MOLECULAR CHARACTERIZATION OF STREPTOCOCCUS UBERIS CAMP FACTOR, LACTOFERRIN BINDING PROTEIN AND THEIR UPSTREAM GENES

A Thesis

Submitted to the Faculty of Graduate Studies and Research

in Partial Fulfilment of the Requirements

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in the Department of Veterinary Microbiology

University of Saskatchewan

by

Min Jiang

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of the requirements for

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by

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ABSTRACT

The gene coding for the CAMP factor from a strain of *Streptococcus uberis* (*S. uberis*) was cloned in *E. coli*. Chromosomal DNA from *S. uberis* was used to construct a gene library in plasmid pTZ18R and six CAMP-reaction positive clones were obtained from a total of 10,000 transformants. One clone, pJLD21, was subcloned and the CAMP factor gene (*cfu*) was localized to a 3.2 kb *Bam*HI fragment. The nucleotide sequence of *cfu* was determined and the deduced amino acid sequence shown to be homologous to the corresponding *Streptococcus agalactiae* (*S. agalactiae*) protein. Immunoblot analysis revealed that the recombinant strain containing pJLD21 expressed a protein with a molecular weight of 28,000. Antibodies raised against purified *S. uberis* CAMP factor cross-reacted with *S. agalactiae* protein B. Southern blot analysis demonstrated that the six CAMP-reaction positive *E. coli* clones contained the same CAMP factor gene, and this gene existed in three out of eight *S. uberis* strains.

An ORF encoding a 277-residue protein was identified upstream of the CAMP factor gene. Sequence analysis indicated that the gene product is potentially a polar amino acid and opine binding protein of an ABC-type

transport system.

The interaction between *S. uberis* and bovine lactoferrin (bLf) has been characterized. The binding of ^{125}I-bLf to *S. uberis* was time-dependent and displaceable by unlabelled bLf. Apo-bLf could inhibit ^{125}I-bLf binding as effectively as iron-saturated bLf. Bovine transferrin, human lactoferrin and human transferrin did not interfere with bLf binding. The Scatchard plot was linear and approximately 7800 binding sites were expressed by each bacterial cell, with an affinity of 1.0×10^{-7} M. Reduced iron availability induced by the presence of iron chelators in the growth medium did not notably modify the saturation of *S. uberis* by bLf. Heat- or protease treatment of bacterial cells reduced bLf binding to a great degree. Two components with estimated molecular weights of 165,000 and 76,000 were originally identified from the cell wall as the functionally active bLf binding proteins.

The gene coding for the bLf binding protein (*lbp*) of *S. uberis* has been cloned and sequenced. A single ORF encoding 561 amino acid residues resulted in the presence of two proteins in the recombinant *E. coli* cell. These proteins were able to bind bovine lactoferrin and had molecular weights of 76,000 and 165,000, similar to those detected in *S. uberis*. The 165 kDa protein was likely a dimer of the 76 kDa protein. Consistent with this, urea treatment resulted in only one protein species, and Northern blot analysis showed

only one major transcript in both *S. uberis* and recombinant *E. coli*. A putative signal peptide was found at the N terminus of the deduced amino acid sequence and the C terminus had the features of the membrane anchor motif found in other surface proteins from Gram positive bacteria. Deletion analysis located the bLf binding domain to a 200 amino acid region at the N terminus of this protein. Analysis of the primary and secondary structure suggested that bLf binding protein of *S. uberis* is a M-like protein. Consistent with this, a gene (*mga*) homologous to that coding for the group A streptococcal Mga, a positive regulator of M and M-like proteins was found in the adjacent region of the *lbp*. The transcript of *mga* was detected in recombinant *E. coli* but not in *S. uberis*. Southern blot analysis revealed that *mga* was distributed in all *S. uberis* strains that contained the *lbp*. However, the relationship of *mga* and *lbp* needs to be studied.

The vaccine potential of recombinant CAMP factor and lactoferrin binding protein has been evaluated. Animals vaccinated with CAMP factor generated high levels of specific antibodies in serum and milk, and possessed normal milk somatic cell counts after challenge. In contrast, lactoferrin binding protein did not induce high levels of specific antibodies in the vaccinated animals, and the mean somatic cell count of these cows was considerably higher than normal level following challenge.

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ABBREVIATIONS USED IN THIS WORK

Abp	amino acid-binding protein
ABTS	2,2'-amino-di(3-ethylbenzthiozoline sulfonate
ATCC	American type culture collection
bLf	bovine lactoferrin
BSA	bovine serum albumin
CAT	staphylococcal chloramphenicol transacetylase
CIP	calf intestinal phosphatase
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDDA	ethylenediamine-di-(o-hydroxyphenyl acetic acid)
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunoadsorbent assay
g	gram
x g	gravity
GAS	group A streptococci
GBS	group B streptococci
h	hour
H. influenzae	*Hemophilus influenzae*
hLf	human lactoferrin
hTf	human transferrin

Ig	immunoglobulin
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobases
kDa	kilodalton
Lbp	lactoferrin-binding protein
Lf	lactoferrin
LB	Luria broth
M	molar
Mga	multigene regulator of group A Streptococcus'
MHC	major histocompatibility complex
ml	millilitre(s)
mM	millimolar
min	minute(s)
N. meningitidis	*Neisseria meningitidis*
NaCl	sodium chloride
O.D.	optical density
OF	serum opacity factor
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PMNs	polymorphonuclear leukocytes
RBC	red blood cell
RNA	ribonucleic acid
S. agalactiae	*Streptococcus agalactiae*
S. aureus	*Staphylococcus aureus*
SDOC	sodium desoxycholate

SDS	sodium dodecyl sulphate
S. pneumoniae	*Streptococcus pneumoniae*
S. pyogenes	*Streptococcus pyogenes*
S. uberis	*Streptococcus uberis*
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TCA	trichloroacetic acid
TCR	T-cell receptor
Tf	transferrin
Tbp	transferrin-binding protein
µg	microgram(s)
µl	microlitre(s)

1.0 INTRODUCTION AND OBJECTIVES

Streptococcus uberis (S. uberis) is an important cause of bovine mastitis, a disease of considerable economic concern to the dairy industry. This Gram positive bacterium is widely distributed in the environment and antimicrobial treatment is generally ineffective in treating disease caused by this organism. Therefore, the development of control measures must be based on an understanding of virulence factors and protective antigens involved in invasion and protection of the mammary gland. It is known that some *S. uberis* strains can produce hyaluronic acid capsule, hyaluronidase, R-like protein, and a cohemolysin, the CAMP factor. However, very little is known of their roles in pathogenicity or inducing protection against *S. uberis* mastitis.

The research described in this thesis is part of a larger study undertaken to investigate the pathogenesis of *S. uberis* in bovine mastitis and to identify potential virulence factors and protective antigens. The long term goal is to develop a protective subunit vaccine. The specific aims of this study were to characterize the gene coding for *S. uberis* CAMP factor, to identify bovine lactoferrin binding

protein(s) and characterize the respective gene(s), and to evaluate the protective capacity of the recombinant CAMP factor and lactoferrin binding protein against challenge by *S. uberis*.

This thesis describes the achievement of our specific objectives. In addition, it describes the identification of two additional genes, *abp* and *mga*, in the upstream regions of the CAMP factor gene and lactoferrin binding protein gene, respectively. The *abp* locus could encode a protein homologous to polar amino acid and opine binding proteins of Gram negative bacteria, and *mga* is a homologue of *virR* (or *mry*), the positive regulator gene of group A streptococci.

In this dissertation, a literature review precedes four chapters which describe the major studies in manuscript form. Therefore, each chapter contains a summary, a short introduction, materials and methods, results, and a concise discussion. The four chapters are followed by a general discussion which interprets the results of the entire research project. The references for the thesis are presented in the last chapter of the dissertation.

2.0 LITERATURE REVIEW

2.1 *Streptococcus uberis*

2.1.1 Bovine Mastitis

Mastitis has been and continues to be recognized as one of the major disease problems concerning the dairy industry. It is generally defined as inflammation of the mammary gland with a resultant increase in somatic cell counts in excess of 500,000/ml in the milk, and all the clinical signs associated with inflammation (Bramley, 1991). Mastitis is generally considered the most costly disease of dairy cows. Economic loss resulting from mastitis is difficult to estimate because of the many levels of infection and other factors. It is mainly attributed to decreased milk production, discarded milk, early culling, drug costs, veterinary fees, and increased labour (Fetrow, 1980). Economic losses from mastitis of $200 per cow per year or $2 billion per year have been estimated for the United States (Jasper *et al.*, 1982; Philpot, 1984).

As early as the 1920s and 30s, *Streptococcus uberis*, along with *S. agalactiae* and *S. dysgalactiae* were identified as the three common species of streptococci

associated with bovine mastitis. The wide application of mastitis control measures, such as post-milking teat disinfection and dry-cow antibiotic therapy, has led to a fall in the incidence of mastitis caused by contagious *S. agalactiae*, but the nonagalactiae streptococci are now responsible for a greater proportion of clinical disease. Reports on the frequency of isolation of nonagalactiae streptococci indicated *S. uberis* to be most common (Bramley, 1990; Bramley, 1984; Smith *et al.*, 1985). It constitutes around 33% of the isolates obtained from clinical cases of bovine mastitis (Hillerton *et al.*, 1993).

S. uberis is not dependent upon the mammary gland for survival in nature. Instead, it is widely distributed in the environments, such as bedding, pastures and many sites on the cow, including the body surface, gut, tonsils and urogenital tract (Bramley, 1982; Kruze and Bramley, 1982). However, this organism does not contribute significantly to diseases outside the mammary gland. It appears that *S. uberis* is a specialised opportunist, taking advantage of weakness within a mammary quarter favourable to its survival. The rate of new intramammary infection caused by *S. uberis* is higher in the dry period than during the lactation, and is highest during the first two weeks of the dry period and again during the two weeks before parturition (Harmon and Crist, 1994).

Mastitis resulting from infection with *S. uberis* is commonly subclinical, characterized by apparently normal milk

with an increase in somatic cell counts due to the influx of leukocytes. The chemical composition of milk is changed due to suppression of secretion with the transfer of sodium chloride and bicarbonate from blood to milk bringing about a shift of pH to a more alkaline level. *S. uberis* mastitis may also take the form of an acute clinical condition, with obvious signs of disease such as clots or discolouration of the milk, swelling or hardness of the mammary gland. Some cases of the clinical disease can be severe and pyrexia may be present. The clinical forms of *S. uberis* mastitis were reviewed by Bramley (1991) and Schalm *et al.* (1971).

The pathogenesis of *S. uberis* infection is poorly understood. Generally, for an organism to infect and persist within the mammary gland, it must be able to gain access to the gland and multiply at a rate sufficient to prevent its elimination during milking. Adherence of bacteria to host cells is an essential feature in the process of colonization. Studies showed that *S. uberis* was capable of adhering to extracellular matrix proteins such as collagen, fibronectin, and laminin, plus bovine mammary epithelial cells (Almeida *et al.*, 1993). More interestingly, *S. uberis* was observed in membrane-bound vacuoles within the cytoplasm of epithelial cells (Matthews *et al.*, 1994b), suggesting a post-adherence cell invasion, a common event among pathogenic bacteria that are not generally considered as "classical invading bacteria" (Donnenberg *et al.*, 1990; Ewanovich *et al.*, 1989; Janda *et*

al., 1991; Sreenivasan *et al.*, 1993). Entry of the pathogen into host cells and further invasion of tissue underlying mucosal membranes enable pathogenic bacteria to spread and avoid host defense mechanisms and the action of most antimicrobial agents (Falkow, 1991). Invasion of bovine mammary secretory epithelial cells may also affect secretory cell function, and this may be related to decreased milk production associated with mastitis.

Phagocytosis and killing of bacteria by neutrophils constitutes a major defence mechanism of the mammary gland. This system is responsible for controlling infections caused by *S. aureus* (Schalm *et al.*, 1976) and the elimination of infections caused by *E. coli* (Hill *et al.*, 1978). The role of phagocytic cells in control of infection by *S. uberis* appears less clear (Thomas *et al.*, 1990). However, a strain of *S. uberis* which was able to resist phagocytosis *in vitro* was also found to be more capable of establishing an infection in the lactating gland than an organism which was readily phagocytosed and killed (Hill, 1988a; Leigh *et al.*, 1990). Infection of the mammary gland with virulent strains of *S. uberis* results in the rapid growth of bacteria and the concomitant appearance of large numbers of neutrophils in secretions (Hill *et al.*, 1994). However, unlike infection with *E. coli*, the presence of neutrophils does not reduce the number of *S. uberis* present in the secretions. Microscopic examination of milk and tissues from animals infected with *S.*

uberis confirms these findings, as free bacteria are clearly detected in the secretion but are only rarely found in association with phagocytic cells (Thomas *et al.*, 1990).

Information on the therapy of *S. uberis* infection is not extensive. Usually, intramammary therapy with antibiotics is the first treatment for acute mastitis. *S. uberis* is very sensitive *in vitro* to many antibiotic agents, especially penicillin. In one study, 100% of *S. uberis* isolates were sensitive to this antibiotic (McDonald *et al.*, 1976). The rates for successful treatment of clinical *S. uberis* mastitis are generally high. Using penicillin or semisynthetic penicillins, cure rates exceeding 70% have usually been obtained (Griffin *et al.*, 1982). However, Tyler *et al.* (1992) stated that response of clinical *S. uberis* infections to antibiotic therapy during lactation is poor, although a combination of parenteral and intramammary erythromycin appears to be efficacious.

The approaches to control *S. uberis* mastitis must involve prevention by either decreased exposure to pathogens or enhanced resistance of the dairy cow. Post-milking teat dipping with germicidal dips, dry cow therapy with antibiotics and other hygienic milking techniques which reduce the residual contamination of teat skin by pathogens only have limited efficacy against *S. uberis* infection (Crist and Harmon, 1991; Oliver *et al.*, 1989). This is likely related to the constant exposure of the teat to the

extramammary environment, the primary reservoir of *S. uberis*.

Vaccination is one of the approaches that enhances the resistance of the mammary gland to new infection and can reduce clinical severity of the disease. The prospects for the control of *S. uberis* infection by means of vaccination would appear promising. Previous studies showed that a primary infection with *S. uberis* can considerably reduce the rate of infection following a second challenge with the same strain (Hill, 1988b). Local vaccination with killed *S. uberis* protects the bovine mammary gland against intramammary challenge with the homologous strain (Finch *et al.*, 1994). Similarly, subcutaneous vaccination with live *S. uberis* caused a dramatic modification of the pathogenesis of mastitis with the same strain (Hill *et al.*, 1994). Animals vaccinated in this way shed many fewer bacteria in their milk and many quarters remained free of infection.

The potential application of cytokines for disease prevention in dairy cattle has been described (Babiuk *et al.*, 1991; Daley *et al.*, 1991; Kehrli *et al.*, 1991). With the production of recombinant interferons, colony-stimulating factors, and interleukins, considerable research is in progress to address their effects on modulating the disease process in the mammary gland. Although this area shows promise for future application to animal health, many details of the mechanisms involved must be investigated.

2.1.2 Identification

S. uberis is Gram stain positive, with cells being spherical and occurring in short chains. They grow readily on blood agar at 30 to 37°C. Most *S. uberis* strains are non-haemolytic or show slight α-haemolysis, and, after 24 to 48 h incubation, colonies are 1 to 2 mm in diameter. Identification is aided by the addition of 0.1% aesculin to the blood agar. *S. uberis* hydrolyses aesculin and this is detectable as a darkening or browning of the medium around the colony. Detailed physiological and biochemical characteristics of *S. uberis* were reviewed by Bramley (1984).

S. uberis is serologically heterogeneous. The serological grouping of *S. uberis* with Lancefield grouping sera has primarily resulted in approximately 20% of these organisms reacting with group E-specific serum (Sweeney, 1964). Occasional reactions, however, have been reported with groups B, G, P, and U (Garvie and Bramley, 1979; Roguinsky, 1969; Roguinsky, 1971; Shuman *et al.*, 1969; Shuman *et al.*, 1972a; 1972b). At least 3, and as many as 11 or more, different serological subtypes within the species have been detected by slide agglutination and precipitation tests (Cullen, 1969). Therefore, serology is of little value in the identification or classification of *S. uberis*.

A common antigen was demonstrated by rocket-line immunoelectrophoresis from *S. uberis* isolates, but was not further characterized (Jones and Norcross, 1983). Other study

showed that *S. uberis* strains exhibited almost identical immunoblot fingerprints of the major antigens of approximately 40-41, 59-61 and 118-122 kDa following reaction with rabbit hyperimmune sera (Groschup *et al.*, 1991). However, variations in the molecular weight of particular bands were too unevenly distributed to permit formation of subgroups.

DNA or genome fingerprinting has been used to distinguish between isolates of *S. uberis*. Comparison of the restriction patterns from *S. uberis* showed that the fragment length of the discriminating region was between 9 and 23 kb (Hill and Leigh, 1989). Hence, sufficient separation of large chromosomal fragments is required to obtain precise comparison.

DNA-DNA hybridization has revealed two subtypes (designated type I and type II) of *S. uberis*, with 80-100% homology among members of a given subtype and 45-65% homology between them (Collins *et al.*, 1984; Garvie and Bramley, 1979). Comparison of 16S rRNA sequences has confirmed this distinction, and type II strains have now been assigned to a separate species, *S. parauberis* (Williams and Collins, 1990). Methods to differentiate the two species based on Southern hybridization analysis of chromosomal digests, polymerase chain reaction (PCR) amplification of 16S ribosomal DNA followed by analysis of restriction fragment length polymorphisms (Jayarao *et al.*, 1991), hybridization by using

oligonucleotide probes specific for 16S rRNA of each species (Bentley *et al.*, 1993), and PCR amplification of 23S ribosomal DNA followed by hybridization with probes specific for 23S rRNA gene sequences of each species (Harland *et al.*, 1993) have been described. Biochemical and serological differentiation of *S. parauberis* from *S. uberis* is not possible at present.

2.1.3 Potential Virulence Factors and pathogenicity

The research into the pathogenic determinants of *S. uberis* is in its infancy compared to that on other mastitis-causing bacteria such as *S. aureus* and *E. coli*, and therefore the influence of *S. uberis* virulence factors on host defense mechanisms and mammary gland physiology is poorly defined. Known virulence factors associated with *S. uberis* include a hyaluronic acid capsule, hyaluronidase, R-like protein, plasminogen activator and CAMP factor.

