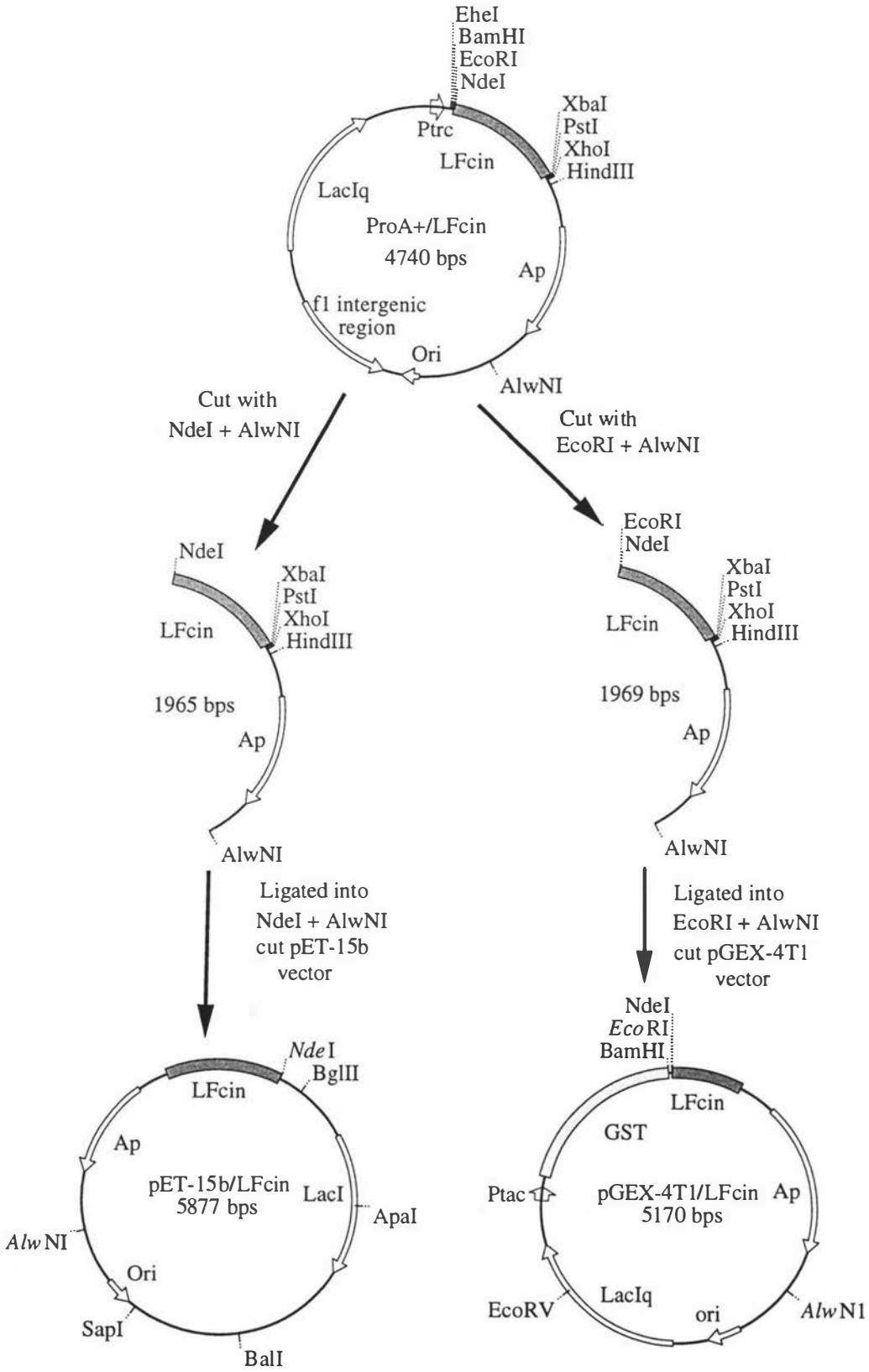
When the coding regions for LFcin-H & B were removed from the pPROEXHTa vector constructs and transferred to the pGEX-4T1 and pET-15b vectors, the reading frame should be preserved. Consequently, these vectors should produce the same lactoferricin sequences including the C-terminal extensions.

4.2.6 Sub cloning of LFcin-H & B fragments from ProA+/LFcin constructs into pGEX-4T1 and pET-15b *E. coli* expression vectors

The *EcoRI/AlwNI* fragment that includes the lactoferricin-coding insert and the ampicillin cassette in ProA+/LFcin constructs (both human and bovine) was sub-cloned into *EcoRI* and *AlwNI* digested pGEX-4T1 vector. Similarly, the *NdeI/AlwNI* fragment that includes the insert and ampicillin cassette in ProA+/LFcin constructs (both human and bovine) was sub-cloned into *NdeI* and *AlwNI* cut pET-15b vector (Fig 4.5). The ligation mixes were transformed into *E. coli* DH5α and the transformants were selected on LB^{amp} agar media. This strategy was designed to obviate the need to repeatedly sequence the DNA after insertion into the different expression vectors. Restriction digestion analysis of the plasmid DNA isolated from these transformants showed the presence of the desired inserts. The presence of *EcoRI/AlwNI* fragments from pPROEXHTa/ LFcin-H & B constructs sub-cloned into the pGEX4T-1 vector were confirmed by digesting the plasmid DNA with *NdeI* and *AlwNI* enzymes. The introduction of a *NdeI* site into these constructs confirmed the presence of lactoferricin coding sequences (*NdeI* site is not present in the pGEX-4T1 vector). Similarly, the presence of approximately 100 bp long lactoferricin inserts in the pET-15b vector constructs was confirmed by a double digestion with *NdeI* and *BamHI* restriction enzymes and analysing on a 2% agarose gel.

The C-terminal modified fragments of LFcin-H & B DNA with codons for four hydrophilic amino acids (Gly-Ser_3) before a stop-codon at the 3′- end of the insert were not sub-cloned into the pGEX4T-1 and pET-15b vectors. This was because the pPROEXHTa constructs of these fragments did not produce any protein product upon induction with IPTG as assessed by SDS-PAGE analysis by the method described in section 2.4.3.

Fig. 4.5 Schematic diagram of sub-cloning strategy for introduction of lactoferricin DNA into *E. coli* expression vectors pGEX-4T1 and pET-15b from pPROEXHTa/LFcin-H & B constructs



The constructs of LFcin-H & B in the three *E. coli* expression vectors were designated as follows (Table 4.3).

Table 4.3 Expression constructs of LFcin-B & H

Vector	Fusion Partner	Peptide	Construct Designation
pPROEXHTa	Poly-His	LFcin-B LFcin-H (without a stop-codon in the DNA insert)	ProA+bp ProA+hH
		LFcin-B LFcin-H (with a stop-codon at the 3′-end of the insert)	ProA+bp# ProA+hH#
		LFcin-B LFcin-H (with codons for four hydrophilic amino acids before a stop-codon at the 3′-end of the insert)	ProA+mbp ProA+mhH
pGEX4T-1	GST	LFcin-B LFcin-H (without a stop-codon in the DNA insert)	nT1+bp nT1+hH
		LFcin-B LFcin-H (with a stop-codon at the 3′-end of the insert)	nT1+bp# nT1+hH#
pET-15b	Poly-His	LFcin-B LFcin-H (without a stop-codon in the DNA insert)	nET+bp nET+hH
		LFcin-B LFcin-H (with a stop-codon at the 3′-end of the insert))	nET+bp# nET+hH#

4.3 Analysis of all the constructs of LFcin-H & B

The *trc* promoter in pPROEXHTa vector, the *tac* promoter in pGEX4T-1 vector and the *lac*I^q gene in both of them enable inducible expression of cloned gene in both vectors with isopropyl-β-D-thiogalactopyranoside (IPTG) in any *E. coli* host. On the other hand, the pET-15b vector utilises T7 promoter, which transcribes the cloned DNA by T7 RNA polymerase in *E. coli* BL21 (DE3). The T7 RNA polymerase gene in *E. coli* BL21 (DE3) is transcribed by the *lac*UV5 promoter, which is inducible by IPTG. All the LFcin-H & B constructs shown in Table 4.3 were analysed for the expression of the recombinant proteins by IPTG induction and subsequent purification. Since these constructs showed considerable differences with respect to expression and solubility of the recombinant proteins, each construct or a group of similar constructs will be discussed separately in the following sections. All expression studies were carried out in the *E. coli* host BL21 (DE3). This *E. coli* is a B strain, deficient in the *lon* protease (*lon*$^-$) and lacks the *ompT* outer membrane protease that can degrade proteins during purification (Grodberg and Dunn, 1988). *E. coli* BL21 (DE3) uses bacteriophage T7 RNA polymerase (induced by IPTG) to direct selective high-level expression of cloned genes and is the preferred strain for the heterologous expression of proteins (Studier and Moffatt, 1986).

4.3.1 Expression studies of the ProA+bp construct

A culture of *E. coli* BL21 (DE3) transformed with ProA+bp construct was grown at 37°C with aeration in LB^{amp} liquid media to mid-log phase (OD_{600} ~0.6), induced by the addition of 1mM IPTG and shaken at 37°C for a further 3 hrs. An uninduced culture was treated as above except no IPTG was added. Cell pellets from uninduced and induced cultures were sonicated and centrifuged to separate soluble and insoluble fractions. These fractions were analysed by electrophoresis on a 16.5% Tris-tricine SDS-PAGE gel as described in section 2.4.3. The recombinant fusion protein was expressed in inclusion bodies, as it was associated with the pellet.

Since these preliminary studies showed that the fusion protein was expressed in inclusion bodies, studies were carried out in an attempt to optimise the expression and to obtain the protein in a soluble form. These studies were carried out using a combination of

different IPTG concentrations, incubation temperatures and different periods of induction (Schein and Noteborn, 1988).

4.3.1.1 Optimisation of expression of the ProA+bp construct

A culture of *E. coli* BL21 (DE3) transformed with ProA+bp construct was grown at 37°C with aeration in LB^{amp} liquid media to mid-log phase (OD_{600} ~0.6) and divided into different tubes. Final IPTG concentrations of 0.1 mM, 1 mM and 2 mM were used for induction and the induction was carried out at 16°C, 25°C, 30°C and 37°C. The cultures were incubated for a further 1, 2 and 3 hrs at the respective temperatures and the cells collected. The uninduced and induced cells were sonicated and centrifuged to separate soluble and insoluble fractions. All fractions were analysed on 16.5% Tris-tricine SDS-PAGE gels. At all the combinations of IPTG concentration, incubation temperature and period of induction tested, the recombinant fusion protein was expressed exclusively in inclusion bodies. Since inclusion bodies were produced under all the conditions tested, subsequent cultures were grown at 37°C and induced with 1 mM IPTG. These conditions were chosen because they gave maximum cell density and yield of recombinant protein.

4.3.1.2 Isolation and solubilisation of inclusion bodies

The induction was carried out using from 50 ml to 500 ml of culture grown at 37°C and induced using 1 mM final IPTG concentration. After induction, the culture was centrifuged at 6000 g and the pellet washed with 1x binding buffer. The washed pellet was then resuspended in 1x binding buffer (40 ml per 100 ml of culture) with PMSF added to a final concentration of 1 mM to inactivate serine proteases. The cells were disrupted by sonication on ice. Sonication was carried out in short bursts at a high power setting until more than 70% cell lysis was achieved. The progress of cell lysis was monitored at 590 nm using the following equation (adapted from the Invitrogen manual).

$$\% \text{ Cells lysed} = [1 - A_{final} / A_{initial}] \times 100$$

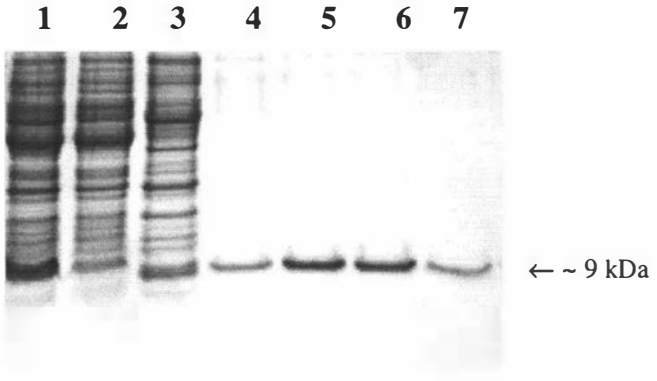
$A_{initial}$ is the initial absorbance of the cell suspension at 590 nm and
A_{final} is the final absorbance, after sonication, of the cell suspension at 590 nm.

The sonicated cell suspension was centrifuged for 20 min (17,000 g). The supernatant was carefully decanted and the pellet was washed twice in 1x binding buffer by sonicating briefly and centrifuging for 20 min at 17,000 g. The washed pellet was resuspended in 5 ml 1x binding buffer containing 8 M urea and incubated on ice for 1 hr to completely dissolve the inclusion bodies. Then it was centrifuged for 30 min at 17,000 g to remove any remaining insoluble material. Following centrifugation, the supernatant was collected carefully. The supernatant was filtered through a 0.45 μm filter before loading on to the metal-chelation (Ni-NTA) column.

4.3.1.3 Purification of solubilised inclusion bodies by column chromatography

The recombinant proteins were purified under denaturing conditions with all buffers containing 8 M urea. PMSF was included in all the buffers at a final concentration of 1 mM. The Ni-NTA column was equilibrated with 1x binding buffer containing 8 M urea before the urea-denatured recombinant protein was loaded on to the column. The column was washed with 10 volumes of 1x binding buffer and then with 10 volumes of 1x wash buffer (60 mM imidazole final concentration). The protein was then eluted using a step-gradient of increasing imidazole concentration with one column volume of buffer at each concentration of imidazole. Samples were collected at each step during the column purification and analysed on a 16.5% Tris-tricine SDS-PAGE gel (Fig. 4.6). The protein started eluting at 80 mM imidazole, most of the protein was eluted by 160 mM and no further material was eluted beyond 300 mM imidazole. The expression levels of poly-His tagged protein was in the range of 10-12 mg per litre of culture, though variable slightly from batch to batch.

Fig. 4.6 Purification of poly-His tagged recombinant LFcin-B expressed in the pPROEXHTa expression vector on Ni-NTA column



Lane

(1) Urea extract of inclusion bodies (supernatant)

(2) Unbound fraction at 5 mM imidazole

(3) Wash fraction at 60 mM imidazole

(4) Elution fraction at 80 mM imidazole

(5) Elution fraction at 100 mM imidazole

(6) Elution fraction at 120 mM imidazole

(7) Elution fraction at 160 mM imidazole

4.3.1.4 Processing of the purified recombinant protein

Since the protein was purified under denaturing conditions, the next task was to remove urea in an attempt to allow refolding of the protein and reconstitution of its activity.

Removal of urea, salt and imidazole by dialysis

The fractions eluted between 100 and 160 mM imidazole were pooled and processed for the removal of urea, salts and imidazole. Various buffers, as listed below, were tried.

a. Dialysis against Milli-Q water at pH 5.5 and pH 7.0

The native lactoferricin-B obtained by acid pepsin hydrolysis of bovine lactoferrin and purification by reverse phase chromatography is soluble in Milli-Q water (Bellamy *et*

al., 1992). For this reason it was thought that soluble recombinant protein might be obtained by dialysis against Milli-Q water. It would then be possible to concentrate the protein by removing water without concentrating salts. The pooled fractions of recombinant LFcin-B were dialysed (Spectra/Por 3 membranes, MWCO 3,500) separately against Milli-Q water at pH 5.5 or pH 7.0 at 4°C, overnight with 3 to 4 changes. Most of the protein precipitated during this treatment. The soluble fraction showed the presence of some recombinant protein, but upon concentration, this also precipitated. When the pH was adjusted to either very acidic (pH ~ 2) or very basic (pH ~ 10) the recombinant peptide stayed in soluble form. However, as the objective was to produce physiologically active lactoferricin, this solubility at extreme of pH was not useful for subsequent work. At neutral pH conditions where lactoferricin is active as an antibacterial agent, the protein was insoluble.

b. Dialysis against 100 mM ammonium acetate

This buffer was used, as both components are volatile and could be removed by lyophilisation. The recombinant protein solubilised by 8 M urea was dialysed against 100 mM ammonium acetate buffer. Dialysis was carried out at 4°C, overnight with 3 to 4 changes. A considerable amount of precipitation was observed. Though some protein remained soluble, after lyophilisation, it was not possible to redissolve the protein.

c. Dialysis against 20 mM Tris-HCl buffer with and without 0.5 M NaCl

The recombinant protein solubilised by 8 M urea was dialysed separately against these buffers at 4°C, overnight with 3 to 4 changes of the buffer. In both cases the recombinant protein precipitated leaving a very low concentration of soluble protein.