Exopolysaccharide capsule or slime is a major virulence factor of many *Streptococcus* species (Van de Rijn, 1983). Few reports have described encapsulated *S. uberis* isolated from bovine mammary secretions (Hill, 1988a; Leigh and Field, 1991). The capsule of *S. uberis* appears to be composed of hyaluronic acid, a linear polysaccharide composed of β 1-4-linked repeating disaccharide units of glucuronic acid, β 1-3-linked to N-acetylglucosamine (Van de Rijn,

1983). Hill (1988a) investigated the pathogenicity of two strains of *S. uberis*, one of which was clearly encapsulated, the other had no demonstrable capsule. The encapsulated strain was more pathogenic and more resistant to neutrophil phagocytosis than the unencapsulated strain. The removal of the capsule layer from the bacterial cell by treatment with hyaluronidase significantly reduced the ability of *S. uberis* to resist phagocytosis by neutrophils (Thomson and Leigh, 1993). Evidence would therefore suggest that the capsule is the mediator of phagocytic resistance for *S. uberis*. However, the mechanism by which the capsular polysaccharide mediates resistance of *S. uberis* to phagocytosis has not been identified. Studies found that this resistance even occurred in the presence of potentially opsonic immunoglobulin bound to the bacterium (Leigh and Field, 1994). Both the phagocyte-resistant (with capsule) and -susceptible (without capsule) phenotypes of individual strains bound equal quantities of antibody. In each case this bound antibody is intact, is in the correct orientation and presents the Fc terminus for interaction with immunoglobulin receptors on the neutrophil. It is clear that the capsule layer of *S.uberis* does not deter immunoglobulin from binding to the bacterium and does not behave in a similar manner as the capsules of *E. coli* (Horowitz and Silverstein, 1980) and *S.aureus* (Sutra *et al.*, 1990), both of which prevent the binding of potentially opsonic ligands to subcapsular components. Furthermore, it is

unlikely that the capsule structure of *S. uberis* acts as a significant barrier to the interaction between the bound immunoglobulin and receptors on the neutrophils. Therefore, it is not yet known how the presence of the capsule on *S. uberis* determines resistance to opsonophagocytosis by neutrophils.

Hyaluronidase was isolated and characterized from *S. uberis* by Schaufuss *et al.* (1989). It is an enzyme that depolymerizes hyaluronic acid, which is an essential component of connective tissues. Although hyaluronidase has been confirmed as an invasiveness factor of pneumococci (Volkova *et al.*, 1994), its pathogenic role in bovine mastitis is not clear. It has been suggested that the hyaluronidase produced by *S. uberis* may influence mammary epithelial cell proliferation (Matthews *et al.*, 1994a), which could be important during the periparturient period, when mammary tissue undergoes rapid differentiation and growth.

Streptococcal R antigens are surface proteins that were first described on the group A *Streptococcus pyogenes* (Lancefield and Perlmann, 1952). They have also been observed on groups B, C, F, G, and L (Lancefield and Perlmann, 1952; Maekawa *et al.*, 1978; Maxted, 1948; Wilkinson, 1972). Like the R-antigens from all these groups, the R-like protein of *S. uberis* is pepsin-sensitive and trypsin-resistant (Groschup and Timoney, 1993). It has a molecular mass of 65 kDa and a similar amino acid composition to that reported for the R

antigen of group C streptococci (Groschup and Timoney, 1993).

For a long period of time, R antigens had been thought to have no biological significance or activity, since anti-R antibody was not protective against reinfection (Lancefield and Perlmann, 1952; Johnson, 1975). However, in the case of group B type III streptococci (Linden, 1983; Linden *et al,* 1983), clinical studies and mouse protection experiments indicate that R antigen contributes to virulence. On these organisms it extends as filamentous protrusions from the cell surface (Wagner *et al.*, 1982). Group B type III strains expressing R protein are significantly more able to colonize the pharynx of the mouse than strains that lack this antigen (Kurl *et al.*, 1984). It is not known whether the R-like antigen of *S. uberis* has a function in colonizing the bacterium to the epithelial surface of mammary gland. However, it has been found that this protein could stimulate opsonic antibody in guinea pigs (Groschup and Timoney, 1993), and react with sera from infected cows, indicating that it may play a role in promoting the protective response of the convalescent mammary gland.

S. uberis is capable of activating bovine plasminogen to the caseinolytic enzyme plasmin (Leigh, 1993). This action is brought about by the plasminogen activator, an extracellular protein with a molecular weight of approximately 57 kDa which is composed of two subunits of 29 kDa (Leigh, 1994). The plasminogen activator of *S. uberis* is

markedly different from the streptokinases isolated from group A (Ohkuni *et al.*, 1992) and C streptococci (Jackson and Tand, 1982), which activate human plasminogen and exist as monomeric structures. It is also different from the staphylokinase, a human plasminogen activator from *S. aureus*, which has a subunit molecular weight of between 23 (Jackson *et al.*, 1981) and 15 kDa (Sako and Tsuchida, 1983).

The role of plasminogen activators in the pathogenesis of infection is not clear, although a variety of potential mechanisms have been suggested. These include facilitating invasion of the pathogen due to the indirect lysis of fibrin barriers deposited around the foci of infection (Tillet *et al.*, 1934), and the inappropriate activation of the complement cascade leading to the initiation of a localized inflammatory response with consequent pathological changes (Holm *et al.*, 1988). The role of the plasminogen activator of *S. uberis* in the pathogenesis of bovine mastitis is not clear, although such an activity might facilitate bacterial growth in the bovine mammary gland due to indirect proteolysis of host proteins (Leigh, 1993) or initiate the inflammatory responses characteristic of this disease.

2.2 CAMP factor

A lytic phenomenon exhibited by group B streptococci (GBS; *Streptococcus agalactiae*) on sheep or cow erythrocytes was first described by Christie *et al.* (1944). These authors had found that GBS induced a distinct zone of complete hemolysis when grown near the diffusion zone of the β-toxin of *Staphylococcus aureus*. Later on, this phenomenon was called the CAMP reaction after the initials of the last names of its discoverers (Murphy *et al.*, 1952). CAMP reaction has been shown to be associated with most group B isolates (Phillips *et al.*, 1980). In addition to GBS, *S. uberis* (Skalka and Smola, 1981) and a few other bacteria, including *Listeria monocytogenes* and *Listeria seeligeri* (Rocourt and Grimont, 1983), *Aeromonas* sp. (Figura and Guglielmetti, 1987), *Rhodococcus equi* (Fraser, 1961), *Actinobacillus pleuropneumoniae* (Kilian, 1976), and certain *Vibrio* spp. (Köhler, 1988) produce reactions similar to the CAMP effect.

Bernheimer *et al.* (1979) characterized the CAMP factor responsible for this reaction as an extracellular protein with a molecular weight of 23,500. They described its mode of action as an interaction with cell membranes pretreated with the staphylococcal β-toxin (a sphingomyelinase) leading to instability and subsequent lysis of erythrocyte membranes. Conversion of membrane sphingomyelin to ceramide by a sphingomyelinase was

considered to be a prerequisite for cell lysis induced by the CAMP factor. Fehrenbach and co-workers (1984; 1988; Sterzik *et al.*, 1984; 1985), using liposomes as artificial membranes, demonstrated that the action of the CAMP factor on membranes was dependent on the ratio of cholesterol to phospholipid and that sphingomyelin could be replaced by other phospholipids in this reaction.

The existence of CAMP factor-inactivating antibodies in immunized rabbits and experimentally infected cows (Brown *et al.*, 1974) indicated that CAMP factor is an antigen and is produced during the course of infection. However, the CAMP factor-induced lysis of target cells *in vitro* remained rather an epiphenomenon and was not considered to be of relevance *in vivo*. Hence, the CAMP factor has long been considered to be of no relevance for pathogenicity. In 1981, Skalka and Smola (1981) reported that a partially purified CAMP factor preparation was lethal for experimental animals, especially for rabbits, when injected intravenously. It was also found that intraperitoneal injection of purified CAMP factor into mice significantly raised the pathogenicity of a sublethal dose of GBS (Fehrenbach *et al.*, 1988). These data suggest that CAMP factor is a potential virulence determinant. The role of CAMP factor in pathogenicity was further supported by the findings of Jürgens *et al.* (1987), who showed that the CAMP factor binds to the Fc sites of immunoglobulins in a way similar to that of protein A of

Staphylococcus aureus. By analogy to protein A, the CAMP factor was named protein B.

CAMP factor has been purified from the culture supernatant of *S. agalactiae* by hydrophobic interaction chromatography and chromatofocusing, and characterized as a polypeptide with an apparent molecular weight of 25 kDa and an isoelectric point of 8.9 (Jürgen *et al.*, 1985). The complete amino acid sequence was determined and compared with sequences of other Fc-binding proteins (Rühlmann *et al.*, 1988). Partial sequence homology was found between protein B and the Fc-binding region of protein A.

The genetic determinant of the CAMP factor from a strain of GBS was cloned and expressed in *E. coli* (Schneewind *et al.*, 1988). The *E. coli* clones containing the CAMP factor gene could cause a weak CAMP reaction in the conventional CAMP test and the expressed CAMP protein could be recognized by a specific rabbit antiserum against purified CAMP factor. However, in contrast to native GBS, where the CAMP factor is readily excreted, the CAMP protein was not excreted in *E. coli* clones but was found associated with the cytoplasmic and periplasmic fractions.

The CAMP factor gene of *A. pleuropneumoniae* was also cloned and expressed in *E. coli* (Frey *et al.*, 1989). The recombinant *E. coli* strain showed a positive CAMP reaction and expressed a protein with a molecular mass of 27,000 Da, similar in size to the CAMP protein of GBS. Rabbit antibodies

against the CAMP$^+$ clone neutralized the CAMP reaction mediated by the *E. coli* strain containing the cloned CAMP gene as well as that of *A. pleuropneumoniae*. Antibodies raised against the cloned CAMP protein cross-reacted with *S. agalactiae* CAMP factor.

2.3 Extracellular solute-binding receptors of bacteria

Bacteria may use different types of transport systems to scavenge solutes from the surrounding medium (Poolman *et al.*, 1994). The binding protein-dependent uptake systems have been found to transport a wide variety of substrates, including sugars, amino acids, opines, peptides, organic and inorganic phosphate esters, inorganic ions and vitamins (Tam and Saier, 1993). These binding protein-dependent transport systems belong to the ATP binding cassette-type (ABC-type), which typically consist of one or two transmembrane proteins which span the membrane five or six times each; one or two peripheral membrane ATP-binding proteins localized to the cytoplasmic side of the membrane, which may be anchored to the membrane via a single transmembrane helix; and an extracytoplasmic solute-binding protein (Higgins, 1992; Hoshino *et al.*, 1992; Kerppola and Ames, 1992). The transmembrane protein components are believed to form solute-specific channels; the peripheral

membrane ATP-binding proteins hydrolyse ATP and energize the systems and sometimes serve regulatory roles (Dean *et al.*, 1990), and the solute-binding proteins are known to serve as the initial receptors for transport, delivering substrate to the integral membrane components.

In Gram negative bacteria, the substrate-binding protein is soluble and periplasmic (Berger, 1973; Berger and Heppel, 1974; Higgins, 1992). Several binding proteins have been crystallized and their three-dimensional structures determined (reviewed in Adams and Oxender, 1989; Quiocho, 1990; Vyas *et al.*, 1991). All have a similar structure with two globular domains and a cleft in between that forms the substrate-binding site. Whatever the nature of the substrate (i.e. charged, hydrophobic, etc) it appears to be bound via hydrogen bonds (Quiocho, 1986; Pflugrath and Quiocho, 1988). The proteins undergo a conformational change upon binding of substrate. This conformational change traps the substrate in the cleft between the two domains (Sack *et al.*, 1989; Mao *et al.*, 1982) and enables the binding protein to interact with the appropriate complex of membrane proteins; this interaction does not seem to occur in the absence of bound substrate (Prossnitz *et al.*, 1988). It appears that the ligand-bound binding proteins confer specificity and high affinity for the substrate to the transport systems. Consistent with this, mutant forms of two ABC-type uptake systems, those specific for maltose and histidine, could

function without the periplasmic binding protein, but with low efficiency (Petronilli and Ames, 1991; Treptow and Shuman, 1988).

The presence of the ABC-type transport systems in Gram positive bacteria has only recently been documented. Gilson *et al.* (1988) provided evidence for the presence of such a system in *Streptococcus pneumoniae (S. pneumoniae)*. The gene for a solute-binding protein, MalX, has been sequenced. The MalX is a maltodextrin-binding protein which is 27% identical to the periplasmic MalE-binding protein of *E. coli*. The amino-terminal parts of both *E. coli malE* and *S. pneumoniae malX* gene products represent signal peptides characteristic of proteins which are exported through the cytoplasmic membrane. For MalE, it was directly shown that the first 26 residues are cleaved from the remainder of the protein following export (Duplay *et al.*, 1984). In MalX, however, the region of the potential cleavage site belongs to a well-defined category; it carries a sequence, L-V-A-C-G-S, corresponding to the consensus sequence for the lipoprotein precursors (reviewed in Wu, 1987; Yamaguchi *et al.*, 1988). Thus, the mature MalX protein appears likely to be a lipoprotein.

It is well known that some lipoproteins are exported through the cytoplasmic membrane, as are other secreted proteins, but following cleavage the amino-terminal cysteine is thioacylated to give a lipoamino acid. This

lipophilic modification is thought to be responsible for membrane anchorage of a number of exported proteins (Nielsen and Lampen, 1982). On the basis of the observed sequence comparisons and the biochemical requirements of the system, membrane attachment of MalX is likely to occur through the same mechanism. MalX could thus be exposed to the outer face of the membrane as an anchored but otherwise water-soluble protein. This membrane-anchored solute-binding protein would be expected to function properly, since a mutant *E. coli* MalE protein which is anchored by its uncleaved amino-terminal signal peptide to the external face of the cytoplasmic membrane can still function in transport (Fikes and Bassford, 1987).

The gene for a second solute binding protein, AmiA, was also sequenced from *S. pneumoniae* (Gilson *et al.*, 1988). This protein exhibits 24% identical residues with the oligopeptide-specific periplasmic binding protein OppA of *Salmonella typhimurium* (Hiles *et al.*, 1987). In OppA, an N-terminal peptide of 23 residues is cleaved from the remainder of the protein following export (Hiles and Higgins, 1986). For AmiA of *S. pneumoniae*, the region of the potential cleavage site carries the sequence L-A-A-C-S-S, corresponding exactly to the consensus of the lipoprotein precursors. Recently, an operon encoding a novel ABC-type amino acid transport system was fully characterized in *Bacillus subtilis* (Rodriguez and Grandi, 1995). Orf1, representing the solute-

binding component of the system, shares 31% identity with the glutamine-binding subunit GlnH of *E. coli*, 26% and 25% identity with the arginine- and histidine-binding subunits ArgT and HisJ of *Salmonella typhimurium* respectively, and 21% identity with the glutamine-binding subunit of *Bacillus stearothermophilus*. As expected, Orf1 has a typical leader sequence for secretion and the potential cleavage site (L-A-A-C-G-A) corresponds to the consensus sequence for the precursors of lipoproteins.

Since Gram positive bacteria lack an outer membrane and consequently have no periplasm, the solute-specific binding proteins are lipoproteins with an N-terminal glyceride-cysteine, which allows them to be tethered to the external surface of the cell membrane. This hydrophobic anchor maintains the binding protein in the proximity of the external face of the integral cell membrane components of the transport system.

Tam and Saier (1993) have recently reviewed the structural, functional and evolutionary characteristics of the extracellular solute-binding receptors upon 50 sequenced periplasmic binding proteins of Gram negative bacteria and homologous lipoproteins of Gram positive bacteria. The vast majority of these solute-binding proteins (46 proteins) were grouped into eight families or clusters, which generally correlated with the sequence similarities, molecular sizes and solute-binding specificities. A particular example is

cluster 3, in which binding proteins are specific for polar amino acids and opines and exist in the size range of 248 to 283 residues. The similarity plot for this group of proteins revealed regions of strong conservation in the N-terminal domains. A selected portion of the multiple alignment for the six cluster 3 binding proteins showed that 9 out off 57 residues in alignment were fully conserved, and substantial sequence similarity was exhibited between these proteins. Two signature sequences which characterize cluster 3 binding proteins have been derived from the portion in alignment.

2.4 Lactoferrin binding proteins in bacteria

Lactoferrin (Lf) is a mammalian iron-binding glycoprotein secreted by the polymorphonuclear leukocytes (PMNs) and various exocrine glands (Baggiolini *et al.*, 1970; Masson *et al.*, 1966). This protein is found at high concentrations in milk and also at mucosal surfaces (Masson *et al.*, 1966; Reiter and Oram, 1967). A regulatory function for Lf in various physiological pathways (Birgins, 1984), including the adhesion of PMNs to the endothelial surface (Oseas *et al.*, 1981), feedback inhibition of the granulocyte-monocyte colony-stimulating factor (Broxmeyer and Platzer, 1984; Broxmeyer *et al.*, 1978), and the regulation of antibody production (Duncan and McArthur, 1981), has been suggested. Specific interaction of Lf with certain mammalian cells seems

to be involved in the above pathways, and, accordingly, specific receptors for Lf were identified on macrophages, monocytes, B lymphocytes, PMNs, activated T lymphocytes, and hepatocytes (Bennet and Davis, 1981; Dehanne *et al.*, 1985; Maneva *et al.*, 1983; Rochard *et al.*, 1989; van Snick and Masson, 1976).

Lf inhibits the growth of *E. coli* and certain other microorganisms *in vitro* (Bullen *et al.*, 1972). This Lf-mediated antimicrobial action has mainly been attributed to its iron deprivation capacity with bacteria (Arnold *et al.*, 1977; Law and Reiter, 1977; Oram and Reiter, 1968). It is well known that with few exceptions, iron is essential for microbial growth (Weinberg, 1978). Even though iron is abundant within mammalian tissues, the environment which confronts bacterial pathogens has very little free iron, far below concentrations required to support bacterial growth (Weiberg, 1978). Virtually all iron within the mammalian body is held intracellularly as ferritin or as heme compounds, pools which are generally inaccessible to invading microorganisms. Additionally, the small amount of iron present in extracellular spaces is effectively chelated by high-affinity iron-binding host glycoproteins: transferrin (Tf) present in serum and lymph, and Lf in secretory fluids and milk (Otto *et al.*, 1992). Iron scavenging caused by these proteins reduces the free-iron concentration below $10^{-12}\mu M$ (Bullen, 1981), which is much lower than the iron

concentration required for bacterial growth (0.05 to 0.5 μM). To overcome this deficiency, bacterial pathogens have developed specific iron uptake mechanisms. In many bacterial species, these mechanisms are based on the synthesis and secretion of small compounds usually of <1,000 Da, called siderophores, which display high affinity for ferric iron (FeIII). More than 200 natural siderophores have been described. The majority of them are either hydroxamates or catecholates-phenolates. They are capable of removing transferrin- or lactoferrin-bound iron to form ferrisiderophore complexes which in turn are recognized by specific iron-repressible membrane receptors and internalized into the bacterium where the iron is released (Crosa, 1989). This iron uptake mechanism has been described in many bacterial species, such as *Vibrio anguillarum* (Crosa, 1989), *E. coli* (Köster and Braun, 1989), *Klebsiella pneumoniae* (Perry and Clemente, 1979), and *Pseudomonas aeruginosa* (Poole *et al.*, 1990). Some bacteria, such as *Neisseria gonorrhoeae* (McKenna *et al.*, 1988; Norrod and Williams, 1978), *Neisseria meningitidis* (Schryvers, 1988; Tsai *et al.*, 1988), *Haemophilus influenzae* type b (Morton and Williams, 1989; Schryvers and Gonzalez, 1989), *Actinobacillus pleuropneumoniae* (Gonzales *et al.*, 1990), and *Pasteurella haemolytica* (Ogunnariwo and Schryvers, 1990), do not secrete detectable siderophores when grown in an iron-deficient environment but produce outer membrane proteins that bind

directly and specifically to transferrin or lactoferrin, thereby allowing iron transport into the bacterial cell.

Tf binding appears to be mediated by the activity of two proteins present in the bacterial outer membranes, transferrin-binding protein 1 and 2 (Tbp1 and Tbp2) (Gonzalez *et al.*, 1990; Ogunnariwo and Schryvers, 1990; Schryvers, 1989; Schryvers and Lee, 1989; Schryvers and Morris. 1988b). Tbp1 varies in molecular mass from 98-115 kDa among gonococcal strains tested (Lee and Bryan, 1989; Cornelissen *et al.*, 1992). The meningococcal homologue has a molecular mass of 93-99 kDa (Rokbi *et al.*, 1993). In *H. influenzae*, Tbp1 is similarly estimated to be 90-105 kDa (Schryvers, 1989; Williams and Griffiths, 1992). This protein can be affinity purified from total membrane preparations by Tf affinity procedures (Schryvers, 1989; Schryvers and Lee, 1989; Cornelissen *et al.*, 1992; Williams and Griffiths, 1992). Sequence analysis of the structural gene encoding Tbp1 suggests that this protein is an integral, TonB-dependent outer membrane protein (Cornelissen *et al.*, 1992; Legrain *et al.*, 1993; Gray-Owen *et al.*, 1995). Tbp2 can also be affinity purified using Tf as the ligand (Schryvers, 1989; Schryvers and Lee, 1989). Unlike Tbp1 (Schryvers, 1989; Schryvers and Lee, 1989; Cornelissen *et al.*, 1992), Tbp2 binds Tf following SDS-PAGE and electroblotting (Schryvers, 1989; Schryvers and Lee, 1989; Cornelissen *et al.*, 1992; Anderson *et al.*, 1994). Most clinical gonococcal and meningococcal isolates express

Tbp2 proteins in the molecular mass range of 85-88 kDa (Schryvers and Lee, 1989; Rokbi et al., 1993; Anderson et al., 1994); however, a few isolates express a low molecular mass Tbp2 (68-70 kDa) (Lee and Bryan, 1989; Rokbi *et al.*, 1993). Likewise, most *H. influenzae* isolates express a 90 kDa Tbp2 although some express a Tbp2 of 70-76 kDa (Williams and Griffiths, 1992). Tbp2 is a surface-exposed lipoprotein in that it can be labelled with [^{14}C]-palmitic acid (Anderson *et al.*, 1994; Gerlach *et al.*, 1992). Consistent with this observation, the N-terminus of the predicted protein sequence contains a consensus cleavage site for signal peptidase II which catalyzes lipid modification of bacterial proteins (Anderson *et al.*, 1994; Gray-Owen *et al.*, 1995; Wu and Tokunaga, 1986). A highly structured and extremely stable Tbp2 domain of about 270 to 290 amino acids, which is involved in the binding to transferrin has been characterized in *Neisseria meningitidis* (Vonder Haar *et al.*, 1994). In *A. pleuropneumoniae*, it was found that the N-terminal half of the transferrin-binding protein (TfbA, Tbp2) is responsible for transferrin binding and three domains containing 13 or 14 amino acids in this region possessed transferrin-binding activity (Strutzberg *et al.*, 1995). Tbp2 and Tbp1 are likely co-expressed from the tandemly arranged, co-transcribed genes, *tbpB* and *tbpA*, respectively (Legrain *et al.*, 1993; Anderson *et al.*, 1994; Gray-Owen *et al.*, 1995). The *tbpB* gene is preceded by a consensus Fur-binding site (Legrain *et al.*,

1993; Anderson *et al.*, 1994; Gray-Owen *et al.*, 1995) suggesting the involvement of the Fur regulatory protein in gene expression. Characterization of isogenic Tbp mutants has confirmed the importance of both receptor proteins in Tf-mediated iron acquisition and growth. Tbp1 appears to play an essential role, since Tbp1$^-$ Tbp2$^+$ mutants are unable to grow on media supplemented with Tf as the sole iron source (Anderson *et al.*, 1994; Cornelissen *et al.*, 1992; Irwin *et al.*, 1993; Gray-Owen *et al.*, 1995). Tbp2, however, is not strictly necessary for growth on Tf but makes iron utilization more efficient (Anderson *et al.*, 1994; Cornelissen *et al.*, 1992; Gray-Owen *et al.*, 1995). Hence, the primary function of Tbp2 is likely to help bring the large host glycoprotein in the external milieu to the cell surface, thus allowing for the juxtaposition of Tf and Tbp1 directly on the cell surface, at which point iron can be removed.

The mechanism of iron uptake from Lf has not been well characterized. It may resemble that of iron acquisition from Tf. A putative 105 kDa receptor for Lf utilization was identified in the gonococcus by affinity isolation (Schryvers and Lee, 1989; Cornelissen *et al.*, 1992; Lee and Bryan, 1989) and the structural gene (*lbpA*) for this Lf-binding protein (Lbp1) has been isolated (Biswas and Sparling, 1995). The genes for meningococcal lactoferrin receptors have also been characterized (Petterson *et al.*, 1993; 1994a; 1994b). The DNA sequence of *lbpA* and the predicted amino acid sequence of the

Lbp1 in the gonococcus and the meningococcus are highly conserved (94% identity). Lbp1 was 46% identical to Tbp1 (Cornelissen *et al.*, 1992) of the same gonococcal strain but only 18% identical to Tbp2 (Anderson *et al.*, 1994). Both gonococcal and meningococcal genes contain relatively well-conserved Fur boxes and the proteins are homologous to the TonB-dependent family of receptors, as was true for Tbp1 (Cornelissen *et al.*, 1992) but not for Tbp2 (Anderson *et al.*, 1994). The strong similarity between the Lf receptor protein Lbp1 and the Tf receptor protein Tbp1 suggests that the binding of Lf to bacterial cells might be similar to Tf binding. Consistent with this, the putative protein encoded by the open reading frame (*lbpB*) upstream of *lbpA* shows extensive homology to Tbp2 (Pettersson *et al.*, 1994b), suggesting that iron-acquisition from Lf, like from Tf, requires two specific proteins in the outer membrane.

In contrast to our knowledge of the iron uptake systems of Gram negative bacteria, there is comparatively little information concerning the mechanisms by which Gram positive pathogens acquire iron during growth in extracellular body fluids. Both *S. aureus* and the coagulase-negative staphylococci have been reported to produce siderophores (Konetschny-Rapp *et al.*, 1991; Meiwes *et al.*, 1990). Of these, a carboxylate-type siderophore termed staphyloferrin A has been isolated and chemically characterized. The ability of staphyloferrin to compete for

transferrin-bound iron was not, however, reported. *S. aureus* appears capable of binding both human and bovine Lfs and human Tf (Naidu *et al.*, 1991a; 1991b; 1992; Modun *et al.*, 1994), while coagulase-negative staphylococci from bovine mastitis and human peritonitis can bind bovine Lf and human transferrin respectively (Naidu *et al.*, 1990; Modun *et al.*, 1994). The interaction of Lf with a bovine *S. agalactiae* strain has also been reported (Rainard, 1992). However, the iron acquisition functions of these Tf- or Lf- binding proteins need to be studied.

2.5 Streptococcal M and M-like proteins

The group A streptococcal M protein is considered one of the major virulence factors of this organism by virtue of its ability to impede attack by human phagocytes (Lancefield, 1962). The bacteria persist in the infected tissue until antibodies are produced against the M molecule. Type-specific antibodies to the M protein are able to reverse the antiphagocytic effect of the molecule and allow efficient clearance of the invading organism.

M proteins form dimeric fibrils projecting from the cell wall of group A streptococcal cell surfaces (Fischetti, 1989; Robinson and Kehoe, 1992). With the exception of the domain that spans the cell wall and membrane, and a short random coil sequence at the extreme amino terminus, M

proteins contain a seven-residue periodicity that produces an α-helical coiled-coil secondary structure throughout the molecule (Fischetti, 1989). This periodicity tolerates considerable variation in the primary sequence, while maintaining the α-helical coiled-coil structure. It has been found that M proteins share physicochemical properties and significant sequence homology with a number of mammalian fibrillar proteins containing α-helical coil structures. For example, the M 5, M 6, M 12 and M 24 proteins have up to 30% overall sequence homology with myosin heavy chain and type I keratins, and M 5, M 6 and M 24 share a similar level of homology with human α-tropomyosin (Fischetti, 1989). M proteins have also been shown to have limited homology with desmin, laminin and vimentin, but lack homology with human fibrinogen α-chain, actin or type II keratins (Fischetti, 1989; McFadden *et al.*, 1991). These patterns of homology provide a structural basis to explain the induction of cross-reactive antibodies associated with post-streptococcal autoimmune disease.

DNA sequencing and hybridization studies of cloned M protein genes have shown that the carboxy-terminal halves of M proteins proximal to the streptococcal cell surface are highly conserved between different M proteins, whereas the amino-terminal halves, which protrude from the cell, are highly variable (Fischetti, 1989; Kehoe, 1991; Scott *et al.*, 1986). Detailed amino acid sequence comparisons and antibody

reactivities revealed limited differences within the conserved carboxy-terminal regions, suggesting that there may be two major classes (class I and class II) of M proteins (Bessen and Fischetti, 1990b; Bessen *et al.*, 1989). However, the much more extensive variability at the amino terminus forms the basis for serological typing of M proteins, with over 100 distinct M types being identified to date (Kehoe, 1991).

A striking structural feature of all M proteins studied to date is the presence of extensive regions of tandem repeats, in both the conserved and variable halves of the molecule (Fischetti, 1989). The number of distinct repeats and their size vary between M types. The M6 protein, whose gene was first cloned and sequenced in *E. coli* (Scott and Fischetti, 1983; Hollingshead *et al.*, 1986), is composed of four repeat blocks, each differing in size and sequence (Fischetti *et al.*, 1988; Hollingshead *et al.*, 1986). The A repeats are composed of 14 amino acids each in which the central blocks are identical and the end blocks slightly diverge from the central consensus repeats. The B repeats, composed of 25 amino acids each, are arranged the same as the A repeats. The C repeats, composed of 2.5 blocks of 42 amino acids, are not as identical to each other as are the A and B repeats. There are also four short D repeats which show some homology. These repeat regions make up the central helical rod region of the M molecule because of the high helical

potential ascribed to the amino acids found in this region as determined by conformational analysis (Fischetti *et al.*, 1988; Phillips *et al.*, 1981).

Analysis of the DNA sequence of the C-terminal region of the M6 molecule reveals a stretch of 20 essentially hydrophobic amino acids that could act as a membrane anchor and a proline- and glycine-rich region adjacent to the D-repeat blocks that is likely to be located within the streptococcal cell wall (Hollingshead *et al.*, 1986). A short six-amino acid sequence L-P-S-T-G-E is close to the hydrophobic region. Hence, the M protein appears to embody the common characteristics shared by many Gram positive bacterial surface proteins (Fischetti *et al.*, 1991).

M proteins are one of the key virulence factors of *S. pyogenes,* due to their involvement in mediating resistance to phagocytosis (Kehoe, 1991) and their ability to induce potentially harmful host immune responses via their superantigenicity and their capacity to induce host-cross-reactive antibody responses (Bisno, 1991; Froude *et al.*, 1989; Stollerman, 1991).

Inhibition of nonimmune opsonization is due principally to the ability of M proteins to bind fibrinogen (Poirier *et al.*, 1989) or the complement control protein factor H (Horstmann *et al.*, 1988), although other streptococcal cell surface structures, including the hyaluronic acid capsule (Wessels *et al.*, 1991), contribute to

the ability of streptococci to resist phagocytosis. It has been suggested that the interaction of plasma fibrinogen with M proteins masks C3b-binding sites on the streptococcal surface (Poirier *et al.*, 1989), and that binding of factor H to M proteins leads to inhibition of the alternative C3 convertase and the classical C5 convertase of complement (Hong *et al.*, 1990): in each case, subsequent phagocytosis by C3b receptor-bearing neutrophils would be reduced or prevented.

Superantigens are proteins that polyclonally activate T cells by an MHC class-II-dependent, but haplotype-unrestricted, mechanism. Proliferative responses to superantigens are limited to T cells expressing particular TCR V_β gene families and are independent of antigen specificity (Herman *et al.*, 1991). The proliferation of human T cells to pepM5 (the amino-terminal halves of M5 protein cleaved from streptococci with pepsin) is MHC class II dependent but not MHC haplotype restricted (Tomai *et al.*, 1990), indicating that pepM5 has some of the properties of superantigens.

Many M proteins elicit antibodies that cross-react with mammalian tissues, and such cross-reactive antibodies are present in the sera of patients with acute rheumatic fever and post-streptococcal glomerulonephritis (Kehoe, 1991; Froude *et al.*, 1989). Sera from experimental animals immunized with pepM fragments or with synthetic peptides from

M5 and M6 proteins, as well as sera from rheumatic fever patients, contain antibodies that bind M proteins and cross-react with human heart tissue (Fischetti, 1989; Froude *et al.*, 1989; Cunningham *et al.*, 1989; Dell *et al.*, 1991). This cross-reactivity has been localized to myosin (Fischetti, 1989; Froude *et al.*, 1989; Dell *et al.*, 1991), and to a 40-50 kDa protein of the sarcolemmal membrane (Beachey *et al.*, 1988; Sargent *et al.*, 1987). Sera from patients with post-streptococcal glomerulonephritis contain antibodies against basement membrane antigens (Fillit *et al.*, 1985), and sera from rabbits immunized with synthetic peptides from M1 and M12 proteins have been shown to react with mesangial cells of human renal glomeruli (Kraus *et al.*, 1989a). Furthermore, these anti-peptide antibodies react with either vimentin (56 kDa) or a distinct 43 kDa protein isolated from mesangial cells (Kraus *et al.*, 1989a; 1989b).

Attempts have been made to apply the knowledge of the M protein's structure and function to the development of a vaccine for preventing streptococcal infection. There is extensive evidence to suggest that opsonic antibody epitopes, which confer systemic protection against group A streptococcal infections and which are least likely to cross-react with the host, are located in the extreme amino-terminal region of M protein molecules (Fischetti, 1989; Kehoe, 1991). However, these protective antibody epitopes are M type-specific, so that effective vaccine will require the

inclusion of epitopes from several important M types. Opsonic, non-host-cross-reactive epitopes can clearly be separated from host-cross-reactive epitopes in M5 and M6 proteins, with the former located in the amino terminal ten or so amino acids, and the latter largely confined to the central region of the molecule (Kehoe, 1991; Beachey *et al.*, 1988; Jones and Fischetti, 1988). Few attempts have been made to test streptococcal vaccines in humans. PepM fragments of the non-host-cross-reactive M24 protein, or synthetic peptides from the amino terminus of a number of M proteins, have been shown to induce opsonic antibodies in human volunteers (Kehoe, 1991). Rabbits immunized with synthetic peptides, including a combination of antibody epitopes from the amino terminus of M5, M6 and M24, produced type-specific opsonic antibodies (Beachey *et al.*, 1987). An alternative strategy has taken advantage of the conserved region of the M molecule that are not buried within the cell wall or membrane. Antibody epitopes located in the carboxy-terminal half of M proteins can induce non-type-specific mucosal immunity in rodent models when delivered to the mucosal surface (Fischetti, 1989). Immunization with peptides from the carboxy-terminal half of M6 coupled to cholera toxin induced non-type-specific protection of mice against pharyngeal colonization by streptococci (Bessen and Fischetti, 1988). These epitopes are thought to be distinct from host-cross-reactive epitopes, which have not been

reported in this region to date. Hence, it seems possible to sensitize the animals' immune systems against the conserved regions of M proteins. By preventing the initial colonizing step of an infection, it should be possible to circumvent the need for type-specific antibodies. Type-specific antibodies become necessary for overcoming the infection only after streptococcal have colonized and invaded tissues.

The M-like protein family is constituted by a number of streptococcal surface proteins that have some sequence and structural similarity to M proteins (Fischetti, 1989; Kehoe, 1994; Whatmore and Kehoe, 1994; Frithz *et al.*, 1989; Gomi *et al.*, 1990; Haanes and Cleary, 1989; Heath and Cleary, 1989; Bessen and Fischetti, 1992; Jeppson *et al.*, 1992). As in the case of M protein itself, the M-like proteins have nonhelical extreme amino termini, an α helical, coiled-coil conformation that forms central rods, proline- and glycine-rich regions that thread through the peptidoglycan layer, hydrophobic membrane anchors and short cytoplasmic tails at their carboxyl termini. The central rod region is divided into several internal repeated blocks, termed the A, B and C repeats, which vary in number. Studies showed that in all group A streptococcal strains, *emm* and *emm*-like genes are located at the same position in the chromosome, between the positive regulator *virR* gene and the C5a peptidase gene (*scpA*), in a locus called the *vir* regulon (Haanes *et al.*, 1992; Hollingshead *et al.*, 1993; Podbielski,

1993).