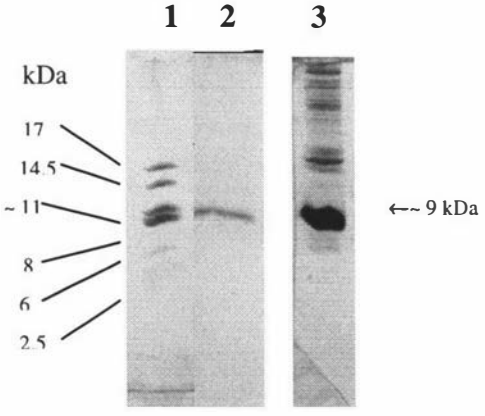
d. Dialysis against 20 mM Tris-HCl buffer (pH 8.0) with a gradual decrease in urea concentration

The recombinant protein solubilised by 8 M urea was dialysed against this buffer by gradually decreasing the concentration of urea from 8 M urea to 0 M urea over a period of 24 hrs at 4°C. The recombinant protein could not be kept in soluble form.

e. Dialysis against Milli-Q water and 20 mM Tris-HCl buffer (pH 8.0) both with 1 mM DTT (final concentration)

In both cases more soluble recombinant protein was obtained compared to dialysis against other buffers. But, on concentration and storage the protein precipitated as shown in Fig. 4.7 (a representative gel for all the dialysis attempts made to remove 8 M urea). The higher molecular weight material seen in lane 3 might due to aggregation of recombinant protein formed after precipitation, or the concentration of high molecular weight impurities in the partially purified protein preparation.

Fig. 4.7 Analysis of the purified poly-His tagged recombinant LFcin-B in the presence of DTT



Lane

(1) Peptide molecular weight markers

(2) Dialysed poly-His fused LFcin-B peptide in the presence of 1 mM DTT

(3) Poly-His fused LFcin-B peptide upon concentration and storage

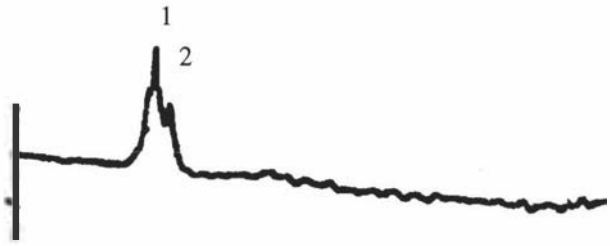
The insolubility of the recombinant protein after removal of urea by dialysis in different buffers might be due to an intrinsic property of the protein that influenced its solubility. The fusion protein expressed by the construct, ProA+bp (poly-His LFcin-B fusion protein with a hydrophobic C-terminal extension added to the peptide from the vector sequences) contained three additional cysteines besides the two cysteines already

present in the coding region of LFcin-B DNA [refer to section 4.2.4 (a) for sequence]. The presence of a total of five cysteine residues that would have contributed to the inter- and intra-molecular disulphide bridges and also the presence of hydrophobic amino acids in the C-terminal tail of the recombinant protein might have decreased its solubility.

4.3.1.5 HPLC purification of recombinant LFcin–B fusion protein

An attempt was made to use HPLC to purify the recombinant protein solubilised by 8 M urea on a reverse phase column (RPC C-18 column). A 100 µl sample was loaded on to the column. The protein was eluted with a gradient of acetonitrile/water and the elution was monitored at 220 nm. The purification was satisfactory (Fig 4.8). The fractions corresponding to peak 1 and peak 2 were collected and lyophilised to remove acetonitrile. However, lyophilisation caused precipitation of protein from both fractions. The precipitated protein could not be resolubilised.

Fig. 4.8 Purification of poly-His tagged recombinant LFcin-B on a reverse-phase HPLC column

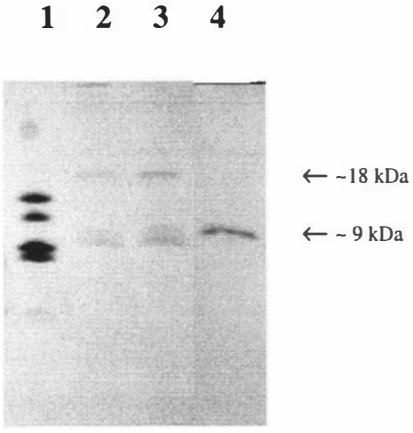


Poly-His fusion protein eluted at 58% gradient of acetonitrile/water. Elution was monitored at 220 nm wavelength and at 0.5 absorbance units (AU) setting.

Units
X-axis Absorbance at 220 nm
Y-axis Time in minutes

The lyophilised fractions corresponding to peak 1 and peak 2 were run on SDS-PAGE gels with and without DTT treatment (Fig. 4.9). When DTT was not added both fractions showed the presence of ~ 9 kDa monomer and ~ 18 kDa dimer. However, the DTT treated peak 1 fraction gave a single band corresponding the monomer ~ 9 kDa. This confirmed the presence of inter-molecular disulphide bridges that might be contributing to the aggregation of the recombinant protein and hence reducing its solubility.

Fig. 4.9 SDS-PAGE analysis of the lyophilised peak fractions of the HPLC purified poly-His tagged recombinant LFcin-B



Lane

(1) Peptide molecular weight markers (sizes as shown in Fig. 4.7, lane 1)

(2) Concentrated peak 1 fraction without DTT

(3) Concentrated peak 2 fraction without DTT

(4) Concentrated peak 1 fraction with DTT

4.3.1.6 N-terminal sequencing of recombinant fusion protein expressed from the pPROEXHTa vector (ProA+bp construct)

Poly-His fusion protein was run on a 16.5% Tris-tricine SDS-PAGE gel. The protein was blotted on to a PVDF membrane as described in section 2.3.5 and stained with Coomassie brilliant blue. A single band corresponding to poly-His tagged recombinant

LFcin-B (~ 9 kDa) was used for the N-terminal sequencing. The N-terminus of the fusion protein was sequenced using an Applied Biosystems' 477A Protein Sequencer. The sequence of the first five residues of poly-His fusion protein was as predicted from the DNA sequence. The sequence is given in Fig. 4.10.

Fig 4.10 N-terminal sequence of the poly-His fusion protein obtained from construct ProA+bp

Ser-Tyr-Tyr-His-His

4.3.2 Expression studies using the constructs Pro+hH, ProA+hH#, ProA+bp#, ProA+mbp and ProA+mhH

Cultures carrying these constructs were grown at 37°C with aeration till mid-log phase (OD_{600} ~ 0.6), induced with 1 mM IPTG and cultured for a further 3 hrs at 37°C. The cell density continued to increase during the induction phase and reached a final OD_{600} of approximately 2.0. The induced and uninduced cell pellets were sonicated, centrifuged and separated into supernatant and pellet fractions. These fractions were examined by electrophoresis on a 16.5% Tris-tricine SDS-PAGE gel. There was no difference between the induced and uninduced supernatant and pellet fractions. These constructs did not yield any recombinant protein. The absence of protein product could be because of proteolysis within the host cell in case if the recombinant protein was expressed in a soluble form (Piers *et al.*, 1993). The recombinant proteins expressed as inclusion bodies might be partially protected from proteolysis within the bacterial cell. It is also possible that these proteins were not expressed at all by the host cell.

4.3.3 Expression studies using the constructs nT1+bp, nT1+hH, nT1+bp# and nT1+hH#

The cultures were grown to mid-log phase at 37°C, induced with 1 mM IPTG and cultured for a further 3 hrs at 37°C with aeration. All constructs expressed the GST-fusion protein as inclusion bodies except for the nT1+hH# construct, which expressed soluble fusion protein. Extracts, which contained the GST-fusion protein also, contained a protein of the same size as the GST protein itself. This protein was suspected to have arisen by proteolysis of the GST-fusion protein. Proteolysis was also observed by earlier investigators studying the heterologous expression of cationic peptides as GST-fusions (Piers *et al.*, 1993). The proteolysis that generated this degradation product might even have occurred after the formation of inclusion bodies. It has been observed that inclusion bodies also undergo proteolysis, by other investigators (Piers *et al.*, 1993) and us.

The constructs nT1+bp# and nT1+hH# were further studied for the purification and characterisation of the recombinant GST-fusion proteins.

4.3.3.1 Purification of GST-fused proteins expressed by the nT1+bp# and nT1+hH# constructs

Cultures carrying these constructs were grown at 37°C with aeration to mid-log phase (OD_{600} ~ 0.6), induced with 1 mM IPTG and incubated for a further 3 hrs at 37°C. The cell pellets were sonicated, centrifuged and separated into soluble and insoluble fractions.

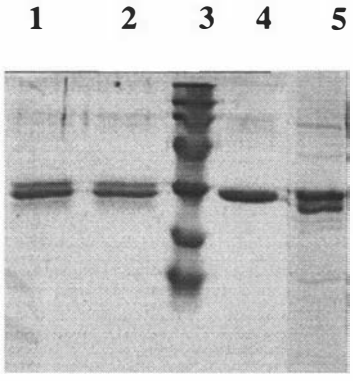
The soluble GST-fusion protein obtained from the construct nT1+hH# was purified on glutathione-Sepharose 4B column using a standard Pharmacia protocol.

The inclusion bodies produced by the construct nT1+bp# were solubilised in 1x PBS containing 8 M urea (5 ml of 8 M urea solution for a pellet from 100 ml of culture) by incubating on ice for 1 hr. Sometimes, a brief sonication helped in resuspending the pellet in the urea solution. The urea-solubilised protein was centrifuged at 17,000 g for 20 min. The supernatant was carefully collected. The conditions, under which the urea solubilised GST-fusion protein would bind to the glutathione-Sepharose 4B resin, were worked out by

diluting the 8 M urea solubilised protein to different concentrations of urea (based on the suggestions given in the Pharmacia GST-purification module manual). The fusion protein bound the glutathione-Sepharose 4B resin satisfactorily between 2.5 M and 3 M urea concentration. Taking advantage of this, the 8 M urea solubilised protein supernatant was diluted to 3 M urea with 1x PBS, filtered through a 0.45 μM filter to remove any precipitate and applied on to a 0.3 ml glutathione-Sepharose 4B resin. The flow-through was collected and the column washed with 1x PBS buffer (20 column volumes).

The GST-fusion proteins in both cases were eluted from the resin with 10 mM reduced glutathione (Fig. 4.11 & Fig. 4.12). The elution was quite inefficient. It was observed that even after eluting the bound recombinant proteins with more than five column volumes of 10 mM reduced glutathione, a major part of the fusion protein was still bound on to the resin. The GST protein itself (the proteolytic degradation product) eluted more efficiently from the resin than the GST-fusion protein. This could be because of the more basic nature of the fusion protein (because of the presence of highly basic lactoferricin in the fusion protein) than the GST protein alone. This might be contributing towards a stronger interaction between the GST-fusion protein and the resin than between the GST protein and the resin. No precipitation of protein on the glutathione-Sepharose 4B resin was observed.

Fig. 4.11 Affinity purification of GST-fused LFcin-B (with a stop-codon at the 3′-end of insert) protein



Lane

(1) Elution fraction 1

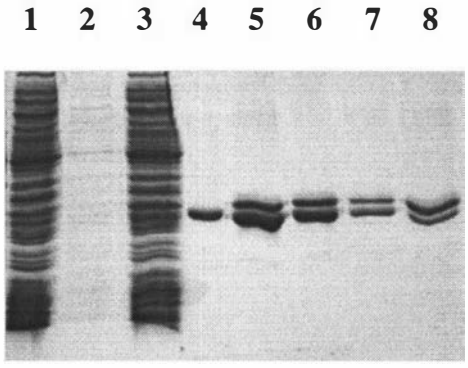
(2) Elution fraction 2

(3) Pharmacia low molecular weight marker

(4) Pure GST

(5) Resin after elution with more than five column volumes of 10 mM reduced glutathione

Fig. 4.12 Affinity purification of GST-fused LFcin-H (with a stop-codon at the 3′-end of insert) protein



Lane

(1) Unbound fraction

(2) Wash fraction

(3) 3 M Urea supernatant used for purification

(4) Pure GST

(5) Elution fraction 1

(6) Elution fraction 2

(7) Elution fraction 3

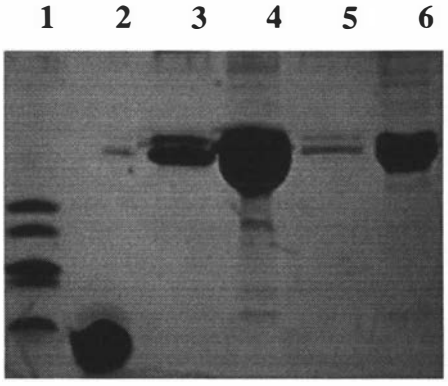
(8) Resin after elution with more than five column volumes of 10 mM reduced glutathione

4.3.3.2 Thrombin cleavage of the GST-fusion protein

Cleavage of the protein/peptide of interest from the GST-fusion protein can be carried out with thrombin because the linker between the fusion partner (GST) and the protein/peptide has a specific cleavage site for thrombin. Proteolytic cleavage of the lactoferricin peptides from both the eluted and the resin-bound GST-fusion proteins expressed from the nT1+bp# and nT1+hH# constructs was attempted using 10 U of thrombin protease per milligram of fusion protein. No peptide product could be seen in the

thrombin digests of the eluted fusion protein. Proteolytic digestion of the GST-fusion protein still bound to the resin released a peptide corresponding in size to lactoferricin. The recombinant LFcin-B & H peptides cleaved from the resin bound fusion protein could be seen on a 16.5% Tris-tricine SDS-PAGE gel. These peptides were not soluble. They remained associated with the resin even after cleavage of the fusion protein by thrombin (Fig.4.13).

Fig. 4.13 Thrombin cleavage of recombinant LFcin-H & B peptides from GST-fusions



Lane

(1) Peptide molecular weight markers
(2) Insulin standard
(3) Supernatant from the GST-fused Lfcin H resin digested with thrombin
(4) Resin bound GST-fused LFcin H after thrombin digestion
(5) Supernatant from the GST-fused Lfcin B resin digested with thrombin
(6) Resin bound GST-fused LFcin B after thrombin digestion

In Fig. 4.13, lanes (3) and (5) contained GST-protein and also GST-fusion protein because of the leakage of some resin into the supernatant as all the thrombin digestions were carried out on small volumes (20 µl). Also, lanes (4) and (6) in the gel were

overloaded to show the cleaved lactoferricin peptides on the gel with Coomassie brilliant blue stain.

There could be several reasons for the association of the thrombin-cleaved peptide with the resin.

(a) Precipitation of the peptide on the resin following cleavage by thrombin from the fusion protein. Precipitation of cleaved protein on the resin has also been observed by Dr. C.L.Day while working with GST-fusion proteins (Day, C.L, personal communication).

(b) Due to the small size of the lactoferricin peptides (~ 3 kDa), the peptides might be getting sieved by GST-Sepharose 4B, which has an approximate sieving molecular weight limit of 2×10^7 Daltons. Because of this, small peptides liberated following cleavage with site-specific protease would require larger elution volumes (reference to Pharmacia GST-purification module manual). This could not be checked with small lactoferricin peptides, as it was not practical to elute them with a large volume of 1x PBS. If it were eluted in large volume of 1x PBS buffer, the concentration of lactoferricin would have been very low. Hence, upon lyophilisation, salt gets concentrated many-fold and further handling of the peptide would be difficult.

(c) There is a possibility that the released lactoferricin peptide, because of its basic nature, might be interacting with the resin upon cleavage from the fusion protein. The isoelectric point (pI) of the GST-moiety is 5.0. The basic lactoferricin peptide with a calculated pI of ~9.0, might be interacting electrostatically with GST after cleavage with thrombin. Because of inefficient proteolytic cleavage with thrombin and low yields of lactoferricin peptides, it was hard to prove this experimentally.

Characterisation of these recombinant peptides was attempted by western blot analysis as described in section 2.4.6. None of the peptides were detected using polyclonal antibodies to bovine and human lactoferrins. Only the pure lactoferrin control showed reaction on the western blots. This confirmed an earlier report that hydrolysed lactoferrin loses its antigenicity (Saito *et al.*, 1991). However, monoclonal antibodies have been raised

against native lactoferricin B in mice and the amino acid sequence which binds to the monoclonal antibody has been identified (Shimazaki *et al.*, 1996). These authors reported that the monoclonal antibodies against native LFcin-B did not react with the C-lobe of bovine lactoferrin, with human lactoferrin or with LFcin-H.

The lactoferricin peptides were not analysed further as they could not be obtained in a soluble form.

4.3.4 Expression studies using the constructs nET+bp, nET+hH, nET+bp# and nET+hH#

The cultures were grown at 37°C with aeration to mid-log phase (OD_{600} ~ 0.6), induced with 1 mM IPTG and incubation continued for a further 3 hrs at 37°C. Control cultures were not induced with IPTG. The cell density increased throughout the induction phase. The induced and uninduced cell pellets were sonicated, centrifuged and separated into soluble and insoluble fractions. These fractions were run on a 16.5% Tris-tricine SDS-PAGE gel. There was no difference between the induced and uninduced fractions. These constructs also did not yield any recombinant protein.