M-like proteins are not considered to be M proteins because an antiphagocytic property has not been demonstrated. Some of these proteins bind fibrinogen, others bind the Fc-domains of IgG and/or IgA rather than fibrinogen, and some bind both fibrinogen and IgG Fc-domains (Bessen and Fischetti, 1992; Gomi *et al.*, 1990; Heath and Cleary, 1989; Lindahl and Åkerström, 1989; Retnoningrum *et al.*, 1993; Schmidt and Wadström, 1990; Stenberg *et al.*, 1992). The possible roles of these proteins in virulence have been reviewed recently by Cleary and Retnoningrum (1994).

2.6 Mga regulatory proteins

In group A streptococci (GAS), the genes for M protein (*emm*) and C5a peptidase (*scpA*) and, if present, the genes encoding the M protein-related IgG- and IgA-binding proteins (*fcrA* and *enn*, respectively) are clustered on the chromosome (Haanes *et al.*, 1992; Hollingshead *et al.*, 1993; Podbielski, 1993). Expression of these virulence-associated surface proteins is coregulated at the level of transcription by the protein Mga (stands for multigene regulator of group A Streptococcus'), formerly called Mry or VirR (Caparon and Scott, 1987; Chen *et al.*, 1993; Haanes and Cleary, 1989; McIver *et al.*, 1995a; Perez-Casal *et al.*, 1991; Podbielski *et al.*, 1995; Podbielski, 1992; Robbins *et al.*, 1987). The

complete cluster was tentatively termed the *vir* regulon for consistency with the nomenclature established for other bacterial pathogens (Cleary *et al.*, 1991).

With increasing data on the GAS genomic organization (Haanes *et al.*, 1992; Hollingshead *et al.*, 1993; Podbielski, 1993), it became obvious that OF^+ (serum opacity factor-producing) strains, which express class II M proteins and Ig-binding proteins (Bessen and Fischetti, 1990; Wannamaker, 1970) exhibit a uniform structure of their *vir* regulons, which always harbour *fcrA*, class II *emm*, and *enn* genes. In contrast, the *vir* regulon of OF^- (serum opacity factor-nonproducing) GAS which express class I M proteins, but do not always produce Ig-binding proteins (Bessen and Fischetti, 1990; Wannamaker, 1970) could be associated with several different forms of architecture. The loci of these strains always contain a class I *emm* gene and frequently do not include *fcrA* and/or *enn* genes. The relationship of the genes in representative GAS *vir* regulons is shown in Fig. 2-1.

The *virR* genes of OF^- GAS serotypes not harbouring *fcrA* or *enn* genes were first analyzed. In a serotype M12 strain isolated from a pharyngitis patient, the expression of *emm* and *scpA* was shown to be under the control of a positive regulator gene *virR* (Robbins *et al.*, 1987; Simpson *et al.*, 1990). The *virR* gene was found to be located upstream of *emm* and *scpA* (Simpson and Cleary, 1987). In addition to *virR12*,

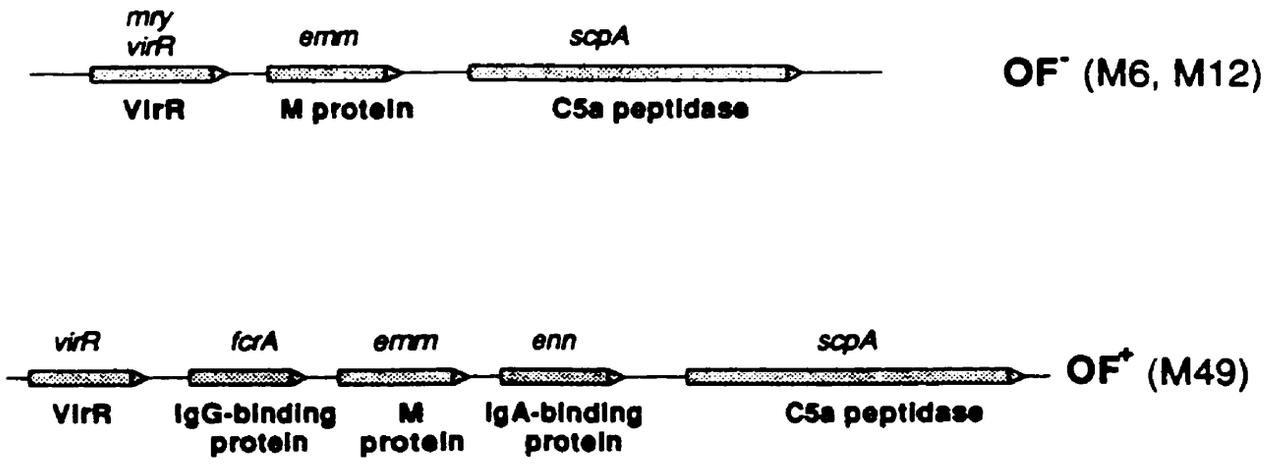


Figure 2-1. Maps of representative GAS *vir* regulons. Map of a *vir* regulon from OF⁻ GAS, e.g., serotypes M6 and M12 is shown on the top. The product of the *virR* gene (equivalent name, *mry*) regulates positively the expression of itself and of the genes encoding M protein (*emm*) and C5a peptidase (*scpA*). Map of a *vir* regulon from OF⁺ GAS, e.g. serotype M49 is presented at the bottom. Here the product of the *virR* gene controls the expression of the genes encoding the IgG-binding protein (*fcrA*) and IgA-binding protein (*enn*) in addition to *emm*, *scpA*, and probably *virR*.

an equivalent locus termed *mry* (for M protein RNA yield) was identified as regulating *emm* expression in a serotype M6 GAS strain (Caparon and Scott, 1987). Comparison of the

nucleotide sequence of *mry* (Perez-Casal *et al.*, 1991) and *virR12* (Chen *et al.*, 1993) revealed 97% homology. Also, a partial *virR1* sequence (Haanes-Fritz *et al.*, 1988) was nearly identical to the *virR12* sequence. The presence of *virR* geneshomologous to *mry* has been demonstrated in one strain each of serotypes M1 and M3 (Perez-Casal *et al.*, 1993).

The *mry* promoters were localized subsequent to the identification of the 5′ ends of the specific mRNAs by performing S1 nuclease protection assays (Okada *et al.*, 1993). The *mry* gene was found to possess two promoters, P1 and P2, located approximately 410 and 120 bp, respectively, upstream of the *mry* start codon. P1 exhibited a weak constitutive activity, whereas P2 was demonstrated to be a comparatively strong promoter which needed induction by the Mry protein. Recently, *mry* expression was described as being potentially even more complex since under special experimental conditions the P1 promoter showed no constitutive activity (Geist *et al.*, 1993).

A recombinant *mry* gene was the first *virR*-type gene which was expressed in an *in vitro* transcription-translation system (Perez-Casal *et al.*, 1991). However, only after establishing recombinant reporter plasmids containing the *emm12* and *scpA12* promoters in the serotype M12 GAS could the trans-regulating activity of VirR be unambiguously demonstrated (Podbielski, 1992). In addition, potential VirR-binding boxes immediately upstream of the -35 boxes of *emm*

and *scpA* promoters of OF$^-$ strains were identified by homology comparisons. Sequences nearly identical to such boxes were also found in intergenic regions upstream of *fcrA, emm, enn,* and *scpA* genes from OF$^+$ strains, indicating a similar control in OF$^+$ strains.

In OF$^+$ GAS strains, the presence of genomic regions homologous to *virR* has been documented by using two different approaches: hybridization with recombinant internal *virR* fragments (Haanes *et al.*, 1992; Hollingshead *et al.*, 1993) and a series of PCR assays (Podbielski *et al.*, 1992). A conclusion of these studies was that sequences of potential *virR* genes from OF$^+$ strains diverged considerably from corresponding ones harboured by OF$^-$ strains. Recently, the *virR* gene region of the OF$^+$ GAS M49 strain CS101 was amplified by PCR, and 2,650 bp were directly sequenced (Podbielski *et al.*, 1995). An open reading frame of 1,599 bp exhibited 76% overall homology to the *virR12* and *mry* sequences. When comparing the deduced amino acid sequences of these three ORFs, the first 484 residues showed 82.3% homology, whereas the 40 C-terminal residues of VirR49 differed completely from the VirR12 and Mry (VirR6) sequences. The C-terminal 40 codons of *virR49* were found to be perfectly conserved in more than 20 other OF$^+$ serotypes and thus could be generally regarded as specific for *virR* genes of OF$^+$ GAS strains. The 5′ ends of two specific transcripts were mapped 370 and 174 bp upstream of the start codon of

this open reading frame. The deduced sequences of the corresponding promoters and their locations differed from those of previously reported *virR* promoters. Transcripts from wild-type *fcrA49, emm49, enn49,* and *scpA49* genes located downstream of *virR49* were characterized as being monocistronic. Insertional inactivation provided evidence that *virR49* controlled expression of *fcrA49, emm49, enn49,* and *scpA49* and that it probably regulated its own expression. Upon the loss of *virR49* function, the mutant did not express Ig-binding proteins and became sensitive to phagocytosis by human leukocytes.

In many bacterial pathogens, the synthesis of virulence factors is influenced by the environment. This adaptation to environmental changes is often mediated by a two-component signal transducing system (Albright *et al.*, 1989; Gross *et al.*, 1989; Miller *et al.*, 1989; Stock *et al.*, 1989). In such a system, the first component is a membrane-spanning sensor protein which, in response to environmental change, transmits a signal (usually phosphorylation) to the second component or receptor protein. The latter regulates transcription of genes under environmental control. Examples of these systems used by bacterial pathogens include the VirA (sensor) (Jin *et al.*, 1990) and VirG (receptor) (Powell *et al.*, 1989) proteins of *Agrobacterium tumefaciens*, ToxR of *Vibrio cholerae*, which has both a sensor and a receptor motif separated by a region that spans the cytoplasmic membrane

(Miller *et al.*, 1987), the *vir* region of *Bordetella pertussis,* which encodes three products with homology to the signal transducing proteins (Arico *et al.*, 1989), and the AgrA protein of *Staphylococcus aureus*, which shows homology with the receptor components (Peng *et al.*, 1988; Stock *et al.*, 1989).

Like other bacterial pathogens, GAS are exposed to a differing environmental conditions as they reside on the skin, mucous membranes or in the bloodstream of the human host. Accordingly, the functional requirements for expression of the different streptococcal surface proteins should vary. In fact, in parallel experiments in which GAS strains were exposed to human blood or isolated from the spleen of animals with experimental skin infections, a variation of the expression patterns of M proteins and Ig-binding proteins were demonstrated by Raeder and Boyle (1993a; 1993b). It was found that transcription of the genes encoding M protein (*emm*) and Scp (*scpA*) was stimulated by growth in elevated carbon dioxide and slightly decreased in elevated oxygen (Caparon *et al.*, 1992; Podbielski *et al.*, 1992). A recent study showed that transcription of *emm* was also influenced by osmolarity, temperature, gas exchange, and iron limitation (McIver *et al.*, 1995b). Since transcription of *mry* is environmentally regulated by the same signals (i.e the CO_2 level) that regulate expression of M protein gene (Okada *et al.*, 1993), it is thought that Mga is a part of a crucial

regulatory system in GAS, possibly functioning as the second component in a two-component regulatory system, even though the one or several Mry-interacting sensors for external stimuli remain unidentified.

Consistent with its role as a two-component response regulator, Mry shows two regions that resemble the conserved sensor-recognizing domain in the receptor protein (Perez-Casal *et al.*, 1991; Stock *et al.*, 1989). Moreover, it includes two potential Helix-turn-helix DNA binding domains which are used by many other receptor components in regulating transcription of the operons they control (Perez-Casal *et al.*, 1991; Stock *et al.*, 1989). However, no other receptor-type proteins with two of each type of domains have been described. It is possible that each receptor domain of Mry responds to a different group of environmental signals and that each DNA-binding domain regulates a different subset of genes. Unlike Mry, VirR12 was found to contain only one similar helix-turn-helix DNA binding domain at the N-terminus (Chen *et al.*, 1993). Interestingly, while studying the *virR49* gene, Podbielski *et al.* (1995) predicted a potential DNA-binding helix-turn-helix motif with a different sequence from those of Mry, VirR12 and other DNA binding proteins (Sauer *et al.*, 1982; Pabo and Sauer, 1984). However, in this region Mry and VirR12 exhibited the largest stretch of contiguous sequence identity to VirR49 (Podbielski *et al.*, 1995).

3.0 CHARACTERIZATION OF *S. UBERIS* CAMP FACTOR GENE AND ITS UPSTREAM ABP LOCUS (ENCODING A PROTEIN HOMOLOGOUS TO POLAR AMINO ACID AND OPINE BINDING PROTEINS OF GRAM NEGATIVE BACTERIA)

3.1 Summary

The gene coding for the CAMP factor from a strain of *Streptococcus uberis* (ATCC 9927) was cloned in *Escherichia coli*. Chromosomal DNA from *Streptococcus uberis* was used to construct a gene library in plasmid pTZ18R and six CAMP-reaction positive clones were obtained from a total of 10,000 transformants. One clone, pJLD21, was subcloned and the CAMP factor gene was located in a 3.2 kb *Bam*HI fragment. The nucleotide sequence of *Streptococcus uberis* CAMP factor gene was determined and the deduced amino acid sequence is highly homologous to the corresponding *Streptococcus agalactiae* protein. Immunoblot analysis revealed that the recombinant strain pJLD21 expressed a protein with a molecular weight of 28,000. Antibodies raised against purified *Streptococcus uberis* CAMP factor cross-reacted with *Streptococcus agalactiae* protein B. Southern blot analysis demonstrated

that the six CAMP-reaction positive *E. coli* clones contained the same CAMP factor gene, and this gene existed in three out of eight *S. uberis* strains.

An ORF was identified upstream of the CAMP factor gene, which could encode a 277-residue protein. On the basis of sequence characteristics, this gene product is potentially a polar amino acid and opine binding protein of an ABC-type transport system.

3.2 Introduction

Streptococcus uberis is an important cause of mastitis in dairy cattle and is responsible for about 20% of all clinical cases (Bramley, 1987; Bramley and Dodd, 1984; Watts, 1988). Since antimicrobial treatment is generally ineffective in control of *S. uberis* mastitis, the development of new control measures must be based on an understanding of virulence factors and protective antigens involved in invasion and protection of the mammary gland (Collins *et al.*, 1988; Leigh *et al.*, 1990; Marshall *et al.*, 1986). It is known that some *S. uberis* strains can produce a hyaluronic acid capsule (Hill, 1988), hyaluronidase (Schaufuss *et al.*, 1989), R-like protein (Groschup and Timoney, 1993), and a cohemolysin, the CAMP factor (Skalka and Smola, 1981). However, very little is known of their roles in pathogenicity.

The effect of CAMP factor was first described by Christie *et al* in 1944 (Christie *et al.*, 1944). These authors found that group B streptococci (*S. agalactiae*) produced a distinct zone of complete hemolysis when grown near the diffusion zone of the *Staphylococcus aureus* β-toxin (sphingomyelinase). This phenomenon was called the CAMP reaction and the compound responsible for it was named as the CAMP factor, an extracellular protein with a molecular weight of 23,500 (Bernheimer *et al.*, 1979) and a pI of 8.9 (Jürgens

et al., 1985). The amino acid sequence was determined by Rühlmann *et al.*(1988).

The mechanism of the CAMP reaction was disclosed by the work of Bernheimer *et al.* (1979), Sterzik *et al.* (1984; 1985), and Fehrenbach *et al.* (1984; 1988). The CAMP factor has lytic activity on a variety of target cells in addition to sheep and bovine erythrocytes and artificial membranes in which membrane phospholipids and sphingomyelin were previously hydrolyzed by phospholipase or sphingomyelinase.

The role of CAMP factor in pathogenicity is unclear, but the following observations suggest that it is a virulence determinant. A partially purified CAMP factor from *S. agalactiae* was lethal to rabbits when injected intravenously (Skalka and Smola, 1981); intraperitoneal injection of purified CAMP factor into mice significantly raised the pathogenicity of sublethal dose of group B streptococci (Fehrenbach *et al.*, 1988); and, like protein A of *S. aureus*, CAMP factor can bind the Fc sites of immunoglobulins and was therefore designed protein B (Jürgens *et al.*, 1987).

In addition to group B streptococci and *S. uberis*, a few other bacteria, including *Listeria monocytogenes* and *Listeria seeligeri* (Rocourt and Grimont, 1983), *Aeromonas* sp. (Figura and Guglielmetti, 1987), *Rhodococcus equi* (Fraser, 1961), *A. pleuropneumoniae* (Kilian, 1976), and certain *Vibro* spp. (Köhler, 1988) produce reactions similar to the CAMP

effect. The CAMP factor genes of group B streptococci and *A. pleuropneumoniae* have been cloned and expressed in *Escherichia coli* (Frey *et al.*, 1989; Schneewind *et al.*, 1988).

It is well known that a wide variety of substrates such as sugars, amino acids, opines, peptides, anions and metals are imported into bacteria by binding protein-dependent ABC-type (or ATP-binding cassette type) transport systems. In Gram negative bacteria, the periplasmic binding protein interacts with the import complex in the inner membrane. The import complex usually consists of two hydrophobic transmembrane proteins which form the translocation pathway and channel the substrate to the cytoplasm. The peripheral membrane ATP-binding proteins localized to the cytoplasmic side of the membrane are tightly associated with the transmembrane proteins and provide energy to the transport system by driving ATP hydrolysis (Higgins, 1992; Fath and Kolter, 1993).

The presence of binding protein-dependent transport systems in Gram positive bacteria has only recently been documented. The oligopeptide transport systems *amiA* of *Streptococcus pneumoniae* (Alloing *et al.*, 1990), *spoOK* and *app* of *Bacillus subtilis* (Koide and Hoch, 1994; Perego *et al.*, 1991; Rudner *et al.*, 1991); the ribose and dipeptide transport systems *rbs* and *dciA* of *Bacillus subtilis* (Mathiopoulos *et al.*, 1991; Woodson and Devine, 1994); the

glutamine transport system of *Bacillus stearothermophilus* (Wu and Welker, 1991) and the amino acid transport system of *Bacillus subtilis* (Rodriguez and Grandi, 1995) have been identified and characterized. It appears that the overall structural organization of these systems is highly similar to their counterparts in Gram negative bacteria. However, since Gram positive organisms lack an outer membrane and consequently have no periplasm, the solute-specific binding proteins are lipoproteins with an N-terminal glyceride-cysteine, which allows them to be tethered to the external surface of the cell membrane in the proximity of the integral cell membrane components of the transport system (Gilson *et al.*, 1988; Tam and Saier, 1993).

To study the CAMP factor of *S. uberis* and compare it with those of *S. agalactiae* and other bacteria, we cloned, sequenced and expressed the CAMP factor gene of *S. uberis*. Also, we report here on an *abp* gene upstream the CAMP factor gene. This gene encoded a 31 kDa protein which, on the basis of sequence homology, is likely to be an amino acid-binding protein of an ABC-type amino acid transport system. To our knowledge, this is the first putative amino acid-binding protein identified in *S. uberis*.

3.3 Materials and Methods

3.3.1 Bacterial strains, plasmids and growth conditions.

The *Escherichia coli* strains used were JF1754 (*hsdR lac gal metB leuB hisB*) (McNeil and Friesen, 1981) and JM105 (Yanisch-Perron *et al.*, 1985), which are from our laboratory collection. Competent *E. coli* JF1754 and JM105 were made as described (Hanahan, 1985). *E. coli* cells were grown in Luria broth (Difco Laboratories) or on Luria-agar (Difco Laboratories) plates. Terrific broth (Maniatis *et al.*, 1989) was used when preparing protein inclusion bodies. Ampicillin was used at 50 µg/ml for the growth of *E. coli* strains containing recombinant plasmids. Four *S. uberis* strains (su-1, su-2, su-3 and su-4), *S. agalactiae* and *S. aureus* were obtained from the American Type Culture Collection (ATCC numbers are 9927, 13386, 13387, 19436, 27541 and 25923, respectively). Other *S. uberis* strains (su-5, su-6, su-7 and su-8) were field isolates kindly provided by M. Chirino-Trejo, University of Saskatchewan. All streptococcal strains were grown in brain heart infusion broth (BHI, Difco Laboratories) or on base #2 blood agar plates containing 5% sheep blood (PML microbiologicals).

All bacteria were cultured at 37°C except those for inclusion body preparations (see 3.3.5).

The cloning vectors used were pTZ18R (Mead *et al.*,

1986) and pUEX3 (Bressan and Stanley, 1987).

3.3.2 Preparation of *S. aureus* β-toxin.

S. aureus (ATCC 25923) was cultured in BHI for 18 h at 37°C and the supernatant obtained after centrifugation at 5,000 g was sterilized by filtration through a 0.22-μm filter (Nalge company). This material is referred to as crude β-toxin and was stored at -20°C.

3.3.3 CAMP reaction.

Plate assay: This was carried out as described by Schneewind *et al* (1988). Briefly, strains were streaked perpendicular to a streak of β-toxin-producing *S. aureus* on blood agar plates and after 6 h - 20 h incubation at 37°C, they were observed for hemolysis.

Tube assay (liquid assay): This was adapted from the procedure of Phillips *et al.* (1980). A 0.25-ml amount of the 2% sheep RBC suspension was mixed with 0.5 ml of the staphylococcal β-toxin preparation diluted 1:2000. The mixture was incubated at 37°C for 30 min. A 0.25 ml volume of streptococcal supernatant was added to this mixture and incubated at 37°C for 1 h. After centrifugation at 1,300 x g for 2 min, the A540 value of the supernatant was read, which reflected the amount of hemoglobin from RBC lysis. The percentage of hemoglobin release was calculated by the formula: (A_{540} of the RBC suspension exposed to test

supernatant - A_{540} resulting from spontaneous release from untreated RBC)/(A_{540} of completely lysed RBC - A_{540} of spontaneous release) x 100% (Devenish, et al., 1992).

CAMP assay after native PAGE: a native PAGE gel strip containing purified CAMP factor was aligned in the β-toxin zone of *S. aureus* on a base #2 blood agar plate. After 2 hours' incubation at 37°C, the hemolytic region was observed. An identical CAMP factor-containing gel strip was stained with coomassie blue, which showed the position of the CAMP factor in the native gel.

3.3.4 Purification of CAMP factor.

CAMP factor was partially purified from the culture supernatant of *S. uberis* by Octyl-Sepharose CL-4B (Pharmacia) chromatography as described by Jürgens *et al.* (1985).

3.3.5 Preparation of protein inclusion bodies.

The procedure used for preparation of protein inclusion bodies was described by Gerlach *et al.* (1992). A mid-log phase broth culture (1 L) grown at 30°C was cultured at 42°C for 2 h with vigorous shaking for protein induction. Cells were harvested by centrifugation, resuspended in 5 ml of 25% sucrose-50 mM Tris-HCl buffer (pH 8.0), and frozen at -70°C. Lysis was achieved by the addition of 1 mg of lysozyme in 250 mM Tris-HCl buffer (pH 8.0), 10 min of incubation on ice, addition of 25 ml of a detergent mix (5 parts of 20 mM

Tris-HCl buffer, pH 7.4, 300 mM NaCl, 2% deoxycholic acid, 2% Nonidet P-40 and 4 parts of 100 mM Tris-HCl buffer, pH 8.0, 50 mM EDTA, 2% Triton X-100), and sonication. Inclusion bodies were harvested by centrifugation for 30 min at 15,000 x g and resuspended in H_2O to a concentration of 5 to 10 mg/ml.

3.3.6 Polyclonal antibodies.

Mouse polyclonal antibodies against the purified CAMP factor were generated as follows. Mice were immunized by intraperitoneal injection with 20 μg of the purified CAMP protein in complete Freund's adjuvant. This primary immunization was followed 3 weeks later by a second intraperitoneal injection of the same amount of CAMP protein with incomplete Freund's adjuvant and another 3 weeks later by a third intravenous injection of 20 μg of CAMP protein. Serum samples were then taken 10 days later.

To generate antiserum against su-1, bacterial cells were killed by 0.3% formalin in 0.1 M phosphate-buffered saline (PBS, pH 7.2) at room temperature overnight. A rabbit was immunized with 2 ml of formalin-killed cells with Emulsigen-Plus (MVP Laboratories), and was boosted on days 21 and 35.

Rabbit antiserum against Abp was raised by subcutaneous injection of 50 μg of purified β-gal fusion protein in complete Freund's adjuvant and a subcutaneous

boost with 50 μg of protein in incomplete Freund's adjuvant.

3.3.7 PAGE and immunoblotting.

Samples from culture supernatants were obtained by trichloroacetic acid (TCA)-precipitation at a final concentration of 10%. Whole cell lysates of *E. coli* were prepared by sonication and those of *S. uberis* were made through mutanolysin (Sigma) lysis at a final concentration of 500 u/ml.

SDS-PAGE of proteins was performed as described by Laemmli (1970). Proteins were electroblotted onto nitrocellulose membranes as recommended by the supplier (Bio-Rad). Nonspecific binding was blocked by incubation in TBS (10 mM Tris-HCl, pH 7.5, 140 mM NaCl)-1% bovine serum albumin (BSA). Blots were incubated with antibody diluted 1:200 in TBS-1% BSA at room temperature for 1 h. After three washes in TBS containing 0.05% Tween 20, seroreactive proteins were detected with goat anti-mouse (or rabbit) IgG coupled to alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc.) at 1:5,000 in TBS-1% BSA. Alkaline phosphatase activity was detected using the Nitro Blue Tetrazolium-5-bromo-4-chloro-3-indolylphosphate toluidinium system as described by the supplier (Promega). Before use, the mouse polyclonal antiserum against partially-purified *S. uberis* CAMP protein was absorbed with antigens of the *E. coli* host strain as described (Frey *et al.*, 1989).

The aggregate protein band was obtained from SDS-PAGE gel by electroelution as described by the supplier (Bio-Rad, model 422 electro-eluter).

Continuous native PAGE was done as described above for SDS-PAGE except SDS was not added to the gel, gel running buffer or sample loading buffer.

3.3.8 DNA manipulations.

All molecular techniques were as recommended by the supplier (Pharmacia Canada Ltd.) or Maniatis *et al.* (1989).

Chromosomal DNA of *S. uberis* was prepared from cells grown in 100 ml BHI plus 5% (w/v) glycine. Cells were pelleted and resuspended in 2.5 ml of TES buffer (30 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl; pH 8.0) with 25% sucrose and 1.6 mg/ml lysozyme (Sigma). The suspension was incubated for 1 h at 37°C, followed by freezing at -70°C. The frozen cells were thawed in a 65°C water bath. EDTA and proteinase K (Pharmacia) were added to final concentrations of 20 mM and 1.2 mg/ml, respectively, before incubation at 65°C for 30 min. To lyse cells completely, sarkosyl was added to 1% and incubated at 37°C for 1 h. Two ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.9) was added prior to phenol: chloroform extraction. DNA was recovered by ethanol precipitation and was treated with RNase (Pharmacia Canada Ltd.).

Size-fractionated *Sau*3AI-digested chromosomal DNA fragments were isolated by sucrose density gradient

centrifugation (Maniatis *et al.*, 1989).

The DNA sequence was determined by the dideoxy-chain termination method of Sanger *et al.* (1977) on double-stranded plasmid templates using a T7 Sequencing kit (Pharmacia Canada Ltd.). The PCGENE software (Release 6.7, IntelliGenetics Inc., Switzerland) was used for sequence analysis.

3.3.9 RNA analyses.

RNA from *E. coli* strains was isolated as described previously (Lloubes *et al.*, 1986) with an additional RNase-free DNase I digestion.

RNA from *S. uberis* was prepared as follows. The cell pellet from a 10 ml culture (OD_{600}=0.6) was resuspended in 250 µl of TE buffer (pH 8.0) containing 500 u of mutanolysin (Sigma) and incubated at 37°C for 30 min. Lysis buffer (250 µl) (60 mM Tris-HCl pH 7.4, 200 mM NaCl, 10 mM EDTA, 2% SDS) and 100 µg/ml (final concentration) of proteinase K was added and the incubation continued for 1 h. The sample was extracted once with 65°C phenol (water saturated, pH 4.0) and twice with room temperature phenol. RNA was recovered by ethanol precipitation and treated with DNase I (Pharmacia Canada Ltd.).

Northern blot analysis was carried out as described by Maniatis *et al.* (1989), and primer extension assay was performed as described by Miller *et al.* (1986) except that

α^{32}P-dCTP was used instead of α^{32}P-dATP. RNasin and moloney murine leukemia virus reverse transcriptase were obtained from Pharmacia Canada Ltd.

3.3.10 Nucleotide sequence accession number.

The nucleotide sequence reported in this section has been submitted to GenBank and has been assigned accession number U34322.

3.4 Results

3.4.1 Identification and purification of the CAMP factor from *S. uberis*.

The CAMP reaction for four *S. uberis* strains was tested on a blood agar plate as described in Materials and Methods, and the result is shown in Fig. 3-1. Like CAMP reaction positive *S. agalactiae* (*Strep. ag.*), su-1 mediated a distinct CAMP reaction, while su-2, su-3, su-4 and negative control *E. coli* JF1754 (*E. coli*1754) did not cause visible hemolysis.

In liquid assays, the supernatant of su-1 was hemolytic in the presence of β-toxin, which caused 97% hemoglobin release. The A_{540} values of β-toxin control (hemolysis caused by β-toxin alone) and supernatant control (hemolysis caused by supernatant alone) were lower than that of spontaneous release, indicating that β-toxin or supernatant alone did not cause hemolysis.

During the purification of the CAMP protein from *S. uberis* (su-1), SDS-PAGE was used to screen the CAMP factor-containing fractions. Purification of the CAMP protein was successful when comparing the purified sample (Fig. 3-2 B) with the original concentrated culture supernatant (Fig. 3-2 A). Purified CAMP protein had an apparent MW of 28,000 Da (Fig. 3-2 B and shown by the arrow in Fig. 3-3 B) and retained CAMP activity as shown in Fig. 3-3 A, in which the

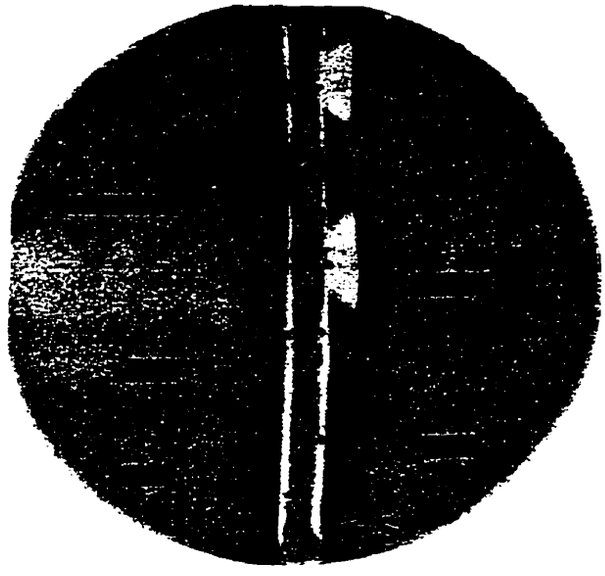


Figure 3-1. CAMP reaction of *S. uberis*. CAMP reaction was done on a base #2 blood plate as described in Materials and Methods. Vertical streak: S, *S. aureus*. Horizontal streak: Su-1, Su-2, Su-3, Su-4, Strep.ag.(*S. agalactiae*, positive control) and *E. coli* 1754 (*E. coli* JF1754, negative control).

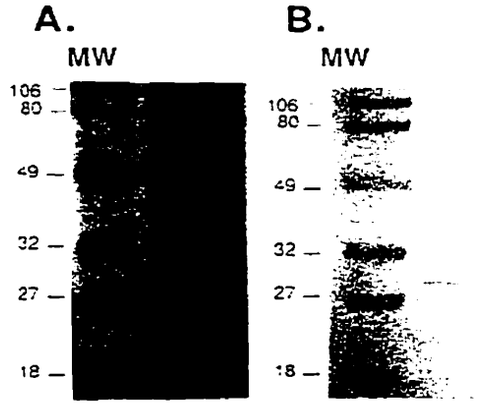


Figure 3-2. SDS-PAGE of culture supernatant and purified CAMP factor of *S. uberis*. 12% polyacrylamide-SDS gels were stained by coomassie blue. A, culture supernatant of *S. uberis*; B, purified CAMP factor of *S. uberis*. Numbers on the left of the figure indicate the positions of the molecular weight markers (in thousands).

purified CAMP protein caused a hemolytic region in the β-toxin zone of *S. aureus* (shown by an arrow).

3.4.2 Cloning and expression of the CAMP factor gene.

Chromosomal DNA of su-1 was partially digested with *Sau*3AI and size fractionated in a sucrose gradient; from this, 2- to 5-kb DNA fragments were recovered. The ends of these fragments were partially filled in with dGTP and dATP and ligated into pTZ18R which was cut with *Sal*I and partially filled in with dTTP and dCTP. Following transformation of *E. coli* JF1754 competent cells, clones expressing the CAMP factor gene were identified on blood plates with ampicillin and β-toxin on the surface. Six clones (Clo1 to Clo6) from a total of 10,000 were phenotypically hemolytic and each one mediated a distinct CAMP reaction (Fig. 3-4). One of them, Clo3, containing recombinant plasmid pJLD21, was selected for further study.

Plasmid pJLD21 contained a 5.2 kb DNA insert and the CAMP factor gene, *cfu*, was localized within a 3.2 kb *Bam*HI fragment in the subclone pJLD21-2 (Fig. 3-5). The CAMP reaction of subclone pJLD21-2 is shown in Fig. 3-6.

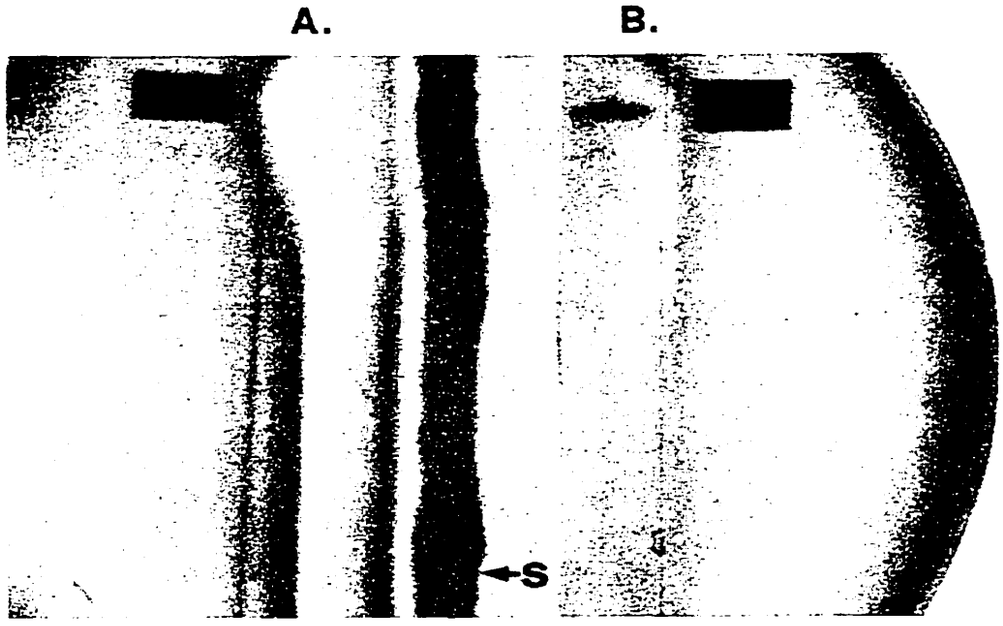


Figure 3-3. Native PAGE and CAMP reaction of purified CAMP factor of *S. uberis*. A, 12% polyacrylamide gel; B, 12% polyacrylamide gel stained by coomassie blue; S, *S. aureus*. Arrows indicate hemolytic region in gel A and purified CAMP factor in gel B.

Figure 3-4. CAMP reaction of recombinant *E. coli* clones. Vertical streak: S, *S. aureus*. Horizontal streak: Su-1, Clo1-Clo6 (CAMP-positive recombinant *E. coli* clones) and *E. coli* 1754 (pTZ18R)(negative control).

Figure 3-5. Restriction enzyme maps of recombinant plasmid pJLD21 and the subclones. Lines indicate *S. uberis* insert DNA, while boxes represent the multiple cloning sites of vector pTZ18R. The CAMP activities of recombinant plasmid pJLD21 and its derived subclone pJLD21-1, pJLD21-2 are indicated on the right (+, CAMP reaction positive; -, CAMP reaction negative). The probe fragment used for Southern or Northern blot experiments is indicated by the hatched bar. The arrows at the bottom indicate the locations of the open reading frames of CAMP factor gene (*cfu*) and polar amino acid binding protein gene (*abp*) of *S. uberis*.