4.3.5 Plasmid stability in the induced cultures

The presence of plasmid and insert was confirmed in all the recombinant cultures after induction with IPTG. Cultures of all the constructs were grown to mid-log phase (OD_{600} ~0.6) at 37°C with aeration. Samples of uninduced cultures were collected and the cells were pelleted by centrifugation. The cultures were then induced with 1 mM IPTG and cultured for a further 3 hrs at 37°C. The host was growing as evidenced by an increase in OD_{600} during the induction phase (from ~ 0.6 to ~ 2.0 in 3 hrs). Cells from both uninduced and induced cultures were used to make plasmid DNA minipreps as described in section 2.2.5. Plasmid DNA was then analysed by restriction digestion with appropriate enzymes. In all cases, the plasmids were present and contained the desired inserts. However, only a few of these constructs expressed recombinant protein upon induction with IPTG (sections 4.3.1 & 4.3.3). To investigate these constructs further, northern blot analysis was carried out to check for the production of a transcript from the cloned DNAs.

4.3.6 Northern blot analysis of the LFcin-H & B constructs

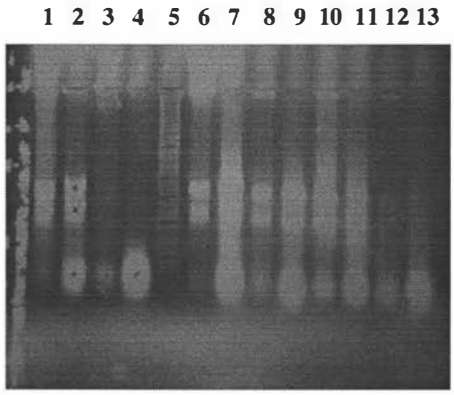
All the LFcin-B & H constructs expressed in the three different *E. coli* expression vectors were tested for the production of a transcript by northern blot analysis. Total RNA was isolated from both induced and uninduced cultures as described in section 2.3.2 and run on denaturing formaldehyde agarose gels as described in section 2.3.3 [Fig.4.14 (a) and 4.15 (a) respectively]. The RNA was blotted onto a nylon membrane (Hybond-N^+) and northern blot analysis was carried out as described in section 2.3.4 with both lactoferricin and GST probes.

4.3.6.1 Analysis of LFcin-H & B constructs with respective probes

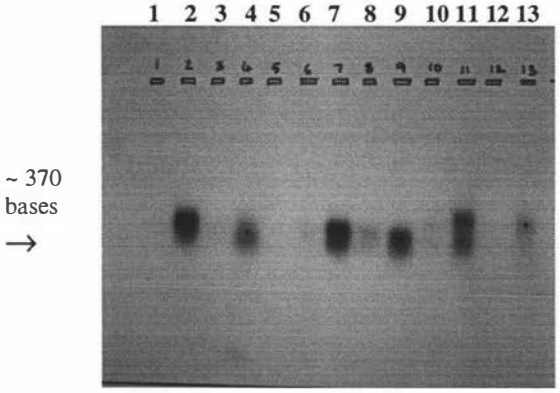
The LFcin-H probe was made by end-filling the annealed overlapping oligonucleotides, Forhlh 65 and Revhlh 63. Similarly, the probe for LFcin-B constructs was made by end-filling the annealed overlapping oligonucleotides, Forblb 70 and Revblb 70. End filling was carried out by using [^{32}P] dCTP and cold dATP, dTTP, dGTP and T4 DNA polymerase. The end-filled probe was purified on a Sephadex G-50 microspin column to remove the free nucleotides and enzyme. Then northern blot analysis was carried out as described in section 2.3.4. Fig. 4.14 (b) and Fig. 4.15 (b) show the northern blots of LFcin-H and LFcin-B constructs respectively.

Fig. 4.14 **Northern blot analysis of total RNA from cells harbouring the LFcin-H constructs**

(a) Ethidium bromide stained gel



(b) Blot probed with LFcin-H DNA

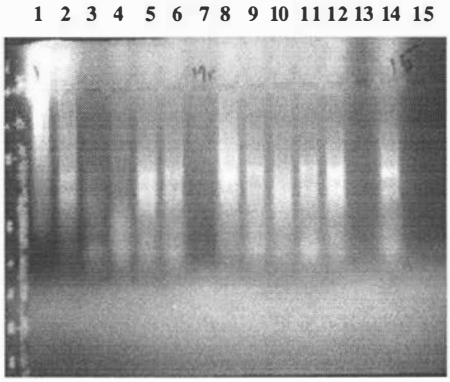


Lane

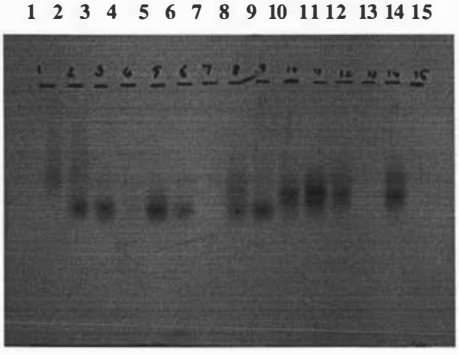
(1)	Uninduced ProA+hH	(8)	Uninduced nET+hH#
(2)	Induced ProA+hH	(9)	Induced nET+hH#
(3)	Uninduced ProA+hH#	(10)	Uninduced nTl+hH
(4)	Induced ProA+hH#	(11)	Induced nTl+hH
(5)	RNA ladder	(12)	Uninduced nTl+hH#
(6)	Uninduced nET+hH	(13)	Induced Tl+hH#
(7)	Induced nET+hH		

Fig. 4.15 Northern blot analysis of total RNA from cells harbouring the LFcin-B constructs

(a) Ethidium bromide stained gel



(b) Blot probed with Lfcin-B DNA



Lane

(1)	Uninduced ProA+bp	(8)	Uninduced nET+bp#
(2)	Induced ProA+bp	(9)	Induced nET+bp#
(3)	Uninduced ProA+bp#	(10)	Uninduced nT1+bp
(4)	Induced ProA+bp#	(11)	Induced nT1+bp
(5)	Uninduced nET+bp	(12)	Uninduced nT1+bp#
(6)	Induced nET+bp	(13)	Empty lane
(7)	RNA ladder	(14)	Induced T1+bp#
		(15)	Empty lane

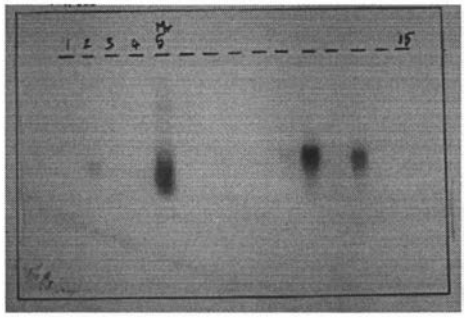
4.3.6.2 Analysis of LFcin-H & B constructs with GST-probe

The GST-region that was selected for making the probe was an *EcoRV/BamHI* fragment from the vector pGEX4T-1. This fragment was approximately 1.8 kb in size and included 280 bp of the $LacI^q$ gene together with the GST gene. The probe was made by using Pharmacia Ready-to go labelling kit with radiolabelled [^{32}P] dCTP. The labelled probe was purified on a Sephadex G-50 microspin column to remove the free nucleotides and enzyme.

The LFcin-H & B hybridised blots were stripped with 0.1% SDS solution at 100°C. This was carried out with three changes of 0.1% SDS solution at 100°C and each change was shaken for 30 min. The stripped blots were exposed to X-ray film overnight at -70°C and developed to make sure that the probes were totally removed. Both the stripped blots were then rehybridised with the GST-probe. After washing, the blots were exposed to X-ray film and developed using standard methods (Fig. 4.16 and Fig. 4.17 for LFcin-H & B constructs respectively). The high molecular weight transcripts that are seen in the blot of LFcin-B probed with GST in Fig. 4.17 could be because of the presence of a 280 bp part of $LacI^q$ gene in the GST-probe. $LacI^q$ gene in the pGEX-4T1 vector is ~ 1 kb in size. Hence transcripts of 1000 nucleotides or more in size on the blot would have arisen because of hybridisation of the $LacI^q$ gene.

Fig. 4.16 Northern blot analysis of total RNA from cells harbouring the LFcin-H constructs with the GST probe

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



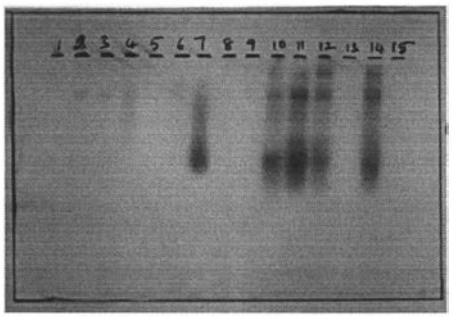
← ~690 Bases

Lane

(1)	Uninduced ProA+hH	(8)	Uninduced nET+hH#
(2)	Induced ProA+hH	(9)	Induced nET+hH#
(3)	Uninduced ProA+hH#	(10)	Uninduced nT1+hH
(4)	Induced ProA+hH#	(11)	Induced nT1+hH
(5)	RNA ladder	(12)	Uninduced nT1+hH#
(6)	Uninduced nET+hH	(13)	Induced T1+hH#
(7)	Induced nET+hH	(14) and (15)	Empty lanes

Fig. 4.17 Northern blot analysis of total RNA from cells harbouring the LFcin-B constructs with the GST probe

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



← ~690 Bases

Lane

(1)	Uninduced ProA+bp	(8)	Uninduced nET+bp#
(2)	Induced ProA+bp	(9)	Induced nET+bp#
(3)	Uninduced ProA+bp#	(10)	Uninduced nT1+bp
(4)	Induced ProA+bp#	(11)	Induced nT1+bp
(5)	Uninduced nET+bp	(12)	Uninduced nT1+bp#
(6)	Induced nET+bp	(13)	Empty lane
(7)	RNA ladder	(14)	Induced T1+bp#
		(15)	Empty lane

4.3.6.3 Discussion of the northern blots

With the northern blot analysis of the LFcin-H constructs in three different *E. coli* expression vectors, as expected, a 370 nucleotide long transcript was seen with the pPROEXHTa and pET-15b constructs and a 690 nucleotide long transcript with the pGEX-4T1 constructs upon hybridisation with the LFcin-H DNA probe. Also, when hybridised with the GST probe a 690 nucleotides transcript was seen with all the pGEX-4T1 constructs and no cross-reaction was seen with any other constructs, except for the RNA marker ladder (Fig. 4.16, lane 5).

Similarly, northern blots of the LFcin-B constructs in three different *E. coli* expression vectors showed a 370 nucleotide long transcript with the pPROEXHTa and

pET-15b constructs and a 690 nucleotide long transcript with the construct in the pGEX-4T1 vector, upon hybridisation with the LFcin-B DNA probe, as expected. When hybridised with the GST probe a 690 nucleotide transcript was seen with all the pGEX-4T1 constructs. There was no cross-reaction with any other constructs, except for the RNA marker ladder (Fig. 4.17, lane 7). The LFcin-B constructs probed with LFcin-B DNA showed the presence of transcripts in the uninduced samples. This could be because of basal level expression of the insert with these constructs. Basal level expression is not uncommon with the *E. coli* BL21 (DE3) expression host. The absence of protein product with some of the LFcin-B constructs could not be attributed to this basal level of mRNA expression. The absence of protein product could be either due to a problem at the translational level or proteolysis within the cell. However, the construct ProA+bp, containing LFcin-B DNA without a stop codon at its 3′-end, expressed poly-His fusion protein in inclusion bodies and seemed to be protected from proteolysis. This suggests that the protein product might not be toxic to the host cells.

The reason that some LFcin-H & B constructs did not produce a protein product might be due to proteolysis within the bacterial cell. Where a protein product was not seen, the protein might be expressed in soluble form and degraded by the intracellular proteases. Although *E. coli* strain BL21 (DE3), a *lon* protease deficient strain was used for the expression of all these constructs, proteolysis may still occur because the host will have other intracellular proteases essential for its survival. The fusion protein expressed in inclusion bodies seemed to be only partially escaping this proteolytic activity inside the cell and hence the protein product was seen. Proteolysis might also explain co-expression of unfused GST protein and GST-fusion protein from the LFcin-H & B constructs in the pGEX-4T1 vector.

Table 4.4 gives an overview of all the lactoferricin constructs made and their expression pattern in the *E. coli* BL21 (DE3).

Table 4.4 Overview of all lactoferricin constructs and their expression pattern in *E. coli* BL21 (DE3)

Expression vector	N-terminal Fusion partner	Peptide	Expression				
			Protein production	mRNA transcript detected	Proteolysis inferred	Cellular location	Fusion protein purified
pPROEXHTa	Poly-His	LFcin-B	++	+	No	IB	Yes
		LFcin-H (with hydrophobic C-terminal extension)	-	+	Yes	ND	No
		LFcin-B	-	+	Yes	ND	No
		LFcin-H (With no C-terminal extension)	-	+	Yes	ND	No
		LFcin-B	-	ND	Yes	ND	No
		LFcin-H (with hydrophilic C-terminal extension)	-	ND	Yes	ND	No
pGEX4T-1	GST	LFcin-B	++	+	Yes	IB	Yes
		LFcin-H (with hydrophobic C-terminal extension)	++	+	Yes	IB	Yes
		LFcin-B	++	+	Yes	IB	Yes
		LFcin-H (with no C-terminal extension)	++	+	Yes	C	Yes
pET-15b	Poly-His	LFcin-B	-	+	Yes	ND	No
		LFcin-H (with hydrophobic C-terminal extension)	-	+	Yes	ND	No
		LFcin-B	-	+	Yes	ND	No
		LFcin-H (with no C-terminal extension)	-	+	Yes	ND	No

(*Abbreviations: ND = not determined; IB = inclusion bodies; C = cytosol*)

(*++ = major cell protein; - = not detected; + = detected*)

4.4 Discussion and conclusions

a. Expression of synthetic gene sequences for LFcin-H & B in *E. coli*

The synthetic genes for the antibacterial domains of both human and bovine lactoferricins were successfully sub-cloned into three different *E. coli* expression vectors. These constructs could be stably maintained in *E. coli* strains DH5α (K12 derivative) and BL21 (DE3) (a B derivative *lon* protease deficient strain). The construct with LFcin-B DNA in the pPROEXHTa vector (ProA+bp) expressed poly-His fusion protein in inclusion bodies upon induction with 1 mM IPTG at 37°C. All LFcin-H & B constructs in the pGEX-4T1 vector expressed the GST-fusion protein in inclusion bodies, except for the LFcin-H construct with a stop-codon immediately following lactoferricin sequence at the 3′-end (nT1+hH#). This construct expressed the fusion protein in soluble form. The GST-fusion proteins were in all cases co-expressed with the unfused GST-moiety whether expressed in inclusion bodies or in soluble form. The other constructs of LFcin-H & B did not show any expression of the protein product. The presence of plasmid containing the desired insert was confirmed by restriction digestion analysis of plasmid DNA isolated from both uninduced and induced cultures for all constructs.

For all constructs, northern blots of RNA from induced cultures showed the presence of transcripts of the expected size. This confirmed that there was no problem at the level of transcription in any of the different *E. coli* vectors used in this study. Therefore the problem might have been at the post-transcriptional level. Transcripts corresponding to the GST protein alone could not be distinguished from transcripts of the GST-fused lactoferricins for constructs of LFcin-H & B in the pGEX-4T1 vector, because their transcripts were similar in size. This difference could be seen with the protein products because they have slightly different mobility on a SDS-PAGE gel.

There have been only a few reports on the heterologous expression of antimicrobial cationic peptides. Most of the expression trials in bacterial expression systems were unsuccessful. Not surprisingly, the potent antimicrobial activity of many of these peptides might be responsible for a failure in their expression in bacterial expression systems. Though we made the first attempt to heterologously express lactoferricins in *E. coli*, an

attempt similar to this has been reported earlier by a group in Canada, who attempted to express other cationic peptides. This group has worked with the bacterial expression of small antimicrobial peptides such as defensin and cecropin/melitin (CEME) hybrid (Piers *et al.*, 1993). These investigators attempted to express the cationic peptides as GST, OmpF and protein A fusion proteins. In all cases they either obtained the expression of fusion proteins in inclusion bodies or no protein product was observed. This is a similar result to that in this study. Biologically active peptides were not obtained after cleaving the peptides from the fusion partner, either by enzymatic or by chemical cleavage methods. The only success these authors have reported was the secretion of protein A fused CEME protein into the supernatant by *Staphylococcus aureus*. Protein A is a naturally secreted protein of *Staph. aureus* and these authors took advantage of this property. The vector pRIT5 that was used for this purpose possessed the protein A secretion signal sequence preceding the protein A gene and the insert. This allowed the fusion protein to be exported to the external medium when expressed in a *Staph. aureus* host. Piers *et al.* (1993) have also suggested that an additional anionic pre-pro sequence besides the fusion partner was required for stabilisation of the heterologously expressed cationic peptides (Piers *et al.*, 1993). They inserted an anionic pre-pro defensin sequence between the GST-fusion partner and defensin peptide, which protected the fusion protein from proteolysis. This may not be applicable in general for the expression of cationic peptides.