BamHI
RsaI
SacI
HindIII
RsaI
EcoRI
HincII
HincII
HpaI
HindIII
RsaI
HincII
RsaI
RsaI
BamHI
abp
ctu
probe
BamHI
HindIII
EcoRI
HindIII
BamHI
BamHI
HindIII
BamHI
HindIII
EcoRI
HindIII
BamHI
HindIII
PLASMID
pJLD21
pJLD21-1
pJLD21-2
CAMP REACTION
+
-
+

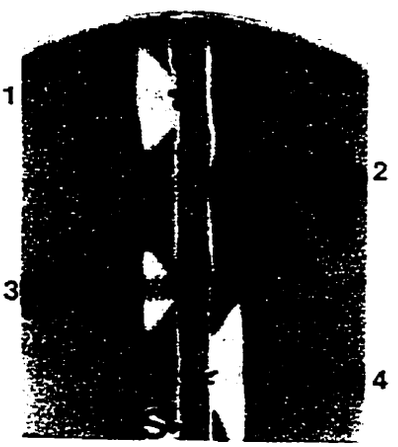


Figure 3-6. CAMP reaction of recombinant *E. coli* subclone. Vertical streak: S, *S. aureus*. Horizontal streak: 1, su-1; 2, *E. coli* JF1754(pTZ18R)(negative control); 3, *E. coli* JF1754(pJLD21)(CAMP-positive recombinant); 4, *E. coli* JF1754(pJLD21-2)(CAMP-positive subclone).

To study the expression of the recombinant CAMP factor, SDS-PAGE analysis of supernatant proteins from Cfu$^+$ *E. coli* JF1754(pJLD21) and host *E. coli* JF1754 (pTZ18R) was performed (Fig. 3-7 A). Compared to the vector control (lane 3), no distinguishable band was observed in the lane containing supernatant from the Cfu$^+$ clone (lane 2), indicating that either expression was at a very low level or the protein was not secreted efficiently. To identify the CAMP factor encoded by pJLD21, proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane and immunoblotted using mouse antiserum against purified *S. uberis* CAMP factor (Fig. 3-7 B). The Cfu$^+$ *E. coli* clone carrying pJLD21 expressed a protein with a molecular weight of 28,000 (lane 2), similar to the native CAMP factor of *S. uberis* (lane 1). Preimmune sera did not react with any proteins of *S. uberis* or *S. agalactiae* (data not shown). The identity of the 49 kD band in lane 4 of Fig. 3-7 B is presently unknown but it could be a dimer of the 26 kD protein.

3.4.3 Nucleotide sequence analysis.

To obtain the nucleotide sequence of *S. uberis* CAMP factor gene, each of the *Eco*RI, *Hin*dIII, *Hin*cII and *Sac*I fragments of pJLD21-2 (Fig. 3-5) was individually cloned into pTZ18R. By using universal and reverse primers, each fragment was sequenced in both orientations. Subsequent primers were

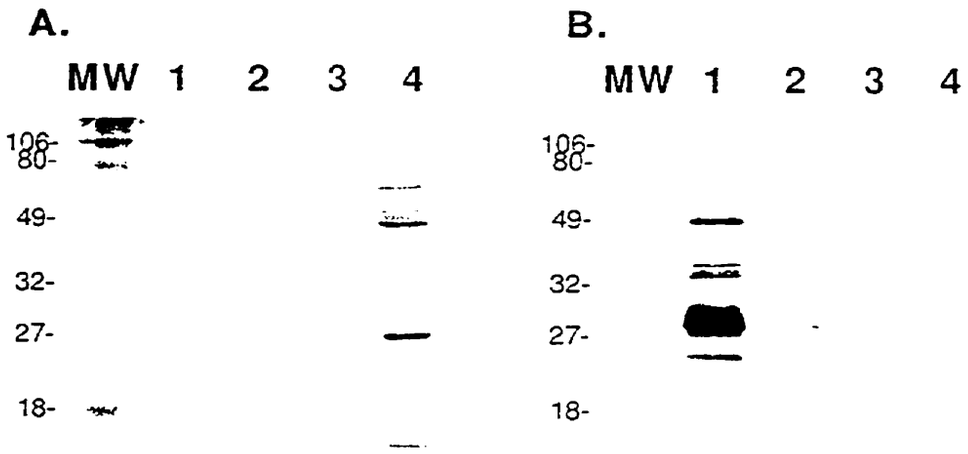


Figure 3-7. SDS-PAGE and immunoblot analysis of the *S. uberis* and *S. agalactiae* CAMP factors. A, Coomassie blue-stained 12% polyacrylamide-SDS gel. Lane 1, partially purified *S. uberis* CAMP factor; lane 2, supernatant of *E. coli* JF1754(pJLD21) (CAMP positive); lane 3, supernatant of *E. coli* JF1754(pTZ18R) (CAMP negative); lane 4, supernatant of *S. agalactiae*. Prestained molecular weight standard (lane MW). Numbers on the left of the figure indicate the positions of the molecular weight markers (in thousands). B, Immunoblot of the gel presented in panel A. Samples were transferred to a nitrocellulose membrane and reacted with mouse antiserum against purified *S. uberis* CAMP factor.

```
ACCGAGCTCGGTACCCGGGGATCCAGAACTACGGCAAGAAGTTGAAAAATTGATTCTTCA     60
AAACAAGGAAATGGGTATGACACAAATTGTCGTAACCCATGATTTAGCTTTTGCTGAATC    120
                 -35                     -10
CATATCGGATCGCATTTTAAAAGTAAATCCGAAATAAGAAAAAAGGCGGATATAGTAATG    180
                                              SD              M
AACTTGAAAAAAATACTCCTAACAACATTAGCATTAGCAAGTACATTGTTTTTAGTAGCA    240
  N  L  K  K  I  L  L  T  T  L  A  L  A  S  T  L  F  L  V  A     21
TGTGGAAAAAGTAGTGCTGCTAAAACTGATCAGTGGGATACTTATAAAAAAGAAAAGAGT    300
  C  G  K  S  S  A  A  K  T  D  Q  W  D  T  Y  K  K  E  K  S     41
ATCACTTTAGGTTTTGATAATACTTTTGTTCCAATGGGATTTAAAGATGAATCAGGTAAG    360
  I  T  L  G  F  D  N  T  F  V  P  M  G  F  K  D  E  S  G  K     61
AACACTGGTTTTGATGTTGAATTAGCCAAAGCCGTTTTCCAGGAATATGGCATTAAAGTC    420
  N  T  G  F  D  V  E  L  A  K  A  V  F  Q  E  Y  G  I  K  V     81
AAGTTTCAACCAATTAACTGGGATTTAAAAGAAACTGAATTAAAAAATGGAAAAATTGAC    480
  K  F  Q  P  I  N  W  D  L  K  E  T  E  L  K  N  G  K  I  D    101
ATGATTTGGAATGGTTACTCTGTGACAAAAGAACGTCAAGCAAAAGTTGCTTTTTCAACT    540
  M  I  W  N  G  Y  S  V  T  K  E  R  Q  A  K  V  A  F  S  T    121
CCTTATATGAAAAATGAACAAGTTTTAGTAACCAAAAAATCCTCAAATATCACGTCGTTT    600
  P  Y  M  K  N  E  Q  V  L  V  T  K  K  S  S  N  I  T  S  F    141
GCTGCTATGAAAGGAAAAGTTTTGGGAGCTCAATCTGGATCATCTGGTTATGATGCATTT    660
  A  A  M  K  G  K  V  L  G  A  Q  S  G  S  S  G  Y  D  A  F    161
ACAAGCAATCCTAAGGTTTTAAAAGATATTGTTAAAGATAATGACGCAACTCAATATGAA    720
  T  S  N  P  K  V  L  K  D  I  V  K  D  N  D  A  T  Q  Y  E    181
ACCTTTATCCAAGCTTTTATTGATTTAAAAAACGACCGTATTGATGGTCTCTTGATTGAT    780
  T  F  I  Q  A  F  I  D  L  K  N  D  R  I  D  G  L  L  I  D    201
AAAGTTTATGCTAACTATTATTTGAAACAAGAAGGCGAATTAACCAATTACAATATCGTC    840
  K  V  Y  A  N  Y  Y  L  K  Q  E  G  E  L  T  N  Y  N  I  V    221
AAGAGTGAATTTGATGGCGAAGATTTTGCAGTTGGCGTTCGTAAAGAAGATAAAATATTA    900
  K  S  E  F  D  G  E  D  F  A  V  G  V  R  K  E  D  K  I  L    241
CTTAAAAATATCAATTCAGCATTTACTAAACTTTATAAGACTGGAAAATTCCAAGAAATT    960
  L  K  N  I  N  S  A  F  T  K  L  Y  K  T  G  K  F  Q  E  I    261
TCTCAAAAATGGTTTGGAGAAGATGTTGCGACGGAAAATGTGAAAAAATAACTAATTACC   1020
  S  Q  K  W  F  G  E  D  V  A  T  E  N  V  K  K  *             277
CCTACTTTAATTGTTTCTTTTAGTTAAAAAAAGAAATATATTGAGTAAGGGTATTTTTTA   1080
ATAATTTTAATAAAAATAATAATATTGTTATTTTTATACTTTTCTACTTAACCAGTTAAG   1140
TTAAAAATTCTGAAATACTTATCAACAAAATAATTTATTATTTTTTTATGGTATAAAACC   1200
TTTATATTGAGATTCTAAATTTTCTGAAAATTACTAAAGATAAGATTGATTCAGGAAGTT   1260
AAAAGGCTCATTTTGTTATAATAAAATGAACATAAAATAAAAATTAATAATTATATATTT   1320
                                                        +1
TTATGATAATCACATATATTTGACTTAAAAAAATTGTTACTGTATGATACAGGCATAAGT   1380
                 -35                     -10
ACTTATTTATTTTATAGATTGCAATTTATAAACAATTATATTTTTCAAAGAGGAATGCTT   1440
                                                 SD
ATGGAATTCAAAAAGTTACTTTATTTAACTGGTTCAATCGCAGGAATTACTTTATTTTCC   1500
  M  E  F  K  K  L  L  Y  L  T  G  S  I  A  G  I  T  L  F  S     20
CCAATTTTAACAAGTGTCCAAGCAAATCAAATAAATGTTAGTCAACCATCTAATAATGAA   1560
  P  I  L  T  S  V  Q  A  N  Q  I  N  V  S  Q  P  S  N  N  E     40
AGTAATGTTATTTCACAGAAAAAAGAAGAAATTGATAATAGTCTAAATCAGGAAAGTGCT   1620
  S  N  V  I  S  Q  K  K  E  E  I  D  N  S  L  N  Q  E  S  A     60
CAACTATATGCCTTGAAAGAAGATGTTAAAGGAACTGAGAAAGAACAATCAGTTAATTCA   1680
  Q  L  Y  A  L  K  E  D  V  K  G  T  E  K  E  Q  S  V  N  S     80
GCAATTTCAGCTGTTGAAAATTTAAAAACTTCACTTAGAGCTAATCCTGAAACAATTTAT   1740
  A  I  S  A  V  E  N  L  K  T  S  L  R  A  N  P  E  T  I  Y    100
GATTTAAATTCGATTGGAACAAGAGTAGAAGCAATCTCTGACGTGATTCAAGCAATTGTT   1800
  D  L  N  S  I  G  T  R  V  E  A  I  S  D  V  I  Q  A  I  V    120
TTTTCAACGCAACAGTTAACAAATAAAGTTGATCAAGCTCACATTGATATGGGATTTGCT   1860
  F  S  T  Q  Q  L  T  N  K  V  D  Q  A  H  I  D  M  G  F  A    140
ATTACGAAATTACTTATTCGCATTGCAGACCCATTTGCTTCAAATGAATCCATTAAAGGG   1920
  I  T  K  L  L  I  R  I  A  D  P  F  A  S  N  E  S  I  K  G    160
CAAGTCGAAGCTGTTAAACAAGTGCAAGCGACTGTGCTTACCTATCCCGATTTGCAGCCT   1980
  Q  V  E  A  V  K  Q  V  Q  A  T  V  L  T  Y  P  D  L  Q  P    180
```

```
ACGGATAGAGCAACTATTTACGTTAAATCAAAATTAGACAAGCTTATTTGGCAAACAAGA  2040
 T  D  R  A  T  I  Y  V  K  S  K  L  D  K  L  I  W  Q  T  R   200
ATTACCAGAGATCAAAAAGTTCTTAATGTAAAGAGTTTTGAAGTTTATCATCAATTAAAT  2100
 I  T  R  D  Q  K  V  L  N  V  K  S  F  E  V  Y  H  Q  L  N   220
AAAGCTATCACACATGCAGTAGGTGTACAATTAAATCCAACTGTAACAGTTGCACAAGTT  2160
 K  A  I  T  H  A  V  G  V  Q  L  N  P  T  V  T  V  A  Q  V   240
GACCAAGAAATCAAAGTGCTACAAGAAGCATTAAATACTGCTCTACAGTAAGGTAGAGAT  2220
 D  Q  E  I  K  V  L  Q  E  A  L  N  T  A  L  Q  *            256
TGAATTGACGTATTAAAAAGGACTGGAATTTATTAATTTCAGTCCTTTAGAATTTTTATT  2280
TAGCTGATTTACTTGTTGAAGAGATTTGGTGGAAAATCAAGTACCATACTTCATTTCTCC  2340
TCCAAATACTTGTATGTCGATTCCCTTCTAAAACATAGCTAATTAGTTTAGTTTTCTGGC  2400
TAATAGATTGTACATGAAATTGTTCAAAATTACTAGGGTAAAAGGTTTTTCTTTTTATAA  2460
ATTCATCATGACTAT                                               2475
```

Figure 3-8. Nucleotide sequence of pJLD21-2. The deduced amino acid sequence of *S. uberis* CAMP factor and Abp is shown in the single-letter code below the sequence. The start site of *cfu* transcription is represented by +1. The -10 and -35 regions of the promoters are also indicated. The putative Shine-Dalgarno sequences are marked as SD, and the secretory signal sequences are double underlined. The putative N terminal glyceride-cysteine of Abp is indicated in italics.

synthesized on the basis of sequence information thereby obtained. The complete DNA sequence is presented in Fig. 3-8.

An open reading frame beginning with an ATG codon located at positions 1441 to 1443 and terminating with the TAA stop codon at positions 2209 to 2211 was found which could encode a 256-residue polypeptide with a calculated molecular weight of 28,363. Analysis of the N-terminus of the predicted amino acid sequence showed a secretory signal sequence, with a potential cleavage site between residue 28 and 29. A purine-rich sequence AAGAGG, which serves as a ribosome binding site in *E. coli* (Stormo *et al.*, 1982) was found in front of the ATG

start codon. Sequence alignment revealed that the protein encoded by this open reading frame showed high homology (66.4% identity) with the protein B sequence of *S. agalactiae* which was determined from the protein directly (Rühlmann *et al.*, 1988) (Fig. 3-9), indicating that this open reading frame encodes *S. uberis* CAMP factor. The N-terminal tail of *S. uberis* CAMP factor lacking matches with the protein B sequence of *S. agalactiae* was from the secretory signal sequence which would be cleaved just before residue N during protein maturation.

A second ORF was identified upstream of the *cfu* gene (Fig. 3-8). This ORF starts with an ATG codon at position 178 to 180 and terminates with the TAA stop codon at position 1009 to 1011 of the DNA sequence and could encode a 277-residue polypeptide with a calculated molecular weight of 31,241 dalton. The ATG start codon is associated with a putative Shine-Dalgarno sequence AGGCGG. A putative -10 and -35 promoter region was also found at -80 and -101 from the ATG start codon. Analysis of the N terminus of the predicted amino acid sequence showed a typical leader sequence for secretion and a potential cleavage site LVA-C, which corresponds to the consensus sequence of the lipoprotein precursors (von Heijne, 1989). A search of the GenBank database revealed that the protein encoded by this ORF shares homologies with a number of the receptor subunits of the polar amino acid and opine transport systems. In particular, it has homology with the

```
MEFKKLLYLTGSIAGITLFSPILTSVQANQINVSQP-----SNNESNVIS   45
                              :     :       :::
                            DQVTTPQVVNHVNSNNQAQQMA   22

QKKEEIDNSLNQESAQLYALKEDVKGTEKEQSVNSAISAVENLKTSLRAN   95
::          : : ::   :  : ::  :  :: ::  :: ::::::::
QKL-------DQDSIQLRNIKDNVQGTDYEKPVNEAITSVEKLKTSLRAN   65


PETIYDLNSIGTRVEAISDVIQAIVFSTQQLTNKVDQAHIDMGFAITKLL  145
 :: ::::::: ::::  ::: :: :::: : ::: :: ::::: ::::
SETVYDLNSIGSRVEALTDVIEAITFSTQHLANKVSQANIDMGFGITKLV  115

IRIADPFASNESIKGQVEAVKQVQATVLTYPDLQPTDRATIYVKSKLDKL  195
::: :::::  ::: ::  ::     ::::::: :::::::: :::::::
IRILDPFASVDSIKAQVNDVKALEQKVLTYPDLKPTDRATIYTKSKLDKL  165

IWQTRITRDQKVLNVKSFEVYHQLNKAITHAVGVQLNPTVTVAQVDQEIK  245
:: :: ::: :::::: : ::  :::::::::::::: ::: ::::::
IWNTRFTRDKKVLNVKEFKVYNTLNKAITHAVGVQLNPNVTVQQVDQEIV  215

VLQEALNTALQ                                         256
 :: :: :::
TLQAALQTALK                                         226
```

Figure 3-9. Homology between *S. uberis* CAMP factor (upper lines) and *S. agalactiae* CAMP factor (low lines). Amino acids are identified in the single-letter code. Identical amino acids are indicated by double dots (:). The aligned sequences show 66.4% identity. Spaces (indicated by dash) were introduced in the sequences to optimize alignment. The secretory signal sequence of *S. uberis* CAMP factor is shown in italics.

glutamine-binding protein GlnH and arginine-binding proteins ArtJ and ArtI of *E.coli* (29%, 30% and 26% identity,

respectively) (Nohno, 1986; Wissenbach, 1993), the histidine-binding protein HisJ and lysine-arginine-ornithine-binding subunit ArgT of *Salmonella typhimurium* (28% and 27% identity, respectively) (Higgins, 1981; 1982), the octopine-binding protein OccT and the nopaline-binding protein NocT of *Agrobacterium tumefaciens* (29% identity) (Valdivia 1991; Zanker, 1992). Hence, this protein is named Abp, standing for amino acid binding protein.

Interestingly, the Abp presented here exhibits substantial sequence similarity at the N-terminal domain to the cluster 3 binding proteins specific for polar amino acids and opines described by Tam and Saier (1993). The alignment of 57 residues (from residue 83 to 139) of the Abp to the cluster 3 proteins showed that Abp sequence contains 8 highly conserved residues (Fig. 3-10) and the two signature sequences which characterize cluster 3 [GF(DE)(LIV)DLX_3(LIVM)(CA)(KE) and P(SA)LX_2(KG)X_2D(LIVMA)$_3$(SA)(GAS)(LIVM)] are partially conserved in Abp. On the basis of sequence homology and the protein size (277 residues), typical for cluster 3 proteins (265±12 residues), the Abp appears to belong to this family of receptors which recognizes and transports polar amino acids and opines.

```
Lao Sty (50) G F D I D L G N E M C K R M Q V K C T W V A S D F D A L I P S L K A K K I D A I I S S L S I T D K R Q Q E I A F S (106)
His Sty (50) G F D I D L A K E L C K R I N T Q C T F V E N P L D A L I P S L K A K K I D A I M S S L S I T E K R Q Q E I A F T (106)
Gln Eco (48) G F D V D L W A A I A K E L K L D Y E L K P M D F S G I I P A L Q T K N V D L A L A G L T I T D E R K K A I D F S (104)
Occ Atu (47) G F E I D L A N A L C E K M K A K C Q I V A Q N W D G I M P S L T G K K Y D A I M A A M S V T P K R Q E V I G F S (103)
Noc Atu (53) G F D I D L G N D L C K R M N I E C K F V E Q A W V G I I P S L T A G R Y D A I M A A M G I Q P A R E K V I A F S (109)
Baa Ngo (61) G F D V D L M N A M A K A G N F K I E F K H Q P W D S L F P A L N N G D A D I V M S G V T I T D D R K Q S M D F S (117)
Apb Sub (83) G F D V E L A K A V E Q E Y G I K V K F Q P I N W D L K E T E L K N G K I D M I W N G Y S I T K E R Q A K V A F S (139)
```

Figure 3-10. Alignment of *S. uberis* Abp to cluster 3 polar amino acid and opine binding proteins. Protein abreviations: LAO Sty, lysine-arginine-ornithine binding protein of *S. typhimurium*. His sty, histidine binding protein of *S. typhimurium*. Gln Eco, glutamine binding protein of *E. coli*. Occ Atu, octopine binding protein of *A. tumefaciens*. Noc Atu, nopaline binding protein of *A. tumefaciens*. Baa Ngo, basic amino acids binding protein of *N. gonorrhoeae*. Residues in boxes are fully conserved. The residue numbers within each protein segment are provided at the beginning and end of each sequence. The alignment of the 6 cluster 3 binding proteins is referred to Tam and Saier (1993).

3.4.4 Northern blot and primer extension analysis of the CAMP factor gene.

To analyse *cfu* transcription, RNA was prepared from su-1, *E. coli* JF1754(pJLD21) and *E. coli* JF1754(pTZ18R), and analysed by Northern blotting. The blot was probed with the 576 bp *Eco*RI-*Hin*dIII fragment of pJLD21 (Fig. 3-5). Similar 1 kb bands were seen in lanes containing RNA from *E. coli* JF1754(pJLD21) and su-1 (Fig. 3-11, lane 2 and 3, respectively), but was absent in lane 1 which contained RNA from *E. coli* JF1754(pTZ18R). This data indicates that only one major transcript was generated by *cfu* in recombinant *E. coli* as well as in native *S. uberis*.

To localize the start site of transcription and the promoter region of the CAMP factor gene, primer extension analysis of RNA from su-1, *E. coli* JF1754(pJLD21) and *E. coli* JF1754(pTZ18R) was done by using a synthetic oligonucleotide complementary to the DNA sequence from position 1485 to 1468. A strong primer extension product corresponding to base 1375 ("T") was identified from both *S. uberis* and *E. coli* JF1754(pJLD21), but not from *E. coli* JF1754(pTZ18R) (Fig. 3-12). This data indicates that the major transcript of *S. uberis* CAMP factor gene is initiated with an "A" residue (+1 in Fig. 3-8). Both -10 and -35 regions, characteristic of *E. coli* promoters (Harley and Reynolds, 1987), were identified at the upstream of the transcriptional start site (Fig. 3-8).

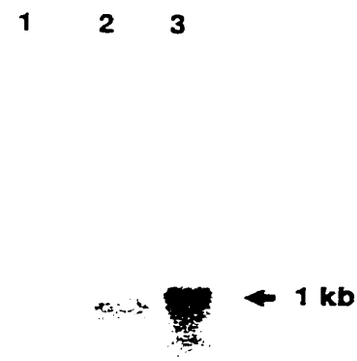


Figure 3-11. Northern blot analysis of RNA from *S. uberis* and CAMP⁻ *E. coli* clone. The origin of the probe used is indicated in Fig. 3-5. Lane 1, 2 and 3 are RNA samples from *E. coli* JF1754(pTZ18R), *E. coli* JF1754(pJLD21) and su-1, respectively. The arrowhead on the right indicates the position and size of the hybridizing bands.

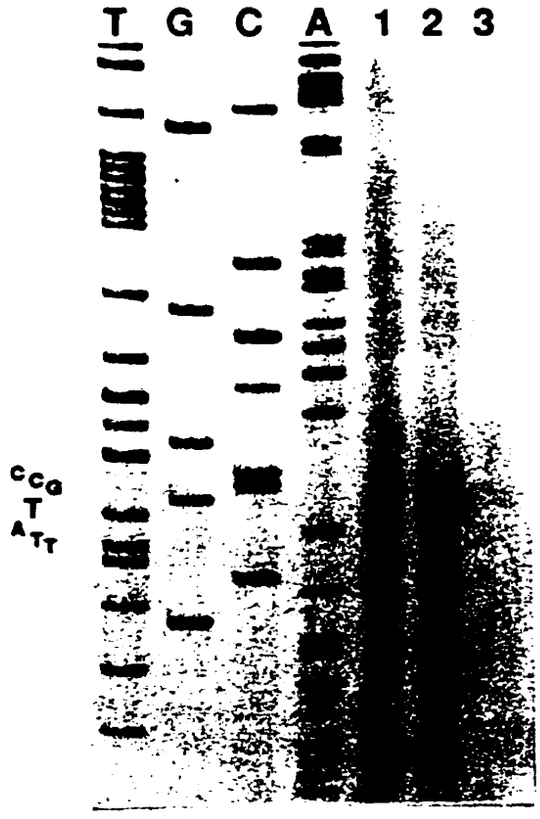


Figure 3-12. Primer extension analysis of the promoter region of *S. uberis* CAMP factor gene. The primer extension reactions were done as described in Materials and Methods. The cellular RNAs used were obtained from su-1 (lane 1), *E. coli* JF1754(pJLD21) (lane 2), and *E. coli* JF1754(pTZ18R) (lane 3). The dideoxy sequencing ladder was generated by using the same primer and is shown as T, G, C, A above each lane.

3.4.5 Comparison of the *S. uberis* CAMP factor with *S. agalactiae* CAMP factor.

To compare the *S. uberis* CAMP factor with protein B of *S. agalactiae*, a concentrated culture supernatant of *S. agalactiae* containing protein B (Jürgens *et al.*, 1985) was separated by SDS-PAGE and analyzed by immunoblotting with antibodies against the purified *S. uberis* CAMP factor. A 25 kDa protein band from the *S. agalactiae* supernatant (Fig. 3-7 A, lane 4) reacted in the immunoblot (Fig. 3-7 B, lane 4). This data indicated that monospecific antibodies raised against the *S. uberis* CAMP factor could cross-react with *S. agalactiae* protein B. This is not surprising, since alignment of the 226-amino acid sequence of the *S. agalactiae* CAMP factor with the deduced 256 amino acids of the *S. uberis* CAMP factor showed 66.4% identical residues as mentioned above (Fig. 3-9).

3.4.6 Southern blot analysis of six CAMP$^+$ *E. coli* clones and the distribution of CAMP factor genes in eight *S. uberis* strains.

Originally, six CAMP reaction positive *E. coli* clones were found from the gene library (Fig. 3-4). Two of them, Clo1 and pJLD21 (Fig. 3-13 A, lane 1 and 3, respectively) had similar restriction patterns when digested with *Hin*dIII, while Clo1, Clo2, Clo4, Clo5 and Clo6 (Fig. 3-13 A, lane 1, 2, 4, 5 and 6, respectively) showed different

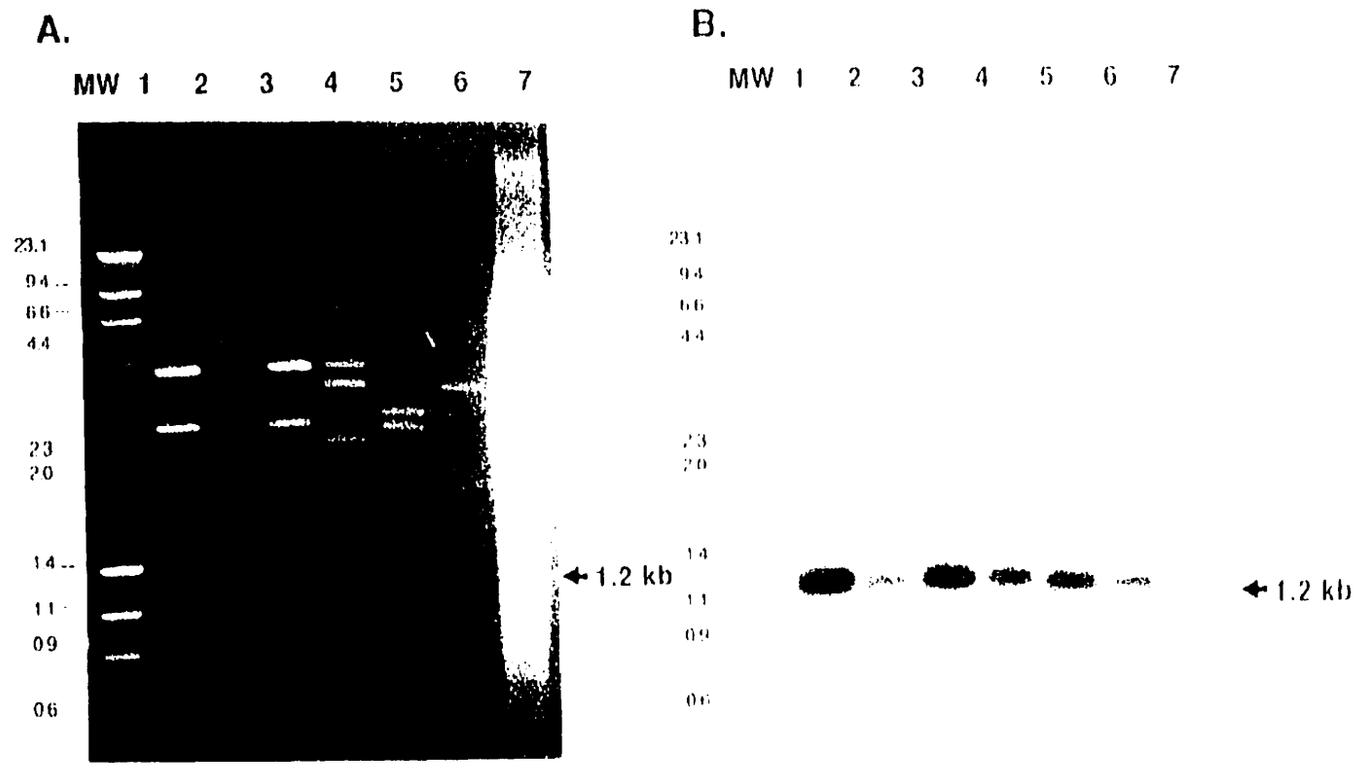


Figure 3-13. Electrophoresis and Southern blot analysis of plasmid DNA from 6 CAMP' *E. coli* clones. A, electrophoresis of plasmid and chromosomal DNA digested with *Hin*dIII in 0.8% agarose. Lane 1 to 6, plasmid DNA from clo1 to clo6, respectively; lane 7, chromosomal DNA of *S. uberis*. MW, molecular weight standard in kilobase. B, Southern blot of the gel presented in panel A. The origin of the probe used is indicated in Fig. 3-5. The arrowheads on the right indicate the position and size of the hybridizing restriction enzyme fragments.

patterns. However, all of them contained a 1.2 kb *Hin*dIII fragment which hybridized with a 576 bp *Eco*RI-*Hin*dIII internal fragment of *cfu* of pJLD21 (Fig. 3-5 and Fig. 3-13 B). A corresponding hybridization band was also present in native su-1 chromosome (Fig. 3-13 B, lane 7) which had been digested with *Hin*dIII (Fig. 3-13 A, lane 7). These data indicate that all the six CAMP reaction positive *E. coli* clones contain the same CAMP factor gene of su-1.

Chromosomal DNA prepared from eight *S. uberis* strains were digested with the restriction endonuclease *Hin*dIII and separated on an agarose gel. Southern blot analysis with the 576 bp *Hin*dIII-*Eco*RI fragment of pJLD21 as a probe (Fig. 3-5) showed that a fragment identical in size to the *Hin*dIII fragment (1.2 kb) in pJLD21 was present in three *S. uberis* strains which were CAMP-reaction positive, while none of the CAMP reaction negative strains reacted with the probe (Fig. 3-14). Thus, the CAMP-negative strains do not contain the *cfu* gene.

3.4.7 Construction of the *abp* gene on plasmids.

One additional ORF identified in pJLD21 was *abp* (Fig. 3-5), which could encode a protein homologous to a number of receptor subunits of the polar amino acid and opine transport systems. To analyse the *abp* gene product, the 1.4 kb *Eco*RI fragment containing the complete *abp* gene was cloned into vector pTZ18R to generate pEB29 (Fig. 3-15). A construct

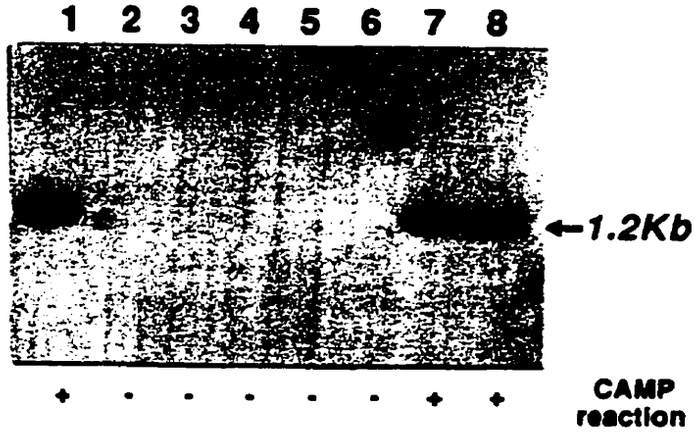


Figure 3-14. Southern blot analysis of different *S. uberis* strains. The origin of the probe used is indicated in Fig. 3-5; chromosomal DNA from each *S. uberis* strain was cut with *Hin*dIII. Lane 1 to 8 are the DNA samples from su-1 to su-8. The arrowhead on the right indicates the position and size of the hybridizing restriction enzyme fragments. CAMP reaction phenotype of each strain is indicated at the bottom by "+" (CAMP reaction positive) and "-" (CAMP reaction negative). (After further analysis, isolates in lane 5 and 6 were demonstrated to be *S. parauberis*.)

Figure 3-15. Construction of plasmid pEB29 and pRF3. Lines represent *S. uberis* insert DNA, while boxes indicate the cloning vectors. The horizontal arrows represent the open reading frames of gene *cfu* and *abp*. The β-gal-Abp fusion protein encoded by pRF3 is indicated by a chimeric bar, with the open bar indicating the *lacZ* and the solid bar representing *abp*.

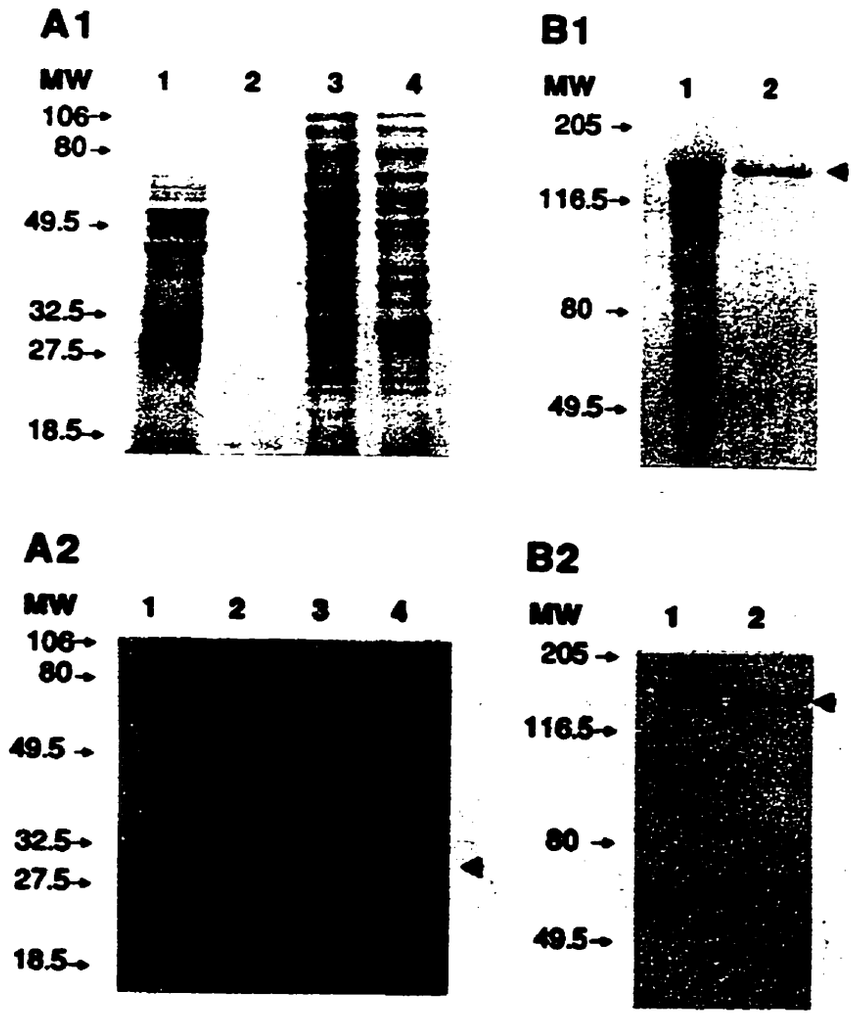
pRF3
pEB29
pJLD21-2
PLASMID
lacZ
abp
abp
abp
ctu
pUC19
pTZ18R
EcoRI
BamHI
(SmaI)
RsaI
SacI
HindIII
HincII
HpaI

pRF3 was also generated by inserting the 1.2 kb *Rsa*I fragment to the *Sma*I site of vector pUEX3 (Fig. 3-15) which is capable of expressing cro'-β-galactosidase fusion proteins at high levels when induced at 42°C for 2 h.