Recently, it has been reported that DNA sequence encoding a chimeric 68 amino acid carrier region (made up of a block of three polypeptides, a truncated RepA, a synthetic cellulose binding domain and a hexa histidine region) fused to the DNA sequence of cationic peptides improved the expression of the fusion protein. These authors reported that this chimeric carrier region helped in the high level expression of recombinant cationic peptides and made purification easy (Zhang *et al.*, 1998). Similarly, another group attempted to express a scorpion insectotoxin in *E. coli*. These authors used a secretion vector that expressed insectotoxin as a secreted fusion protein into the external medium. However, this peptide did not possess any biological activity because of improper processing at the N-terminus (Pang *et al.*, 1992). In contrast, they also reported that only a trace amount of insectotoxin was detected in bacterial cytosol when expressed from a non-secretion expression vector, suggesting that the recombinant protein was unstable because

of proteolysis in the bacterial cells (Pang *et al.*, 1992). These authors also attempted to express a scorpion insectotoxin in yeast, *S. cerevisiae* and tobacco cells, but the peptides did not show any biological activity.

These are the only reports where other investigators have attempted to express cationic peptides in a bacterial expression system. The procedures followed by all these investigators were similar to those that have been used in this study.

On the other hand, a baculovirus expression system has been successfully used for the heterologous expression of the cationic peptides. Cecropin A has been produced in two different baculovirus expression systems (Andersons *et al.*, 1991; Hellers *et al.*, 1991). Also, β-defensin has been successfully expressed in a baculovirus vector system (Valore *et al.*, 1998). In all cases the recombinant peptides retained activity. From these observations it seems that a baculovirus vector system is by far the most preferred heterologous expression system for the production of recombinant antimicrobial peptides. However, a baculovirus system may not be economically viable for heterologous production of cationic peptides if they are intended for use as therapeutic agents.

b. Purification and characterisation of recombinant fusion proteins of lactoferricins

The poly-His LFcin-B fusion protein expressed in inclusion bodies by the construct ProA+bp was successfully solubilised in the presence of 8 M urea and purified on a metal-chelation (Ni-NTA) column under denaturing conditions. Following purification, the fusion protein could not be refolded by removing urea to obtain a biologically active protein. The fusion protein precipitated during dialysis against all buffer systems and under all the conditions investigated.

The GST-fused LFcin-H & B peptides were solubilised in the presence of 8 M urea and could be bound on to the GST-Sepharose 4B resin under denaturing conditions by reducing the concentration of urea to approximately 3 M. The fusion protein was eluted from the resin using 10 mM reduced glutathione. The elution was not very efficient with the fusion protein as it bound more strongly compared to the unfused GST-moiety. This

could be because of the increase in total basic charge on the fusion protein compared to the unfused GST-moiety. Thrombin digestion of GST-fused LFcin-H & B proteins bound to the glutathione- Sepharose 4B released a peptide similar in size to lactoferricin peptides. However, these thrombin cleaved peptides were not soluble and could not be further characterised.

In conclusion, the lactoferricin H & B DNAs were successfully sub-cloned into three different *E. coli* expression vectors and stably maintained in *E. coli* strains DH5α and BL21 (DE3). All the LFcin-H & B constructs expressed the cloned DNA producing transcripts of the expected size, as shown by northern blot analysis. Many of the constructs, though not all, expressed the recombinant fusion protein as inclusion bodies. The exception was the GST-fused LFcin-H protein expressed by the construct nT1+hH#, which was soluble. The fusion proteins expressed both as inclusion bodies and in soluble form were purified on appropriate affinity resins. However, soluble biologically active lactoferricin H & B peptides could not be obtained for further biochemical and antibacterial studies.

5.1 Introduction

Lactoferrin has been shown to have antimicrobial activity (refer to section 1.3.6 of introduction chapter). Initially, the antimicrobial activity of lactoferrin was attributed to its powerful iron sequestering property, which produces an iron-deficient environment that limits microbial growth. Later lactoferrin was shown to have direct bactericidal activity by binding to sensitive Gram-negative bacteria, damaging their outer membrane by the release of lipopolysaccharide (LPS) and altering the permeability properties of the outer membrane (Ellison III *et al.*, 1988).

Similarly, lactoferricin B, an antimicrobial peptide released from bovine lactoferrin upon acid-pepsin hydrolysis, was shown to have antimicrobial activity against a wide range of Gram-negative and Gram-positive bacteria and against yeasts (Table 1.7) (Bellamy *et al.*, 1992). Yamauchi *et al.,* (1993) reported that lactoferricin B demonstrated consistent bactericidal activity against many Gram-negative bacteria. Transmission electron microscopy of *E. coli* strain CL99 1-2 cells exposed to lactoferricin B showed an immediate development of electron-dense 'membrane blisters'(Yamauchi *et al.*, 1993). Later Shin *et al.,* (1998) studied the antibacterial activity of lactoferricin B on the entero-pathogenic *E. coli* strain 0157:H7. Transmission electron microscopy observations of *E. coli* 0157:H7 exposed to lactoferricin B initially showed some 'membrane blisters' on the surface of the cells. Lactoferricin B treatment ultimately caused alterations in the cytoplasmic contents of the bacterial cell (Shin *et al.*, 1998).

Chapple *et al.,* (1998) showed that synthetic antibacterial peptides, corresponding to the helical surface region of the antibacterial domain of human lactoferrin, exhibited potent antibacterial activity against a range of both Gram-positive and Gram-negative bacteria (Chapple *et al.*, 1998). Transmission electron microscopy of *E. coli* 0111 (NCTC 8007) treated with one of these synthetic peptides revealed bacterial distortion, with the outer membrane becoming detached from the inner cytoplasmic membrane. This effect was

different to that was observed with lactoferricin B by other investigators (Chapple *et al.*, 1998).

The strains of *E. coli* used in these studies (Chapple *et al.*, 1998; Shin *et al.*, 1998; Yamauchi *et al.*, 1993) have different minimum inhibitory concentration (MIC) values for lactoferricin B. *E. coli* strain CL99 1-2 has a MIC of ~13 µg/ml while *E. coli* serotypes 0157 and 0111 were more sensitive with a MIC of 6-8 µg/ml at a cell density of ~$1x10^6$ CFU/ml. Differences in the susceptibility of bacteria to cationic peptides are presumably due to differences in the bacterial cell wall and, in the case of Gram-negative bacteria, differences in the structure of LPS, which affects the interaction between the outer membrane and the peptides.

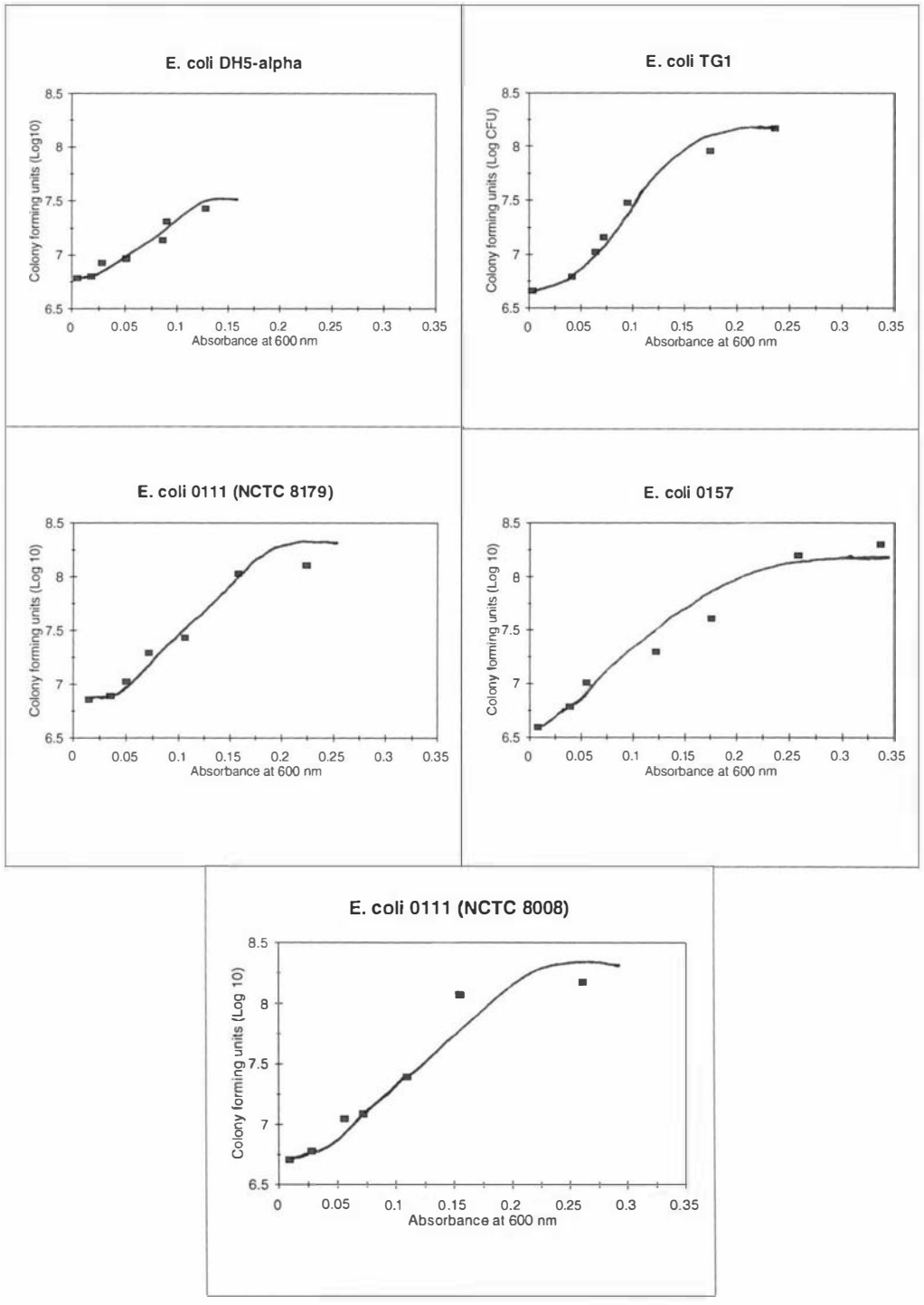
In this study, estimation of the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of native lactoferricin B for five different *E. coli* strains was carried out. Because differences have been reported in the way lactoferricin B interacts with the membranes of different Gram-negative bacteria, we also looked at the ultrastructural changes in the membranes of a very sensitive and a relatively resistant *E. coli* strain upon treatment with native lactoferricin B. The main aim of this study was to eventually use the same methods to characterise recombinant lactoferricins. Unfortunately, soluble recombinant lactoferricins could not be obtained.

5.2 Results

5.2.1 Calibration curves to assess the growth of *E. coli* strains used in this study

Five different *E. coli* strains were selected for this study. They were *E. coli* 0111:K58 (B4):H2 (NCTC 8179, equivalent of NCTC 8007), *E. coli* 0111:K58 (B4):H12 (NCTC 8008), *E. coli* 0157 (food pathogen, New Zealand isolate), *E. coli* DH5α™ (lab strain) and *E. coli* TG1 (lab strain). All these strains had different growth rates when grown on 1% peptone liquid media at 37°C with aeration. To obtain cultures of the different *E. coli* strains at the same initial cell density, calibration curves (OD_{600} vs Log_{10} CFU) were constructed for all these strains during growth on 1% peptone at 37°C as described in section 2.6.1 (Fig 5.1).

Fig. 5.1 Calibration curves (drawn between OD_{600} vs Log_{10} CFU) for the five selected *E. coli* strains



Each data point shown is the mean (± SEM) from triplicate measurements and the standard error for all data points is less than 10% of the mean.

5.2.2 Estimation of native lactoferricin B concentration

Native lactoferricin B was obtained in the form of a lyophilised powder. Stock solutions of native lactoferricin B were prepared in distilled water and filter-sterilised. Lactoferricin B concentration was estimated using the dye-binding assay method as described in section 2.2.3 using BSA as a standard. Then the concentration of the stock solution of native lactoferricin B was adjusted to 1 mg/ml.

5.3 Antibacterial activity of lactoferricin B

The antibacterial activity of cationic peptides has been determined using a number of different assay procedures. Some of them are listed below.

(1) Zones of inhibition of bacterial growth in a thin bacterial plate/lawn
(2) Reduction of colony forming units (CFU) as a loss of viable counts
(3) Reduction of turbidity as recorded with a spectrophotometer

The other ways of assaying the activity of cationic peptides on cell membranes are

(1) Channel formation in artificial membranes
(2) Uncoupling of respiration in mitochondria

The most commonly used and most rigorous measure of antibacterial activity is the reduction of colony forming units (CFU) (as a loss of viable counts). It is however very laborious for routine use. Viable colony count analysis (CFU/ml vs concentration of peptide) typically shows a threshold concentration, below which no effect is seen. This method is insensitive to fewer than a hundred colonies per ml. Variation in the ratio of inoculum size to a specific concentration of antimicrobial peptide would show differences in the rate of killing of the bacterial cells. This is because of the difference in ratios of peptide molecules to bacterial cells and not because of the rapid emergence of resistant organisms. This is in contrast to the situation with conventional antibiotics where resistant organisms arise with high frequency (Yamauchi *et al.*, 1993).

5.3.1 Determination of minimum inhibitory concentration (MIC) of lactoferricin B

Determination of MIC for lactoferricin B for all the selected *E. coli* strains at two different initial inoculum sizes was done using the assay method as described in section 2.6.2.

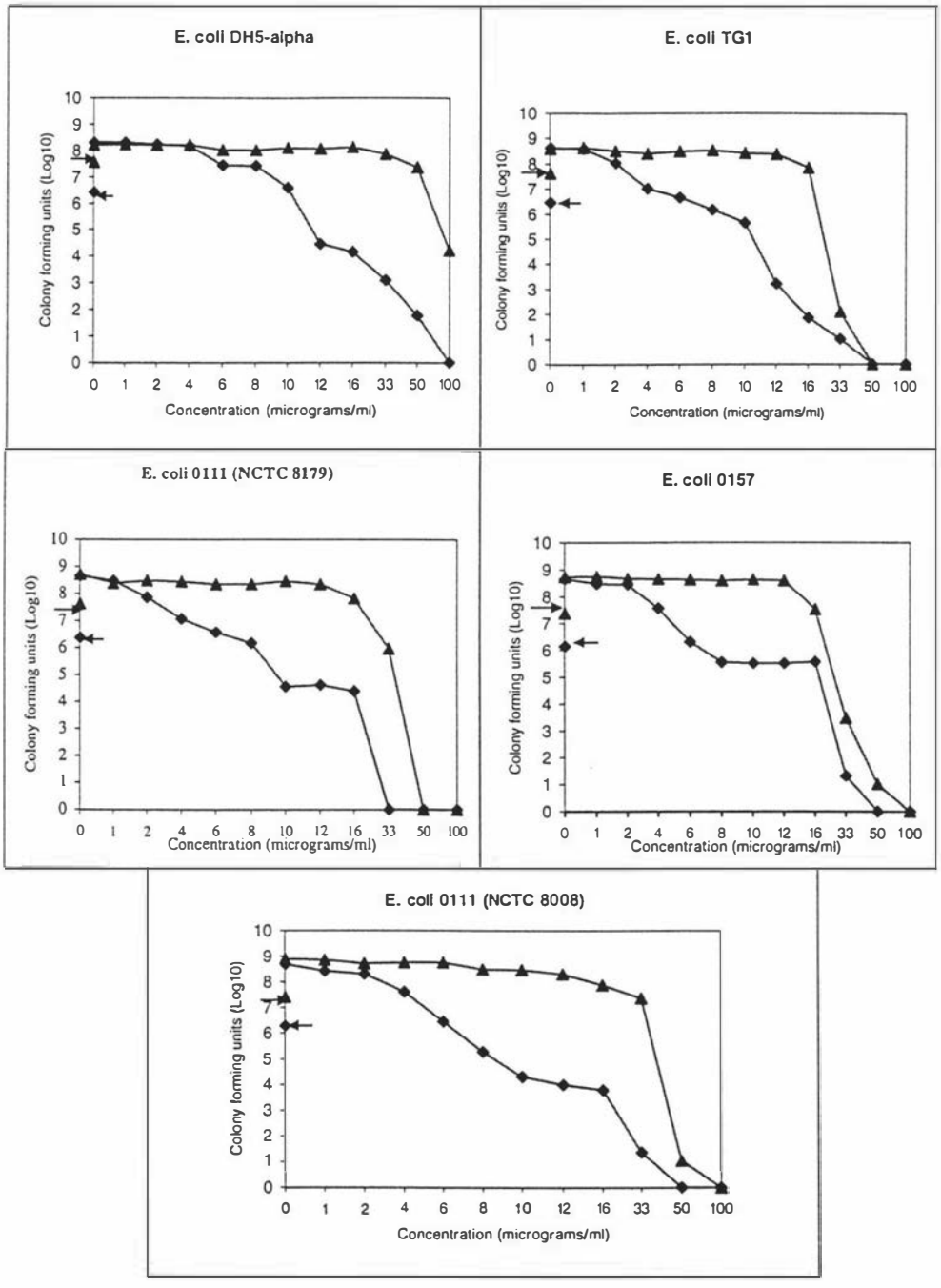
The *E. coli* cultures were grown to the appropriate OD_{600} to obtain initial inoculum sizes of $3x10^6$ CFU/ml and $3x10^7$CFU/ml. The MIC assay for lactoferricin B was performed as described in section 2.6.2. The following are the MIC values obtained for lactoferricin B at these initial cell densities (Table 5.1).

Table 5.1 MIC values of lactoferricin B for the five selected *E. coli* strains grown on 1% peptone media at cell densities of $3x10^6$ CFU/ml and $3x10^7$ CFU/ml

E. coli strain	MIC values of lactoferricin B on 1% peptone (μg/ml)	
	Cell density $3x10^6$ CFU/ml	Cell density $3x10^7$ CFU/ml
DH5α	10-12	33-50
TG1	6-8	16
0111 (NCTC 8179)	6-8	16
0157	6-8	16
0111 (NCTC 8008)	6-8	16-33

Fig 5.2 gives the antibacterial assay curves for the five *E. coli* strains at cell densities of $3x10^6$ CFU/ml and $3x10^7$ CFU/ml from which the MIC values shown above were determined.

Fig. 5.2 Antibacterial assay curves of *E. coli* strains grown on 1% peptone at initial cell densities of $3x10^6$ CFU/ml and $3x10^7$ CFU/ml to determine MIC values of lactoferricin B



Each data point shown is the mean (± SEM) from triplicate determination and the standard error for all data points is less than 10% of the mean. Initial cell densities are indicated by arrows on Y-axis.

Key for cell densities (♦) → $3x10^6$ CFU/ml and (▲) → $3x10^7$ CFU/ml.

5.3.2 Time-kill curves of lactoferricin B

The assessment of bactericidal activity was done by time-kill studies for up to 24 hrs of incubation at 37°C with lactoferricin B and is expressed as the minimum bactericidal concentration (MBC). Selected *E. coli* strains were tested at two different initial inoculum sizes for their killing-rate by various concentrations of lactoferricin B for the first 3 hrs and at 24 hrs of incubation using the method described in section 2.6.3.

The aim was to determine the differences in the rate of killing for the first 3 hrs and the respective MBC values after 24 hrs of incubation at 37°C for the entire five different test strains. This was carried out at two initial inoculum sizes and at different lactoferricin B concentrations. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of lactoferricin B that resulted in at least 99.9% killing after 24 hrs of incubation at 37°C when compared to the culture where no lactoferricin B was added.

Each of the *E. coli* cultures was grown to the appropriate OD_{600} to obtain initial inoculum sizes of $3x10^6$ CFU/ml and $3x10^7$ CFU/ml as described in section 5.2.1. The time-kill studies were performed as described in section 2.6.3.

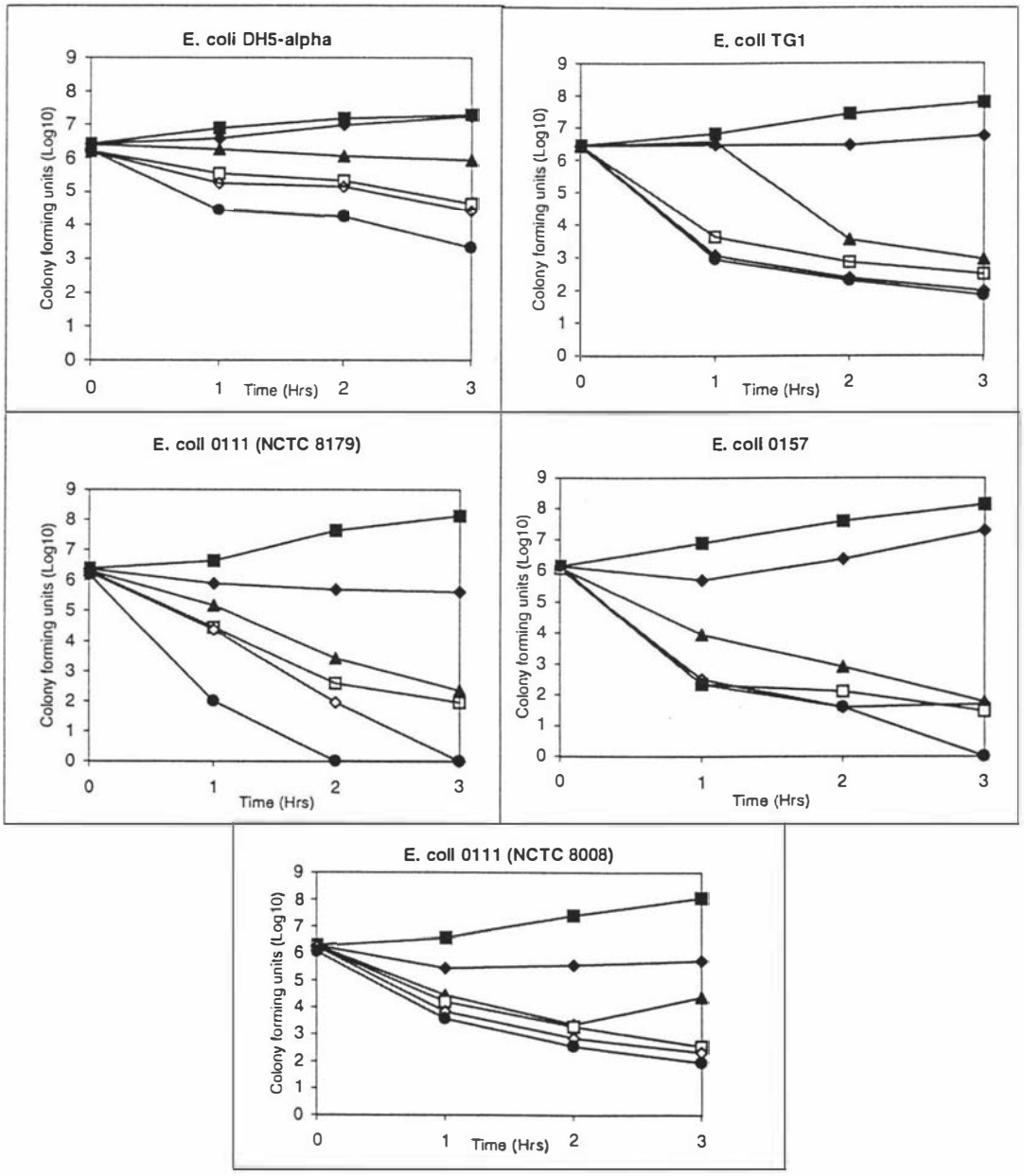
Table 5.2 MBC values of lactoferricin B for the five *E. coli* strains at $3x10^6$ CFU/ml and $3x10^7$ CFU/ml cell densities

E. coli strain	MBC of lactoferricin B on 1% peptone (μg/ml)	
	Cell density $3x10^6$ CFU/ml	Cell density $3x10^7$ CFU/ml
DH5α	100	>100
TG1	33-50	50
0111 (NCTC 8179)	33	50
0157	50	100
0111 (NCTC 8008)	50	50-100

Table 5.2 gives the MBC values of lactoferricin B for the five E. coli strains at $3x10^6$ CFU/ml and $3x10^7$ CFU/ml after 24 hrs of incubation at 37°C.

Fig. 5.3 and Fig. 5.4 represent the Time-kill curves of lactoferricin B for the five selected *E. coli* strains at initial inoculum sizes of $3x10^6$ CFU/ml and $3x10^7$ CFU/ml respectively in the first 3 hrs of incubation at 37°C.

Fig. 5.3 **Time-kill curves of lactoferricin B for the five selected *E. coli* strains at $3x10^6$ CFU/ml cell density**

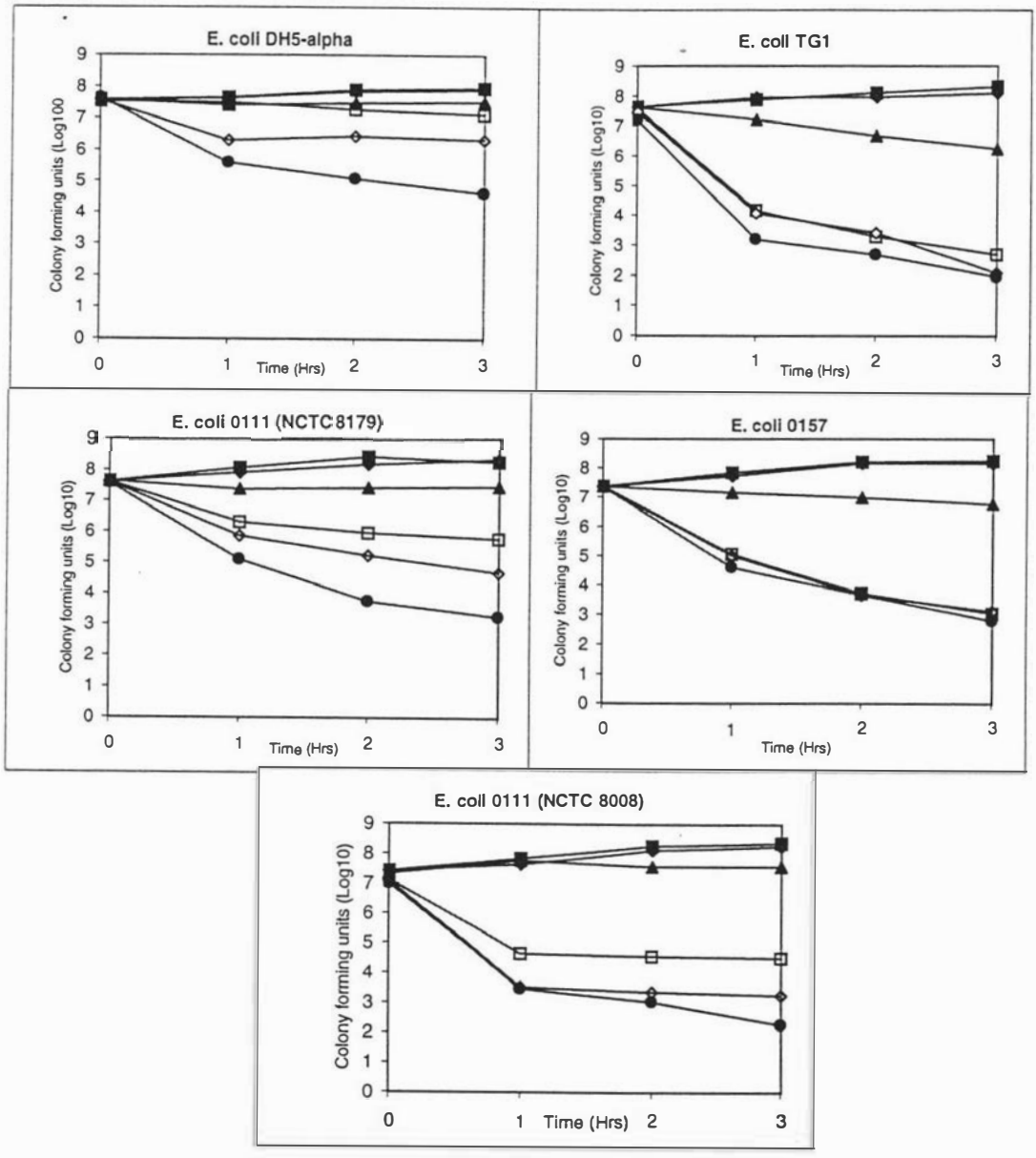


Legend for lactoferricin B concentrations

(■)	0 μg/ml	(♦)	1 μg/ml	(▲)	10 μg/ml
(□)	33 μg/ml	(◇)	50 μg/ml	(●)	100 μg/ml

Each data point shown is the mean (± SEM) from triplicate determination and the standard error for all data points is less than 10% of the mean.

Fig. 5.4 **Time-kill curves of lactoferricin B for the five selected *E. coli* strains at $3x10^7$ CFU/ml cell density**



Legend for lactoferricin B concentrations

(■)	0 μg/ml	(♦)	1 μg/ml	(▲)	10 μg/ml
(□)	33 μg/ml	(◇)	50 μg/ml	(●)	100 μg/ml

Each data point shown is the mean (± SEM) from triplicate determination and the standard error for all data points is less than 10% of the mean.

5.4 Transmission electron microscopy (T.E.M.) studies of selected *E. coli* strains

To visualise changes in the membranes of two different strains of *E. coli* treated with lactoferricin B, both untreated cells and cells treated with lactoferricin B for different time periods were examined by transmission electron microscopy (T.E.M.). The MIC (Table 5.1) and MBC (Table 5.2) studies of lactoferricin B on five different selected *E. coli* strains showed that *E. coli* lab strain DH5α was less susceptible compared to pathogenic *E. coli* strain 0111 to lactoferricin B at the same cell density and concentration of lactoferricin B. So, we decided to check the response of these two strains at the ultrastructural level by T.E.M. upon exposure to lactoferricin B for different time intervals.

An initial concentration of 100 μg/ml of lactoferricin B was used at a cell density of $2x10^9$ CFU/ml. Both strains showed considerable killing in 30 min at this concentration of lactoferricin B and the viable count was reduced to $2x10^3$ CFU/ml. T.E.M. studies of treated cells showed some 'membrane blisters' and outer membrane dislocation but mostly just cell debris. In this experiment, only 0.5 ml of the untreated and treated cell suspensions was used for processing the samples for T.E.M. The number of cells was quite low and limited the number of observations which could be made.

We therefore decided to examine the effect of lactoferricin B at 33 μg/ml, which was sub-bactericidal for *E. coli* strain 0111 (NCTC 8179) at a cell density of $1x10^8$ CFU/ml (Table 5.2). The same concentration of lactoferricin B (33 μg/ml) and initial cell density was also used for *E. coli* strain DH5α. The volume of the treated and untreated cell suspensions was increased to 7 ml to facilitate handling and to provide more material.

5.4.1 T.E.M. studies of lactoferricin B treated *E. coli* strain DH5α

As shown by the MIC and time-kill curve analyses, *E. coli* strain DH5α was the least susceptible to lactoferricin B (Tables 5.1 & 5.2). A culture of *E. coli* strain DH5α was taken to give an initial cell density of $1x10^8$ CFU/ml as enumerated by 10-fold serial dilution plating as described in section 2.6.1. Lactoferricin B was added to the culture at a concentration of 33 μg/ml, incubated at 37°C and samples were collected at zero, 15 min, 30 min, 60 min and 90 min time intervals. By 90 min, the viable cell count had dropped to $4.4x10^7$ CFU/ml. All these samples were processed to produce sections as described in section 2.7. These were observed by transmission electron microscopy as described in section 2.7.2. Fig. 5.5 gives a comparison of the changes in the membranes of the treated and treated cells at different time intervals as observed using T.E.M.

Fig. 5.5 T.E.M. pictures of *E. coli* DH5 α cells treated for different time intervals with lactoferricin B

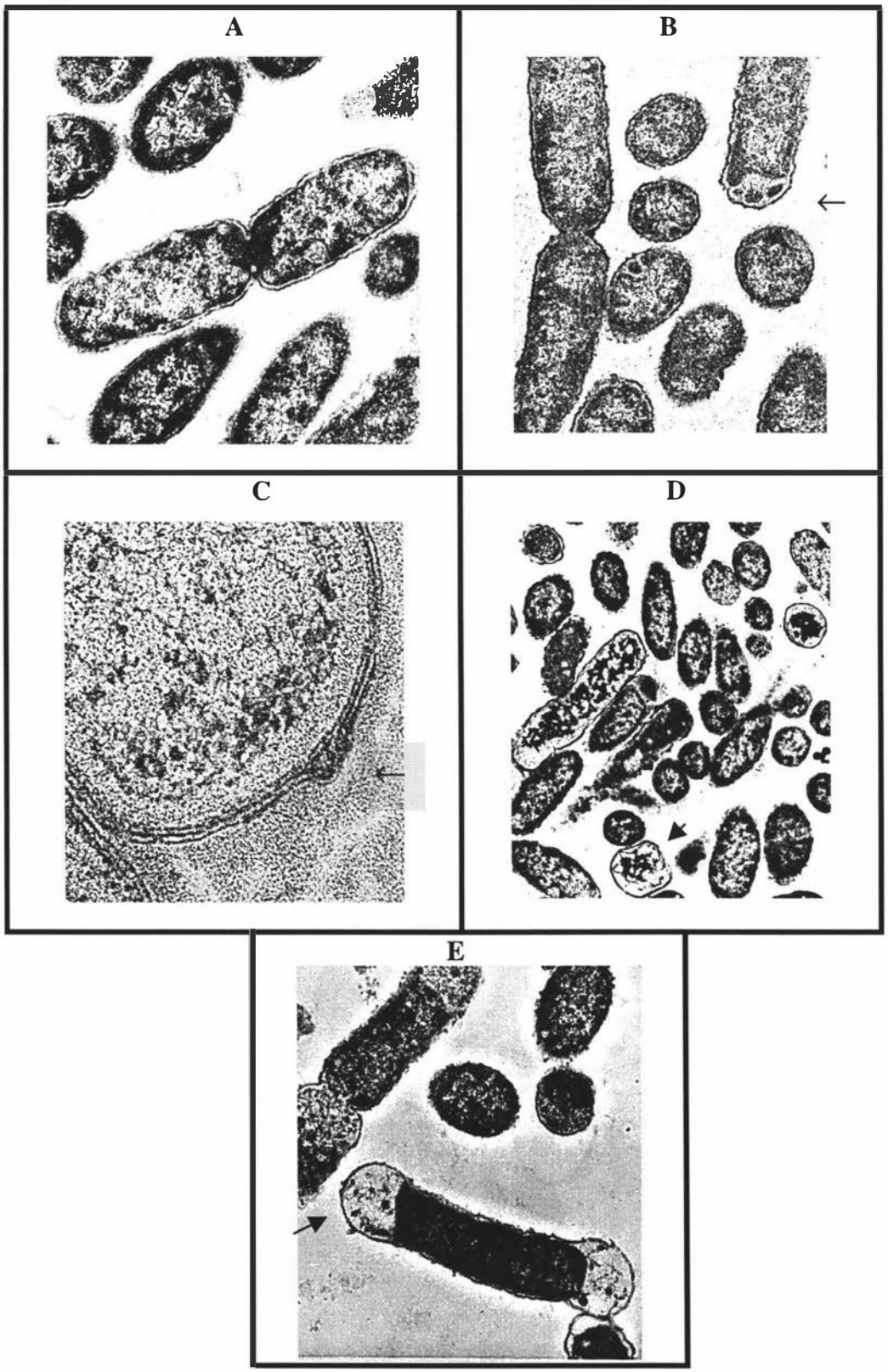


Fig. 5.5. Transmission electron micrographs of E. coli DH5α

(A) control, not treated with lactoferricin B (magnification, 31,800 x).

DH5α cells treated with lactoferricin B and samples taken (B) at 15 min (magnification, 31,800 x) *(C) at 30 min* (magnification, 31,800 x) *(D) at 60 min* (magnification, 15,300 x) *and (E) at 90 min* (magnification, 21,200 x). (→ indicates 'membrane blisters' and ➞ indicates the membrane dislocation).

5.4.2 T.E.M. studies of lactoferricin B treated *E. coli* strain 0111 (NCTC 8179)

E. coli strain 0111 was the most susceptible organism to lactoferricin B when compared to the other tested *E. coli* strains, as shown from the MIC and time-kill curve analyses (Tables 5.1 & 5.2). Lactoferricin B was added at a concentration of 33 μg/ml to the culture ($1x10^8$ CFU/ml) and incubated at 37°C. Samples were collected at zero, 15 min and 30 min. By 30 min the viable cell count had dropped to $2.5x10^7$ CFU/ml. All these samples were processed for making sections for T.E.M. studies as described in section 2.7. Fig. 5.6 gives a comparison of the changes in the membranes of the treated cells at different time intervals as observed using T.E.M.

Fig. 5.6 **Comparison of the changes in the membranes of the untreated and treated *E. coli* 0111 (NCTC 8179) cells at different time intervals (T.E.M. pictures)**

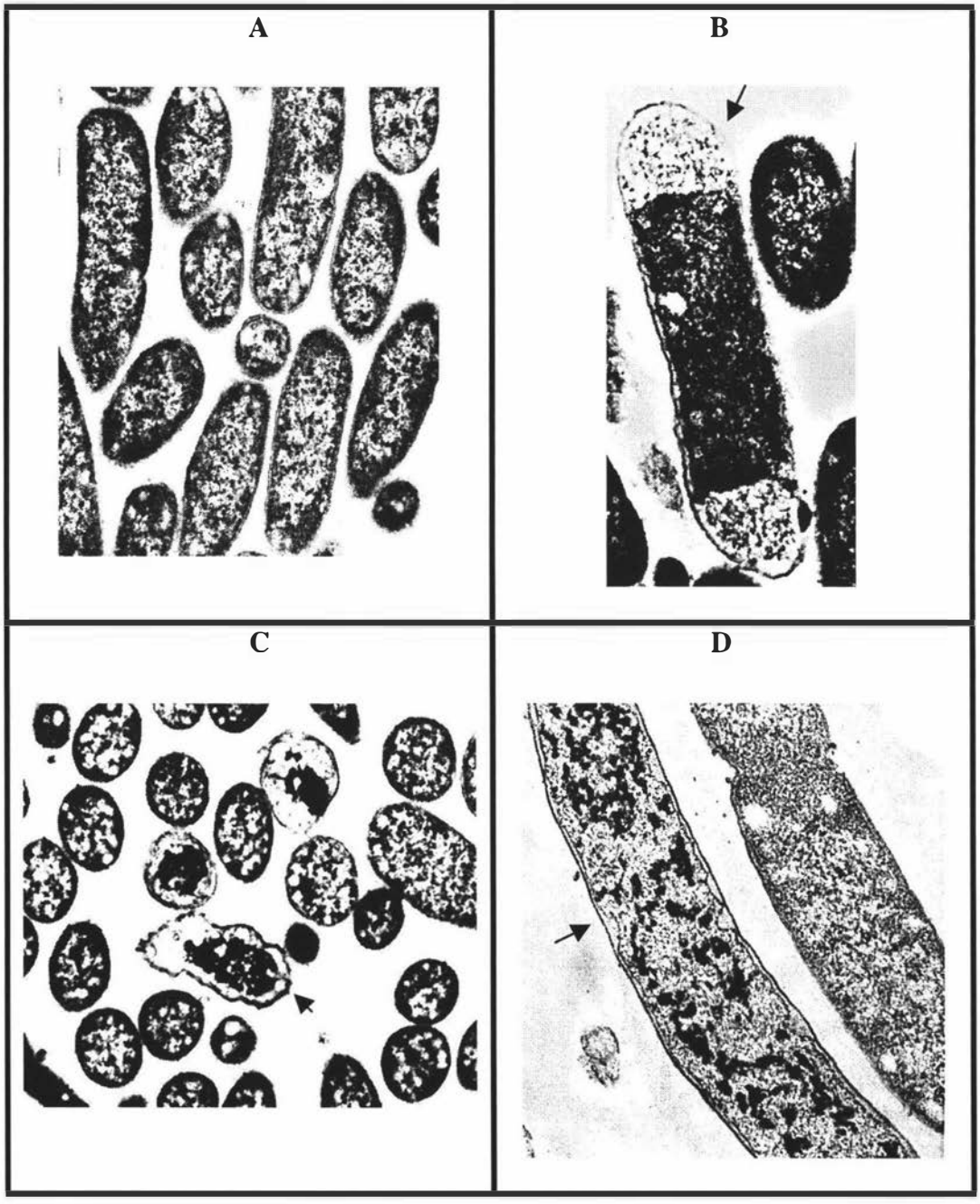
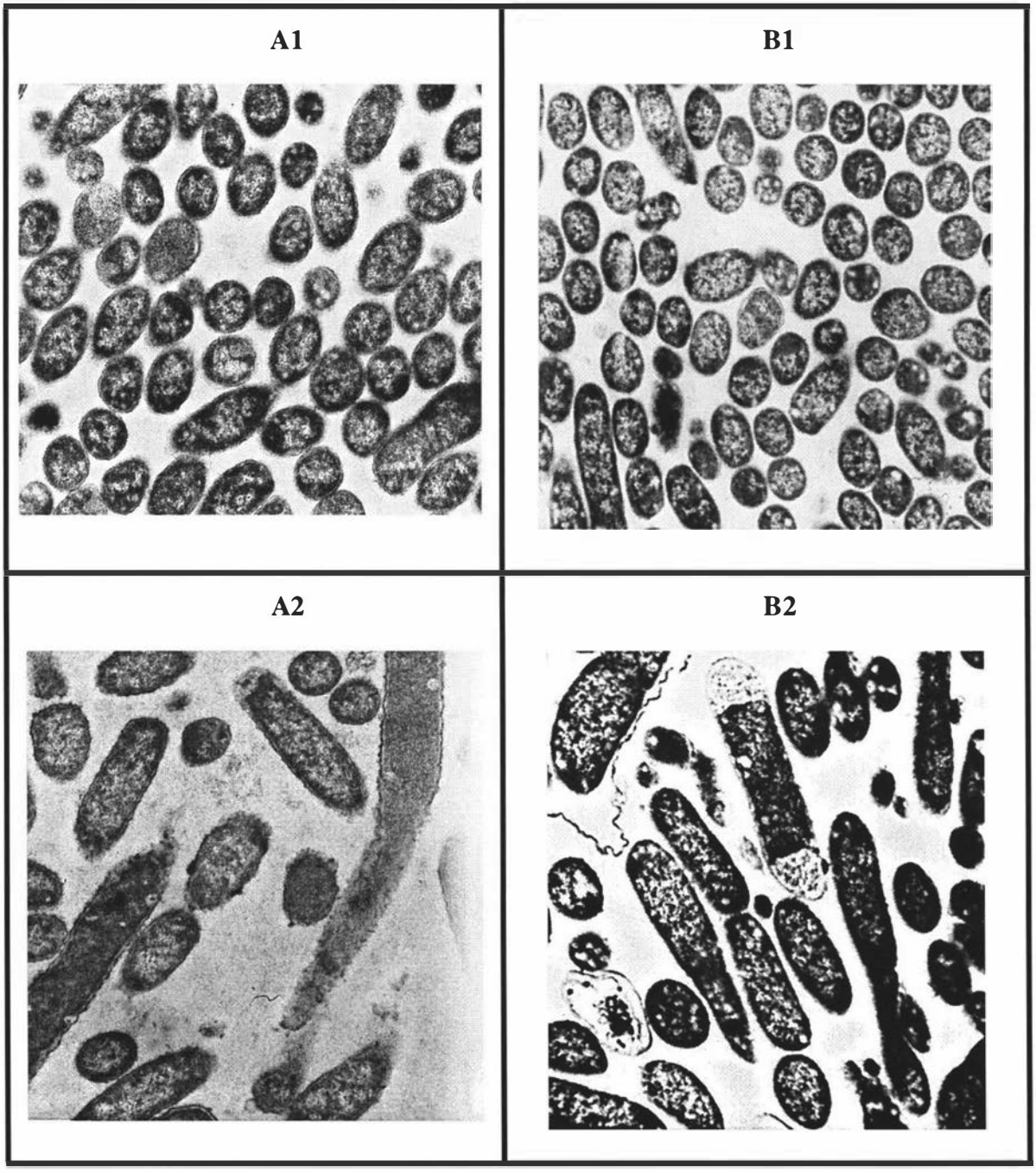


Fig. 5.6. *Transmission electron micrographs of E. coli 0111* (NCTC 8179). *(A) control, not treated with lactoferricin B* (21,200 x). *E. coli 0111 cells treated with lactoferricin B and samples taken (B) at 15 min* (magnification, 31,800 x) *(C) at 30 min* (magnification, 14,300 x) *and (D) at 30 min* (magnification, 48,600 x). (→ indicates dislocation of cell membranes)

5.4.3 Comparison of the T.E.M. pictures of *E. coli* DH5α and 0111 at the same magnification

To show the differences between the lactoferricin B treated *E. coli* strains DH5α and 0111 transmission electron micrographs for the same treatment time and magnification (15,300 x) were compared. These results are shown in Fig. 5.7

Fig 5.7 T.E.M. pictures of *E. coli* strains DH5α and 0111 at the same magnification (15,300 x)



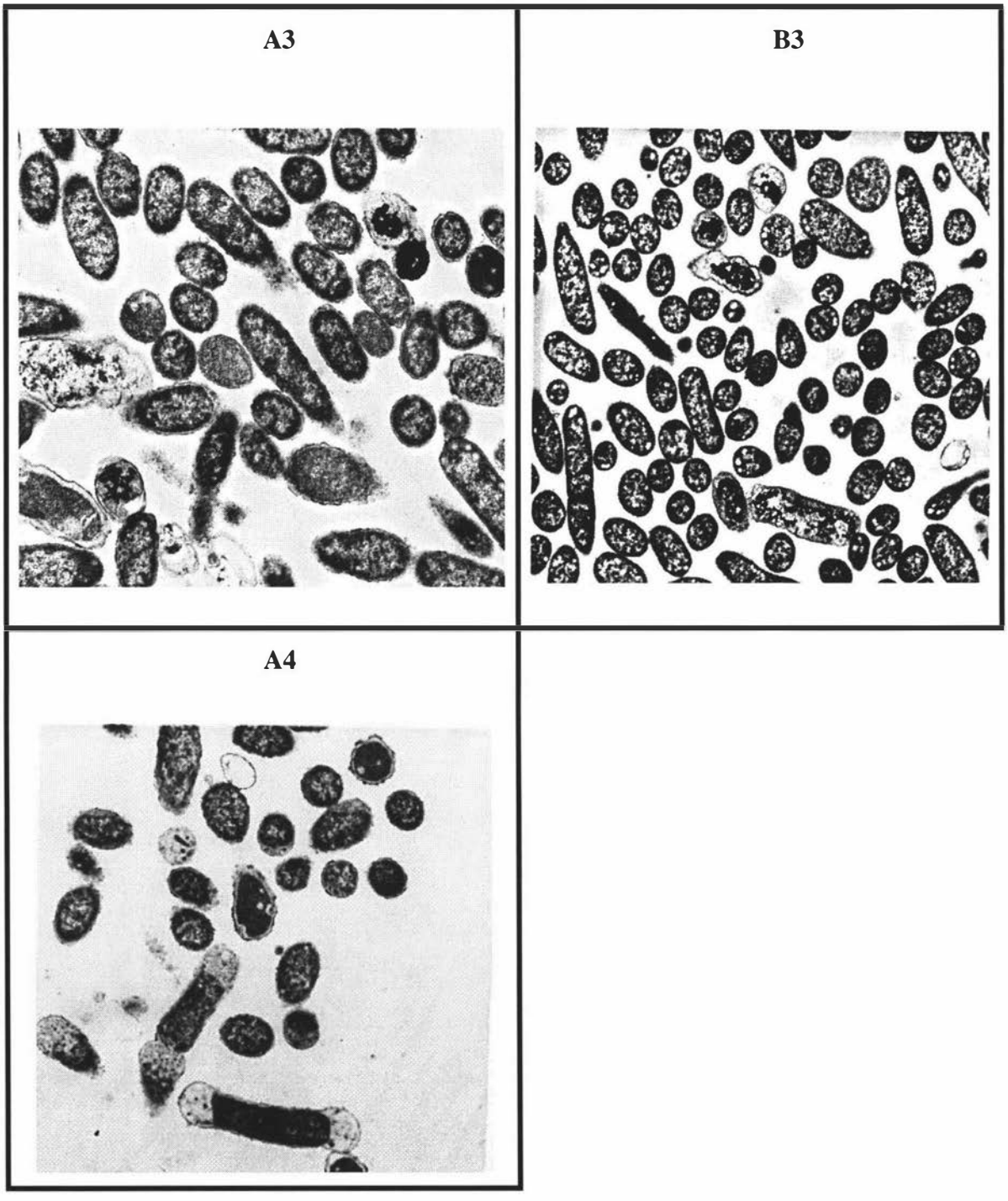


Fig. 5.7 Transmission electron micrographs of E. coli DH5α. (A1) control, not treated with lactoferricin B and cells treated with lactoferricin B and samples taken (A2) at 15 min (A3) at 30 min (A4) at 90 min and of E. coli 0111 (B1) control, not treated with

lactoferricin B and cells treated with lactoferricin B and samples taken (B2) at 15 min (B3) at 30 min (all T.E.M. pictures were taken at a magnification, 15,300 x*).*

5.5 Discussion and conclusions

The five selected *E. coli* strains showed different growth rates on 1% bacto-peptone and different susceptibility towards lactoferricin B as assessed by the MIC and Time-kill curve assays. The pathogenic *E. coli* strains 0111 and 0157 were more susceptible to lactoferricin B. Whereas, the lab *E. coli* strain DH5α showed more resistance towards lactoferricin B when compared to the pathogenic strains *E. coli* 0111 and 0157, which were quite susceptible to lactoferricin B. *E. coli* strain TG1 showed susceptibility to lactoferricin B closer to the pathogenic strains. This difference could be clearly seen with the differences in the MIC and MBC values for lactoferricin B for these strains (Tables 5.1 and 5.2). *E. coli* strain DH5α had higher MIC and MBC values of lactoferricin B when compared to *E. coli* strain 0111 (NCTC 8179) at both initial cell densities used for the antibacterial assays.

The transmission electron microscopy pictures of *E. coli* DH5α (a relatively resistant strain) showed considerable differences (Fig. 5.7) when compared to *E. coli* 0111 (NCTC 8179) (a very sensitive strain) cells (Fig. 5.8). *E. coli* DH5α cells showed 'membrane blisters' even after 30 min exposure to lactoferricin B and eventually showed membrane dislocation at 90 min. Whereas, *E. coli* 0111 showed membrane dislocation in the first 15 min exposure without the formation of any 'membrane blisters'. Lactoferricin B subsequently effected the cytoplasmic content of the cells. There was a progressive increase in the formation of cytoplasmic debris. The formation of 'membrane blisters' may be the response of a relatively resistant strain showing less susceptibility towards lactoferricin B. Ultrastructural differences between strains of *E. coli* with differing susceptibility to lactoferricin B have not been addressed by earlier workers.

We conclude that lactoferricin B has a selective interaction with different strains of *E. coli* depending upon the cell wall structure which confers on them differential susceptibility towards lactoferricin B. This has been shown with both antibacterial assays and transmission electron microscopy studies.

6.1 General discussion

Lactoferrin, a protein present in the milk and other secretory fluids of many, but not all mammalian vertebrates has been implicated in many biological functions. There have been a number of studies on the heterologous expression of human lactoferrin (hLF), an 80 kDa secretory protein. Full length human lactoferrin and a number of variants have been successfully expressed in baby hamster kidney (BHK) cells and have been well characterised by both iron-binding and structural studies (Day *et al.*, 1992; Stowell *et al.*, 1991). Full-length human lactoferrin has also been produced in a number of strains of *Aspergillus* (Ward *et al.*, 1992a; Ward *et al.*, 1992; Ward *et al.*, 1995) and with limited success in the yeast *Saccharomyces cerevisiae* (Liang and Richardson, 1993). Recently, heterologous expression of N-terminal deletion mutants of bovine lactoferrin in *E. coli* has been reported (Maheswari *et al.*, 1998).

6.2 Expression of full-length human lactoferrin (hLF) in *Kluyveromyces lactis*

The main aim at the start of this project was to express full-length human lactoferrin (hLF) in the yeast *Kluyveromyces lactis*. *K. lactis* was chosen for this work because yeasts of this genus have been used for many years in the food industry and are classified as "Generally Regarded As Safe" (GRAS) organisms. *S. cerevisiae* has been the preferred yeast host for the production of heterologous proteins due to its well-characterised molecular biology and genetics. However, several problems have been encountered with this host, such as the lack of very strong, tightly regulated promoters and hyperglycosylation of proteins (Rallabhandi and Yu, 1996a). In addition, *K. lactis* has the ability to grow on cheap substrates, such as lactose and whey, which are abundantly available in New Zealand as by-products of the cheese and casein industries. For this reason *K. lactis* is ideal for the production of low value heterologous proteins. In New Zealand, ethanol production from whey using yeasts of the genus *Kluyveromyces* is a well-established technology (Mawson, 1987).

Many attempts were made to sub-clone the hLF cDNA into the unique *SacI* site of the *K. lactis* expression vector, pEPS1 (Macreadie *et al.*, 1993) and the *S. cerevisiae* expression vector, pYEXS1 (Castelli *et al.*, 1994) and to establish the constructs in *E. coli*. All these attempts were unsuccessful. The difficulty in establishing stable insert-carrying clones in *E. coli* proved to be a major stumbling block in this project. The group, which developed the pEPS1 vector for *K. lactis* and the pYEXS1 vector for *S. cerevisiae* also encountered difficulties in establishing stable clones carrying the β-lactamase gene in *E. coli* (Macredie, I.G., personal communication). These workers transformed yeast *K. lactis* with the ligation mix, obtained transformants on selective media and characterised them by standard assay methods for β-lactamase activity. Similarly, we also electroporated the ligation mix directly into *K. lactis* and selected for transformants. Transformants secreting full-length human lactoferrin or its fragments were obtained as assessed by the lactoferrin ELISA assay (Vilja *et al.*, 1985). Expression of the recombinant protein in *K. lactis* continued for several transfers but, gradually fell below the detection limits of the lactoferrin ELISA both with the working liquid cultures at 4°C and glycerol stocks stored at -70°C by. The process of ligation, electroporation into *K. lactis* and screening for transformants by the lactoferrin ELISA was repeated several times, but no transformants that constantly expressed recombinant hLF were obtained. Southern blot analysis of the plasmid DNA isolated from recombinant *K. lactis* did not show the presence of the insert when probed with hLF cDNA. As this process was becoming quite unpredictable and very laborious, it was abandoned. There are no known reports on the use of these yeast vectors for the heterologous expression of any other mammalian proteins.

6.2.1 Expression studies of the N-terminal lobe of hLF cDNA in *E. coli*

The N-terminal lobe of hLF cDNA was expressed in *E. coli* as GST-and poly-His fusion proteins. Both fusion proteins were produced in *E. coli* as inclusion bodies. The inclusion bodies produced by both of these constructs were isolated, solubilised in 8 M urea and analysed on 10% SDS-PAGE gels. Further purification and characterisation of these fusion proteins was not carried out. Attempts to express the full-length hLF cDNA in *E. coli* in our laboratory have proven to be unsuccessful (Tweedie, J.W., personal communication).

6.3 Heterologous expression of human and bovine lactoferricins in *E. coli*

All attempts to express the full-length hLF cDNA and its variants in yeast and *E. coli* expression systems were unsuccessful. Because of this, the objective of the work became the heterologous expression of synthetic DNA fragments for the antimicrobial domain of both human and bovine lactoferrins in *E. coli*, using commercially available expression vectors.

Gene-encoded antimicrobial peptides have been found in many species of animals, plants and insects. These natural antimicrobial peptides have created considerable interest because of their potential as therapeutic agents in place of conventional antibiotics (Hancock and Lehrer, 1998). The use and misuse of conventional antibiotics has led to the development of plasmid-borne antibiotic resistance genes, which pose an increasing threat to the future medical use of these antibiotics. To date, there does not appear to be any development of resistance to the antimicrobial activity of these peptides. Since these antimicrobial peptides are small, ranging between 15 and 35 amino acid long, the complementary DNA that encodes them could be synthesised as overlapping oligonucleotides, to enable the introduction of the restriction sites of choice. This synthetic DNA could then be sub-cloned into commercially available expression vectors.

Overlapping synthetic oligonucleotides for both human and bovine lactoferricin DNA with three different 3′-ends were designed with restriction sites for sub-cloning into commercially available *E. coli* expression vectors. Both human and bovine lactoferricin peptides were expressed in *E. coli*, though not by all constructs, as poly-His or GST-fusion proteins.

The constructs with LFcin-H DNA in *E. coli* expression vector pGEX4T-1, expressed the GST-fusion proteins either as inclusion bodies (nT1+hH) or in a soluble form (nT1+hH#). The other constructs of LFcin-H DNA in the vectors pPROEXHTa and pET-15b (ProA+hH, ProA+hH#, ProA+mhH, nET+hH and nET+hH#) did not yield any protein product.

The construct with LFcin-B DNA in the pPROEXHTa vector (ProA+bp) expressed poly-His fusion protein in inclusion bodies. The constructs with LFcin-B DNA in the pGEX4T-1 vector (nT1+bp and nT1+bp#) expressed the GST-fusion proteins as inclusion bodies. The other constructs of LFcin-B (ProA+bp#, ProA+mbp, nET+bp and nET+bp#) did not produce any protein product.

6.3.1 Northern blot analysis of LFcin-H & B constructs

Since only some, but not all constructs of LFcin-H & B in different *E. coli* expression vectors expressed the recombinant proteins, their expression pattern was examined at the transcription level. IPTG induced cultures of all the constructs of LFcin-H & B (except ProA+mhH and ProA+mbp) were studied for the expression of cloned DNA at the transcription level by northern blot analysis. The northern blots showed the production of transcripts of the expected size by the IPTG induced cultures of all these constructs.

All the constructs of LFcin-H & B, that expressed the protein product, produced the recombinant proteins as inclusion bodies, except the LFcin-H construct in pGEX-4T1 vector (nT1+hH#), which produced a soluble GST-fusion protein. However, all GST-fusion proteins of both LFcin-H & B constructs in the pGEX-4T1 vector were co-expressed with the unfused GST-moiety whether expressed as inclusion bodies or in a soluble form. Piers *et al.,* (1993) have also reported the presence of the unfused GST-moiety along with the GST-fusion proteins when human neutrophil peptide-1 (HNP-1) was expressed as a GST-fusion in *E. coli* (Piers *et al.*, 1993).

6.3.2 Degradation of recombinant protein inside the *E. coli* cell by proteolysis

Proteolysis is a selective and a highly regulated process that plays an important role in cellular physiology. *E. coli* contains a large number of proteases that are localised in the cytoplasm, the periplasm, and the inner and outer membranes (Goldberg and Goff, 1986). These proteolytic enzymes participate in a host of metabolic activities, including the selective and efficient removal of abnormal proteins such as those that might be expressed by a cloned foreign DNA. To date, the mechanisms of protein degradation are poorly

understood and it is unlikely that all proteolytic pathways or enzymes operating in *E. coli* have been identified as yet (Makrides, 1996).

Proteolysis inside the *E. coli* cell might explain the absence of a protein product in many of the LFcin-H & B constructs unless there was a problem with the expression at the translational level. The northern analysis showed that there was no problem at the transcription level. A problem with the expression of cloned DNA at the translational level *in vivo* is hard to prove experimentally and difficult to distinguish from loss of protein by proteolysis. Proteolysis might also explain the co-expression of unfused GST-protein along with the GST-fusion product (Piers *et al.*, 1993).

There have been very few reports on the expression of antimicrobial peptides in *E. coli* (Pang *et al.*, 1992; Piers *et al.*, 1993; Zhang *et al.*, 1998) and most of the attempts have been unsuccessful. This might be because of the cationic and antimicrobial nature of these peptides. There have been many approaches suggested for the bacterial expression of antimicrobial peptides (Piers *et al.*, 1993; Zhang *et al.*, 1998) but, these approaches are specific for particular antimicrobial peptides and may not be generally applicable.

6.3.3 Production of inclusion bodies in *E. coli*: Problems and prospects

In this study, most of the protein-expressing constructs of LFcin-H & B produced the protein product as inclusion bodies. In spite of the extensive knowledge of the genetics and molecular biology of *E. coli*, not every gene can be expressed efficiently in this organism. This may be due to the unique and subtle structural features of the gene sequence, the stability and translational efficiency of the mRNA, the ease of protein folding, degradation of the protein by host cell proteases, major differences in the codon usage between the foreign gene and native *E. coli* and the potential toxicity of the protein to the host (Makrides, 1996). The main drawback is that many, but not all, proteins expressed in *E. coli* accumulate intracellularly in the form of inclusion bodies from which biologically active protein must be recovered by denaturation and refolding *in vitro* (Hockney, 1994). From a vast number of studies carried out with the heterologous expression of different genes in *E. coli*, it has been concluded that inclusion body formation is the rule rather than

the exception (Marston, 1986). A statistical analysis of the composition of 81 proteins that do and do not form inclusion bodies in *E. coli* showed that there are six parameters that are correlated with inclusion body formation. They are average charge, fraction of turn-forming residues, cysteine content, proline content, hydrophilicity and the total number of residues (Wilkinson *et al.*, 1991). Although there are some advantages in producing recombinant proteins as inclusion bodies, the downstream process of denaturation and *in vitro* refolding into biologically active conformation is very empirical and frustrating. Conventional protein-folding protocols are, in most cases, not applicable for the efficient reconstitution of inclusion bodies into biologically active proteins (Rudolf and Lilie, 1996).

Recently, it has been shown that the efficient post-translational folding of proteins, the assembly of polypeptides into oligomeric structures and the localisation of proteins are mediated by specialised host proteins termed molecular chaperones, such as the GroES and GroEL proteins in *E. coli* (Becker and Craig, 1994; Clarke, 1996; Ellis and Hartl, 1996; Goloubinoff *et al.*, 1989). Several of these chaperones are heat-shock proteins whose synthesis is induced in response to stress. So, attempts have been made to produce recombinant proteins in a soluble form by co-expression of the *E. coli* host chaperones, GroESL. Although, the exact mechanism of chaperone-assisted protein folding is still under debate, it seems clear that these proteins assist folding by preventing unproductive side reactions such as aggregation (Rudolf and Lilie, 1996). However, the experimental results from the use of chaperones have been inconsistent and hence the effects of chaperone co-production on gene expression in *E. coli* appear to be protein specific (Wall and Pluckthun, 1995). It has also been shown that the co-production of thioredoxin in *E. coli* caused a dramatic increase in the solubility of eight vertebrate proteins whereas, the co-production of GroESL chaperones increased the solubilities of only four of those proteins (Yasukawa *et al.*, 1995).

6.4 Solubilisation and purification of recombinant proteins

Poly-His fusion protein expressed by the LFcin-B construct (ProA+bp) and the GST-fusion proteins expressed by the LFcin-H & B constructs (nT1+bp, nT1+bp# and nT1+hH) as inclusion bodies (except for the nT1+hH# construct, which produced a soluble

protein) were solubilised in urea and purified on affinity resins. All attempts to refold the purified recombinant proteins into their active form by removing the urea were unsuccessful. Although lactoferricin peptides could be cleaved from the GST-fusion proteins bound on to the resin by using a site-specific protease, the peptides were not soluble after cleavage from the fusion partner. This is not uncommon for peptides that are cationic and antimicrobial in nature. Observations similar to the ones presented in this study were also made by other investigators working with the heterologous expression of cationic peptides in bacterial systems (Piers *et al.*, 1993).

The failures with the expression of cationic peptides in *E. coli* suggest that this organism might not be a suitable host for the expression of these peptides. Alternative hosts such as yeasts, mammalian or insect cells might offer a better opportunity to avoid the problems of folding and the formation of inclusion bodies. Successful expression of biologically active cationic peptides in the baculovirus expression system has been reported (Andersons *et al.*, 1991; Hellers *et al.*, 1991; Valore *et al.*, 1998).

6.5 Antibacterial assays of lactoferricin B

Five different *E. coli* strains selected for this study showed variations in their susceptibility to lactoferricin B. The laboratory strain *E. coli* DH5α consistently showed more resistance towards lactoferricin B compared to the other strains of *E. coli*, as revealed by the MIC and MBC values. This could be because of differences in the bacterial cell wall and especially in the case of these Gram-negative bacteria, differences in the structure of the lipopolysaccharide (LPS), which has been suggested to act by preventing interaction between the bacterial outer membrane and lactoferricin B (Chapple *et al.*, 1998b). The variations in the MIC and MBC values of lactoferricin B for these strains might correlate with differences in the structure of their cell walls. An understanding of how lactoferricin B is positioned within the lipid bilayer would help in elucidating its bactericidal mechanism.

6.6 Transmission electron microscopy studies of lactoferricin B treated *E. coli* cells

The transmission electron microscopy work was initiated with native lactoferricin B with the hope that the same methods could be used with recombinant lactoferricin peptides. Unfortunately, the T.E.M. studies could not be extended to the recombinant lactoferricins, because they were not obtained in a soluble form.

Transmission electron microscopy (T.E.M.) studies have shown considerable changes in the outer membrane structure of lactoferricin B treated *E. coli* strains DH5α (a relatively resistant strain) and 0111 (NCTC 8179) (a very susceptible strain) at the ultrastructural level. This might be related to the differences in the susceptibility of these two strains to lactoferricin B and in their LPS structure. Lactoferricin B treated *E. coli* DH5α cells mostly showed the initial formation of 'membrane blisters' and eventually membrane dislocation and alterations in the cytoplasmic contents in some cells after longer exposure to lactoferricin B. Formation of 'membrane blisters' and alterations in the cytoplasmic contents have also been observed with the T.E.M. studies of lactoferricin B treated *E. coli* CL99 1-2 cells (Yamauchi *et al.*, 1993) and entero-pathogenic *E. coli* strain 0157:H7 (Shin *et al.*, 1998).

On the other hand, lactoferricin B treated *E. coli* 0111 (NCTC 8179) cells did not show the formation of any 'membrane blisters' and instead showed a direct membrane dislocation and ultimately, an effect on the cytoplasmic contents. This may suggest that in the case of *E. coli* 0111 (NCTC 8179, an equivalent of NCTC 8007), lactoferricin B not only bound to the surface of the cells but also penetrated the cell membranes to cause membrane dislocation and affect the cytoplasmic contents. This may support the earlier suggested mode of antibacterial activity of a synthetic peptide corresponding to the helical region of human lactoferricin against *E. coli* 0111 (NCTC 8007) (Chapple *et al.*, 1998a).

6.6.1 Interaction of lactoferricin B with biological membranes

The 2D ^{1}H NMR structure of lactoferricin B has revealed the peptide to be a somewhat distorted antiparallel β-sheet (Hwang *et al.*, 1998). This contrasts with the X-ray crystallographic structure of bovine lactoferrin, in which residues 18-30 (ie. residues 1-13

of lactoferricin B) form a α-helix (Moore *et al.*, 1997) (Fig. 1.6). It has been postulated that lactoferricin B could adopt either a helical or sheet-like conformation (Hwang *et al.*, 1998). Many hydrophilic and positively charged residues surround the hydrophobic surface giving lactoferricin B an amphipathic character. Lactoferricin B bears numerous similarities to a number of cationic peptides, such as cecropins, magainins and defensins. These cationic peptides exert their antimicrobial activities through membrane disruption. The amphiphilicity observed in the lactoferricin B solution structure suggests that it is capable of interacting with biological membranes in a way similar to the other cationic peptides (Hwang *et al.*, 1998).

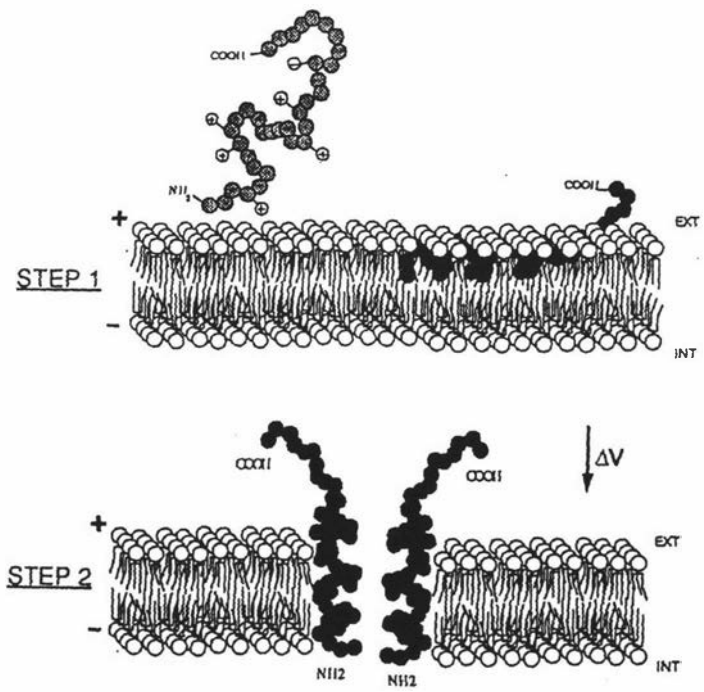
There are different possible modes in which lactoferricin B could bind to cell membranes. The first possibility is by the formation of oligomeric pores, where lactoferricin B would insert into the membrane, so that its extended strands lie perpendicular to the membrane surface. Several lactoferricin B monomers might then multimerise, so that the outer hydrophobic surface would interact with the membrane while the inner hydrophilic surface forms the inside of a transmembrane channel. This mechanism has been proposed for the defensins (Hill *et al.*, 1991). The second possibility is that lactoferricin B would interact with a single leaflet of the lipid bilayer, so that its backbone lies parallel to the membrane surface. This mode of binding has been suggested for magainin 2 and cecropin P1 (Gazit *et al.*, 1996), but other studies have shown that these peptides can also form transmembrane multimeric pores (He *et al.*, 1995).

The T.E.M. results in this study indicate that with a relatively resistant *E. coli* strain, lactoferricin B might be interacting with a single leaflet of the lipid bilayer so that its backbone lies parallel to the membrane surface. The formation of 'membrane blisters' might probably be a result of the aggregation of many lactoferricin B molecules on the membrane surface. Eventually, a long exposure of *E. coli* DH5α cells to lactoferricin B might result in formation of a transmembrane channel causing membrane dislocation and affecting the cytoplasmic contents of some cells. On the other hand, with entero-pathogenic and very susceptible *E. coli* 0111 (NCTC 8179) cells, it appears that lactoferricin B might be directly forming transmembrane channels by inserting itself in to

the membranes perpendicularly to the membrane surface. This may occur without the formation of 'membrane blisters'. These differences in the mode of action of lactoferricin B on *E. coli* strains DH5 α and 0111 cells could be accounted for by the differences in their cell wall structures and the composition of their lipopolysaccharide coat.

Mor & Nicholas (1994) have suggested a tentative molecular model in which dermaseptin (antimicrobial peptide derived from frog skin) would interact with the cell membranes. These authors suggested that it is a two step process as depicted in Fig. 6.1 (Mor and Nicolas, 1994).

Fig. 6.1 Tentative molecular model depicting mode of action of dermaseptin with membranes (adapted from Mor & Nicholas, 1994)



(EXT- exterior and INT- interior)

In step 1 in this figure, the binding of the basic peptide to the plasma membrane's acidic groups induces the folding of the peptide and the generation of an amphipathic helix due to the hydrophobic membranous environment of the lipid bilayer. Dermaseptin would then take on a α-helical structure in which the charged residues occupy one face of the cylinder while the hydrophobic residues segregate on the other face. This conformation generates in turn a dipole moment along the long axis of the helix. In step 2, because of this dipole moment and the difference of potential (ΔV) across the plasma membrane, the oriented insertion of dermaseptin within the bilayer is likely to occur, which will result in the perturbation of the membrane structure. Ultimately, the aggregation of several monomers in a barrel-like formation would result in additional perturbation and might form a pore in the membrane.

Though many possible mechanisms have been suggested, the exact mechanism involved in the interaction between the cationic peptides and the lipid bilayer membranes is still unclear. A better understanding about the binding properties between these peptides and lipid bilayers might come from studies using bacterial LPS preparations and cationic peptides and by following their real time molecular interaction by the Surface plasmon resonance (SPR) technique. Unfortunately, this could not be carried out during this study because of monetary constraints.

6.7 Conclusions

1. The pEPS1 and pYEXS1 expression vectors were not useful for the heterologous expression of hLF cDNA in yeast cells. Although expression and secretion of human lactoferrin protein products by the recombinant *K. lactis* was obtained for a few generations, it could not be sustained.

2. The DNA encoding the antimicrobial lactoferricin H & B peptides were successfully sub-cloned and expressed in *E. coli* using commercially available expression vectors. The fusion proteins expressed both as inclusion bodies and in a soluble form were

purified on suitable affinity resins. But, these fusion proteins could not be obtained in soluble form after the removal of urea. Lactoferricin H & B peptides could be cleaved from the GST-fusion proteins using a site-specific protease, but they were not soluble. These peptides appeared to precipitate on the resin upon cleavage from the GST-fusion proteins.

3. The five different strains of *E. coli* used in this study showed differences in their susceptibility towards lactoferricin B as shown by antibacterial assays. Transmission electron microscopy studies of lactoferricin B treated cells of relatively resistant and sensitive strains of *E. coli* revealed that lactoferricin B has a differential mode of action on their membranes. This might correspond to the differences in their susceptibility to lactoferricin B.

6.8 Future Directions

The results that have been presented in this investigation give a way to answer some future questions that may be raised. In this study, it has been shown that the yeast host-vector system used for the heterologous expression of human lactoferrin was not a suitable system. Also for the expression of cationic peptides like lactoferricin, *E. coli* expression host with the use of commercial expression vectors has not proven to be very productive. Although the sub-cloning, expression in *E. coli* and the purification of the fusion proteins did not pose any problems, ultimately obtaining soluble lactoferricin H & B peptides for the antibacterial activity studies was not possible. The results of transmission electron microscopy studies have clearly shown that lactoferricin B has a differential mode of action on the membranes of relatively resistant and more susceptible strains of *E. coli.*

Based on the good and bad experiences I have had during this investigation, the following suggestions can be made as to what I conceive of as future directions for any further work on this topic.

6.8.1 Expression of human lactoferrin in *K. lactis* using a different set of proven expression vectors

The selection of the yeast host *Kluyveromyces lactis* for the heterologous expression of human lactoferrin (a low value protein) was quite well justified because of the availability of cheap substrates like lactose and whey in New Zealand in abundance. The problem that was faced in this project was with the expression vectors, pEPS1 and pYEXS1 (using a constitutive PGK promoter), which failed to establish the constructs of hLF cDNA in *E. coli*. This made the project very difficult. *K. lactis* has been shown to be a successful yeast host for the production of recombinant human serum albumin (Fleer *et al.*, 1991a) and human interleukin-1β (Fleer *et al.*, 1991b). These authors used vectors that had PGK/LAC4 and PHO5 promoters respectively for high-level expression of these proteins up to 300 mg/L. One could try to use such proven yeast expression vectors to express human lactoferrin on whey-based media. Unfortunately, most of the proven yeast expression vectors such as those mentioned above are covered by a patent or in commercial use, which makes it impossible or very expensive to obtain them.

If many yeast expression vectors are available, it is always a good idea to make as many constructs as possible with different vectors and the insert and its variants and to try the feasibility of establishing them in *E. coli* rather than trying direct electroporation into yeast. Of course, direct electroporation into yeast would be feasible if there is an easy direct screening method available.

6.8.2 Alternative expression systems for the expression of cationic peptides

I faced quite a few problems in all the attempts to express lactoferricin H & B peptides in *E. coli*. The fusion proteins were expressed in large quantities in *E. coli* as inclusion bodies. Though these inclusion bodies were urea solubilised and purified on appropriate affinity resins, soluble proteins could not be obtained after the removal of urea. This prevented any further characterisation of the proteins. Similarly, the *E. coli* system has not proven to be very useful for other investigators who tried to express cationic peptides in this system (Piers *et al.*, 1993; Zhang *et al.*, 1998).

The baculovirus expression system has been used successfully for the heterologous expression of the cecropin A (Andersons *et al.*, 1991; Hellers *et al.*, 1991) and human β-defensin (Valore *et al.*, 1998) with the retention of antimicrobial activity by the recombinant peptides. Similarly, the baculovirus expression system may be useful for the expression of lactoferricin peptides and to obtain them in soluble and active form, which has not been attempted by anybody so far.

From our experiences in expressing lactoferricins and that of other investigators in expressing cationic peptides in bacterial expression systems, it can be suggested that alternative hosts such as yeasts, mammalian or insect cells might offer better opportunity to avoid the problems of folding and the formation of inclusion bodies as encountered with microbial overexpression (Rudolf and Lilie, 1996). The cationic peptides expressed in mammalian or insect cell culture systems would not exert their antibacterial activity on the host cells, which makes the system favourable for producing them. But, the cell culture systems are not economically viable for production of these cationic peptides on a large scale if the final aim is to use them as therapeutic agents. Under these conditions, it may be viable to obtain lactoferricin B peptide by the acid pepsin-hydrolysis of bovine lactoferrin, which is abundantly available. Lactoferricin H has to be produced by chemical synthesis or by the recombinant route, as human lactoferrin is not as abundantly available as bovine lactoferrin. The mutants of both lactoferricin H & B may have to be chemically synthesised to conduct any studies on them.

6.8.3 Studies on the binding characteristics of lactoferricins and their variants to the LPS preparations of different *E. coli* strains

In our studies, we have shown that lactoferricin B has different modes of action on the outer membranes of different strains of *E. coli*. This was shown with both the antibacterial assay and T.E.M. studies. It has been suggested that differences in the composition of the bacterial cell wall, especially in the case of Gram-negative bacteria and differences in the structure of LPS play an important role in differentially controlling the interaction between the bacterial outer membrane and the antimicrobial peptides (Chapple *et al.*, 1998a). A study on the real time interaction between the LPS preparations of

different *E. coli* strains and lactoferricin and its mutant variants (produced either heterologously or synthetically) using the Surface plasmon resonance (SPR) technique would give insights into the binding characteristics of different lactoferricin variants with LPS at the molecular level. The SPR studies can be carried out in combination with antibacterial assay and T.E.M. observations of cells treated with either lactoferricin or its variants for a better understanding of the mode of action of these cationic peptides.

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Appendix 1

E. coli codon bias table

Amino acid	Codon	Number	/1000	Fraction
Gly	GGG	19118.00	11.38	0.16
Gly	GGA	14968.00	8.91	0.12
Gly	GGT	41093.00	24.46	0.33
Gly	GGC	47517.00	28.29	0.39
Glu	GAG	30797.00	18.33	0.32
Glu	GAA	64836.00	38.60	0.68
Asp	GAT	54484.00	32.44	0.63
Asp	GAC	31925.00	19.01	0.37
Val	GTG	42648.00	25.39	0.36
Val	GTA	18780.00	11.18	0.16
Val	GTT	31446.00	18.72	0.27
Val	GTC	24953.00	14.86	0.21
Ala	GCG	53321.00	31.74	0.34
Ala	GCA	33957.00	20.22	0.22
Ala	GCT	26624.00	15.85	0.17
Ala	GCC	42164.00	25.10	0.27
Arg	AGG	3058.00	1.82	0.03
Arg	AGA	4946.00	2.94	0.05
Ser	AGT	15956.00	9.50	0.16
Ser	AGC	26531.00	15.79	0.26
Lys	AAG	19176.00	11.42	0.25
Lys	AAA	56992.00	33.93	0.75
Asn	AAT	32436.00	19.31	0.47
Asn	AAC	35939.00	21.40	0.53

Amino Acid	Codon	Number	/1000	Fraction
Met	ATG	45625.00	27.16	1.00
Ile	ATA	9517.00	5.67	0.09
Ile	ATT	50298.00	29.94	0.50
Ile	ATC	40796.00	24.29	0.41
Thr	ACG	23884.00	14.22	0.26
Thr	ACA	13786.00	8.21	0.15
Thr	ACT	15978.00	9.51	0.17
Thr	ACC	37806.00	22.51	0.41
Trp	TGG	25072.00	14.93	1.00
End	TGA	1724.00	1.03	0.32
Cys	TGT	8911.00	5.30	0.45
Cys	TGC	10851.00	6.46	0.55
End	TAG	462.00	0.28	0.09
End	TAA	3220.00	1.92	0.60
Tyr	TAT	28377.00	16.89	0.58
Tyr	TAC	20620.00	12.28	0.42
Leu	TTG	22665.00	13.49	0.13
Leu	TTA	23919.00	14.24	0.14
Phe	TTT	38054.00	22.65	0.58
Phe	TTC	27491.00	16.37	0.42
Ser	TCG	14782.00	8.80	0.15
Ser	TCA	13682.00	8.15	0.14
Ser	TCT	15486.00	9.22	0.15
Ser	TCC	14693.00	8.75	0.15
Arg	CGG	9925.00	5.91	0.11
Arg	CGA	6527.00	3.89	0.07
Arg	CGT	33711.00	20.07	0.36
Arg	CGC	35344.00	21.04	0.38

Amino Acid	Codon	Number	/1000	Fraction
Gln	CAG	47932.00	28.54	0.65
Gln	CAA	25265.00	15.04	0.35
His	CAT	21719.00	12.93	0.58
His	CAC	15986.00	9.52	0.42
Leu	CTG	84003.00	50.01	0.48
Leu	CTA	6997.00	4.17	0.04
Leu	CTT	19368.00	11.53	0.11
Leu	CTC	18085.00	10.77	0.10
Pro	CCG	37092.00	22.08	0.50
Pro	CCA	14588.00	8.68	0.20
Pro	CCT	12441.00	7.41	0.17
Pro	CCC	9391.00	5.59	0.13

Appendix 2

Consensus *E. coli* stop codon table

(Position T of stop codon = +1)

	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	+1	+2	+3	+4
%G	30	32	19	29	32	21	31	33	21	26	0	32	9	20
%A	24	28	33	24	29	33	23	26	39	27	0	68	91	24
%T	26	17	26	25	16	24	25	16	22	25	100	0	0	39
%C	20	23	21	22	22	22	20	26	18	22	0	0	0	18
Total	5406	5406	5406	5406	5406	5406	5406	5406	5406	5406	5406	5406	5406	5367

	+5	+6	+7	+8	+9	+10	+11	+12	+13
%G	25	22	21	22	22	22	23	22	22
%A	25	33	30	28	31	30	28	27	27
%T	27	23	27	29	26	28	27	27	26
%C	23	22	22	22	22	20	22	23	25
Total	5321	5272	5213	5140	5106	5085	5060	5027	5004

CONSENSUS sequence to a certainty level of 50.0 percent at each position: Length: 23

1 KRWKRWKRWR TAA WKWWWWWWWW

Appendix 3

The genetic code table

First Letter	Second Letter								Third Letter
	T		C		A		G		
T	TTT	Phe	TCT	Ser	TAT	Tyr	TGT	Cys	T
	TTC		TCC		TAC		TGC		C
	TTA	Leu	TCA	Ser	TAA	Stop	TGA	Stop	A
	TTG		TCG		TAG	Stop	TGG	Trp	G
C	CTT	Leu	CCT	Pro	CAT	His	CGT	Arg	T
	CTC		CCC		CAC		CGC		C
	CTA	Leu	CCA	Pro	CAA	Gln	CGA	Arg	A
	CTG		CCG		CAG		CGG		G
A	ATT	Ile	ACT	Thr	AAT	Asn	AGT	Ser	T
	ATC		ACC		AAC		AGC		C
	ATA	Ile	ACA	Thr	AAA	Lys	AGA	Arg	A
	ATG	Met	ACG		AAG		AGG		G
G	GTT	Val	GCT	Ala	GAT	Asp	GGT	Gly	T
	GTC		GCC		GAC		GGC		C
	GTA	Val	GCA	Ala	GAA	Glu	GGA	Gly	A
	GTG		GCG		GAG		GGG		G

Appendix 4

Amino acid abbreviations

Amino Acid	Abbreviation	Symbol
Alanine	Ala	A
Arginine	Arg	R
Aspsragine	Asn	N
Aspartic acid	Asp	D
Asparagine or Aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or Glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V