3.4.8 Identification of the *abp* gene product in recombinant *E.coli* and *S. uberis* strains.

Attempts to identify the *abp* gene product from pEB29 in *E.coli* JM105 were unsuccessful when comparing the whole cell lysates of *E. coli* JM105 (pEB29) and *E.coli* JM105 (pTZ18R) separated by SDS-PAGE (Fig. 3-16 A1, lane 3 and 4, respectively). Therefore, we decided to prepare inclusion bodies from temperature-induced *E.coli* JM105 (pRF3). As expected, a major protein band with molecular weight of 150 kDa was seen on SDS-PAGE gels (Fig. 3-16 B1, lane 1), which was the β-gal (MW, 116.5 kD) fusion product of the *abp* gene. This protein was further purified from the SDS-PAGE gel to a relatively good purity (Fig. 3-16 B1, lane 2). The expressed β-gal fusion protein of the *abp* gene was recognized by rabbit polyclonal antiserum generated against formalin-killed su-1 in an immunoblot (Fig. 3-16 B2), while β-gal was not (data not shown). To further analyse the expression of the *abp* gene in *S. uberis*, serum against the *abp* gene product was raised in rabbits as described in Materials and Methods. A 31 kDa protein was identified from a su-1 whole cell lysate, but not from the TCA (trichloroacetic acid)-

Figure 3-16. SDS-PAGE and immunoblot analysis of the *abp* gene products. A 1, coomassie blue-stained 10 % polyacrylamide-SDS gel. Lane 1, whole cell lysate of *S. uberis;* lane 2, supernatant of *S. uberis;* lane 3, whole cell lysate of *E. coli* JM105 (pEB29); lane 4, *E. coli* JM105 (pTZ18R). A 2, immunoblot of the gel presented in panel A 1 using rabbit antiserum against purified β-gal-Abp fusion protein. B 1, coomassie blue-stained 7.5% polyacrylamide-SDS gel. Lane 1, aggregate of β-gal-Abp fusion protein from *E. coli* JM105 (pRF3); lane 2, purified β-gal-Abp fusion protein. B 2, immunoblot of the gel presented in panel B 1 using rabbit antiserum against the formalin-killed *S. uberis*. The molecular weight (MW) standards (kD) are represented by arrows to the left and the target protein bands are indicated by arrows to the right.


A1
MW
1
2
3
4
106
80
49.5
32.5
27.5
18.5
B1
MW
1
2
205
116.5
80
49.5
A2
MW
1
2
3
4
106
80
49.5
32.5
27.5
18.5
B2
MW
1
2
205
116.5
80
49.5

concentrated culture supernatant, after SDS-PAGE (Fig. 3-16 A1, lane 1 and 2, respectively) and immunoblotting with rabbit antiserum against the *abp* gene product (Fig. 3-16 A2, lane 1 and 2, respectively), indicating that the *abp* gene was expressed in *S. uberis*, but the expressed protein was not released into the medium. Considering the existence of the secretory signal peptide and the consensus cleavage site for lipoproteins in the deduced protein sequence of the *abp* gene, it appears very likely that this protein is exported through the cytoplasmic membrane and anchored on the cell surface through the lipophilic modification of the N terminal cysteine in the mature protein as described in many other lipoproteins (von Heijne, 1989). The 31 kDa protein was also found in the whole cell lysate of *E.coli* JM105 (pEB29) (Fig. 3-16 A2, lane 3) immunoblotted with rabbit antiserum against the *abp* gene product, but not from the *E. coli* JM105 (pTZ18R) (Fig. 3-16 A2, lane 4), indicating that the *abp* gene was indeed expressed, even though the expressed protein could not be visualized on coomassie blue-stained SDS-PAGE gels as mentioned above.

To study whether other *S. uberis* strains could produce the same or related amino acid binding protein, whole cell lysates of eight *S. uberis* strains were blotted with rabbit antiserum against the *abp* gene product of su-1 (Fig. 3-17). Five *S. uberis* strains, su-2, su-3, su-4, su-7 and su-8 (lane 2, 3, 4, 7 and 8, respectively) showed cross-reactive

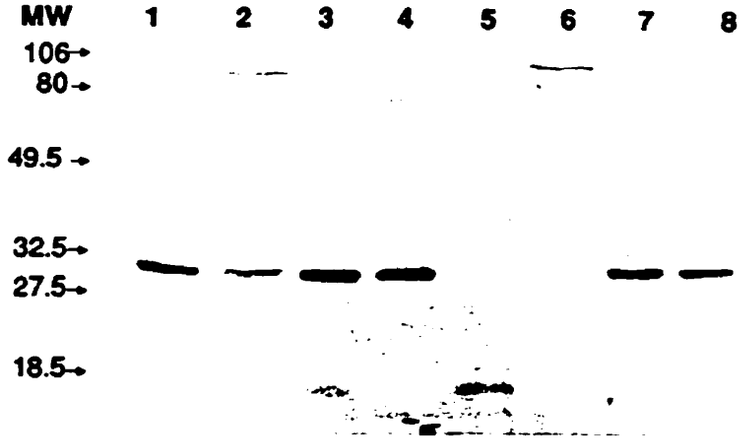


Figure 3-17. Immunoblot analysis of Abp from *S. uberis* strains. Samples were analysed in 12% SDS-PAGE and transferred to a nitrocellulose membrane which was further blotted with rabbit antiserum against *abp* gene product of su-1. Lane 1 to 8 are whole cell lysate from su-1 to su-8, respectively. Numbers on the left of the figure indicate the positions of the molecular weight markers (in thousands).

bands with a molecular weight of 31 kD, similar to the Abp of su-1 (lane 1); while su-5 and su-6 (lane 5 and 6, respectively) did not produce the same sized protein. Therefore, Abp appears to be a common protein in most *S. uberis* strains.

3.5 Discussion

Streptococcus uberis is an increasingly important cause of bovine mastitis and there are no effective measures for its control, due in part to a lack of understanding of the virulence determinants and pathogenesis of this organism. Some *S. uberis* strains produce a CAMP factor, which has been gaining more attention as a potential virulence factor because of its cohemolytic and Fc-binding properties.

In our experiments, one *S. uberis* strain (su-1) was originally identified as CAMP reaction positive in both plate and tube assays. To purify the CAMP protein from the culture supernatant of *S. uberis*, we used ammonium sulphate precipitation, ultrafiltration, and hydrophobic interaction chromatography on Octyl-sepharose as described by Jürgens *et al* (1985). A Lowry assay was originally applied to screen protein-containing fractions. However, no obvious protein peaks were found. Attempts to detect CAMP factor-containing fractions using a CAMP tube assay was also unsuccessful since the presence of the detergent (SDOC) in the eluting buffer

caused non-specific lysis of RBC. Purified materials exhibited considerable homogeneity and CAMP activity as revealed by SDS-PAGE and native PAGE followed by a CAMP assay. To generate antiserum against *S. uberis* CAMP factor, the purified protein was injected into mice.

We have cloned the gene coding for the CAMP factor, *cfu*, from su-1 based on functional activity in *E. coli*. Even though the CAMP activity of clone pJLD21 and pJLD21-2 was observed on blood agar after 6 hours incubation, and a 28,000 Da protein reacting with anti-*S. uberis* antibodies was found in the culture supernatant, we do not know whether the CAMP factor was released by cell lysis or secreted by the recombinant *E. coli*.

In order to localize the *cfu* gene, we established restriction enzyme maps of the recombinant plasmid pJLD21 and generated several subclones of this plasmid for sequencing. An open reading frame encoding a 256-residue polypeptide was identified. A putative signal sequence was found at the N-terminus of this polypeptide. This observation suggests that the CAMP factor is secreted in *S. uberis*. However, we have no experimental evidence that this Gram positive signal sequence can facilitate the secretion of the CAMP factor from recombinant *E. coli*. From primer extension experiments, we found that transcription of the CAMP factor gene is initiated from an "A" residue which is preceded by a -10 box and -35 region (Fig. 3-8).

Since, on the basis of previous experimental data (Frey *et al.*, 1989), the *S. agalactiae* CAMP factor could react with antibodies raised against the CAMP factor of *Actinobacillus pleuropneumoniae*, we analysed the culture supernatant of *S. agalactiae* by immunoblotting with antibodies against the *S. uberis* CAMP factor to see whether the proteins from these two streptococcal species were related. We found that a 25 kDa protein band from the culture supernatant of *S. agalactiae* reacted with antibodies raised against the *S. uberis* protein, indicating that the two CAMP factors are immunologically similar. The similarity was subsequently demonstrated at the amino acid level.

Based on the map location of the *cfu* gene, a *Hind*III-*Eco*RI fragment was chosen as a DNA probe to confirm the identity of the cloned DNA and its distribution in other *S. uberis* strains. The Southern blot analysis demonstrates that the *S. uberis* strains containing *cfu* genes can show a positive CAMP reaction phenotype. Moreover, Southern blotting with the same probe showed that the six CAMP reactive positive *E. coli* clones contained the same CAMP factor gene. The different sizes and *Hind*III maps of these clones may result from *Sau*3A digestions at different sites during library construction. Cloning of different *cfu*-containing fragments generated by *Sau*3A digestion at different sites on chromosomal DNA may result in these *E. coli* clones with different sizes and *Hind*III maps.

An ORF capable of coding for a 277-residue polypeptide has been identified in *S. uberis* and this protein has sequence similarity to a number of polar amino acid and opine binding proteins of Gram negative bacteria. The putative secretory signal sequence and the consensus cleavage site suggest that this protein could be a lipoprotein tethered to the cell membrane with a N terminal glyceride-cysteine. This speculation is supported by the evidence that the Abp was absent from the cultural supernatant of *S. uberis*. On the basis of sequence homology and lipoprotein character, it appears that the Abp belongs to a receptor family which transports polar amino acids and opines.

Unfortunately, we do not have direct experimental evidence to demonstrate the function of this protein. Moreover, if a larger DNA clone had been obtained and the gene loci for the correspondent transmembrane component and the ATP-binding protein had been found, we would feel more confident that the Abp belongs to an ABC-type amino acid transport system.

4.0 IDENTIFICATION OF A BOVINE LACTOFERRIN BINDING PROTEIN IN *S. UBEREIS*

4.1 Summary

S. uberis is commonly associated with clinical and subclinical mastitis in dairy cattle. The interaction between this pathogen and bovine lactoferrin (bLf), an iron-binding protein present at high concentrations in milk has been characterized in this chapter. The binding of ^{125}I-bLf to *S. uberis* (ATCC 9927) was time-dependent and displaceable by unlabelled bLf. Apo-bLf could inhibit ^{125}I-bLf binding as effectively as iron-saturated bLf. Bovine transferrin, human lactoferrin and human transferrin did not interfere with bLf binding. The Scatchard plot was linear and approximately 7800 binding sites were expressed by each bacterial cell, with an affinity of 1.0×10^{-7} M. Reduced iron availability obtained by the presence of iron chelators did not notably modify the saturation of *S. uberis* by bLf. Heat- or protease treatment of bacterial cells reduced bLf binding to a great degree. Two components with apparent molecular weights of 165 and 76 kDa were identified from the cell wall preparations of *S. uberis* as the functionally active bLf binding proteins.

4.2 Introduction

Bovine lactoferrin (bLf) is a 92.1 kDa iron-binding glycoprotein occurring in milk, various biological secretions, and polymorphonuclear leukocytes (PMN) (Masson *et al.*, 1966; 1969; Weiner and Szuchet, 1975). During acute bovine mastitis, the bLf concentration in the lacteal secretions can increase up to 30-fold depending on the severity of infection (Harmon *et al.*, 1976). The biological roles of lactoferrin (Lf) include: the amplification of the inflammatory response by promoting the adhesion and aggregation of PMN to the endothelial surface (Oseas *et al.*, 1981), the stimulation of the phagocytic and cytotoxic properties of macrophages (Broxmeyer *et al.*, 1985; Broxmeyer and Platzer, 1984), and the regulation of myelopoiesis and thereby antibody production by feedback inhibition of granulocyte-monocyte colony-stimulating factor (Broxmeyer *et al.*, 1978; Broxmeyer and Platzer, 1984).

Lf can exert a powerful inhibitory effect on bacterial growth. Iron chelation and the resulting iron limitation for bacteria have been suggested to form the mechanism of Lf-mediated antimicrobial action (Arnold *et al.*, 1977; Law and Reiter, 1977; Oram and Reiter, 1968). However, many Gram negative pathogens, notably *Haemophilus influenzae* and *Neisseria meningitidis*, are capable of using iron-binding glycoproteins such as Lf or transferrin (Tf) as iron sources

(Herrington and Sparling, 1985; Mickelsen *et al.*, 1982). Iron acquisition is mediated by bacterial surface receptors which specifically interact with iron-binding glycoproteins (Cornelissen and Sparling, 1994; Williams and Griffiths, 1992). Therefore, it is believed that the ability to compete with Lf and Tf for iron is essential for the pathogenesis of these bacterial infections and the receptors for iron-binding glycoproteins is an important virulence factor of these bacteria. Some Gram positive bacteria such as *Staphylococcus aureus*, coagulase-negative staphylococci and *Streptococcus agalactiae* also bind Lf through specific receptors on their surface (Naidu *et al.*, 1990; 1991a; 1991b; 1992; Rainard, 1992). However, in contrast to our knowledge of the iron uptake systems of Gram negative bacteria, there is comparatively little information concerning the role of Lf receptors from Gram positive bacteria in the uptake of glycoprotein-bound iron.

S. uberis is an environmental pathogen responsible for a high proportion of cases of clinical and subclinical mastitis in lactating cows and is the predominant organism isolated from mammary glands during the nonlactating period (Bramley, 1984; Bramley and Dodd, 1984; Oliver, 1988). Studies have revealed some potential virulence factors associated with *S. uberis*, including hyaluronic acid capsule (Almeida *et al.*, 1993; Hill, 1988a; Matthews *et al.*, 1994b), hyaluronidase (Schaufuss *et al.*, 1989), uberis factor,

believed to be similar to CAMP factor of *S. agalactiae* (Skalka and Smola, 1981; and our study in chapter 3), R-like protein (Groschup and Timoney, 1993), and plasminogen-activating protein (Leigh, 1993). In the present study, the interaction of Lf with *S. uberis* has been investigated and the specific bLf-binding protein of *S. uberis* has been identified.

4.3 Materials and Methods

4.3.1 Bacterial strains and cultural conditions.

One *S. uberis* strain (su-1) from the American Type Culture Collection (ATCC 9927) was chosen for study. Bacteria were grown on base #2 sheep blood agar plates (PML Microbiologicals) at 37°C for 18 h. Iron-restricted conditions were achieved in Todd-Hewitt broth supplemented with 0.3% yeast extract (THB-YE) by the addition of 800 µM EDDA, 800 µM dipyridyl or 100 µM desferrioxamine mesylate. All of the iron chelators were obtained from Sigma.

4.3.2 Preparation of bacterial cell wall.

Cell wall components of *S. uberis* were extracted as described by Baker *et al.* (1976). Twenty base #2 blood agar plates were inoculated with *S. uberis* and incubated for 18 h at 37°C. Bacterial cells were collected, washed once with 200 ml of 0.85% saline and resuspended in 50 ml of extraction buffer (0.05 M Na_2HPO_4, 0.15 M NaCl, 0.01 M EDTA, pH 7.4). Cell walls were extracted by shaking with glass beads (4-mm diameter) for 20 h at 37°C. After centrifugation at 48,300 g for 20 min, supernatant (cell wall extract) was collected, filtered (0.22 µM Nalgene filter), dialysed against distilled water, lyophilized and resuspended in 1 ml of distilled water.

4.3.3 Preparation of iron-binding proteins.

All iron-binding proteins, including bLf (from bovine milk), bovine transferrin (bTf), human lactoferrin (hLf) and human transferrin (hTf), were purchased from Sigma in the most iron-free form available. Iron-saturated proteins and the apoproteins were prepared by methods described previously (Mazurier and Spik, 1980).

4.3.4 Preparation of ^{125}I-labelled bLF.

Bovine Lf was iodinated by the lactoperoxidase method of Thorell and Johansson (1971). Approximately 70 µg of bLf (33% iron-saturated) was used for iodination; ^{125}I-labelled protein was separated from free Na^{125}I by chromatography on a Sephadex G-25 column. The labelled protein was aliquoted and stored at -70°C until use. Lactoperoxidase was purchased from Boehringer-Mannheim; and Na^{125}I from Amersham.

4.3.5 Lactoferrin binding assays.

The binding assays were performed as described by Naidu *et al* (1990; 1991a; 1991b; 1992). Bacterial cells were harvested from culture media, washed once in 0.1 M phosphate-buffered saline (PBS), pH 7.2, and resuspended in PBS containing 1% bovine serum albumin (PBS-1% BSA) to a density of 10^{10} bacteria/ml (c. OD_{600}=1.5). To determine saturation time, 10^9 bacteria (in 0.1 ml of PBS-1% BSA) were mixed and

incubated with 0.1 ml of ^{125}I-bLf solution (6.9 nM in PBS-1 % BSA) for periods of 5, 10, 15, 20, 25, 30, 60, 90, 120, 150 min at room temperature. Bacteria were pelleted and washed three times with 1 ml of ice-cold PBS containing 0.1% Tween 20. Radioactivity bound to the bacterial pellet was measured in a γ-counter. In competitive binding experiments, 10^9 bacteria were mixed with 2 x 10^5 cpm ^{125}I-bLf in the presence of serially-diluted unlabelled bLf and incubated at room temperature for 2 h. Total input, cell bound and free proteins were calculated and subjected to Scatchard analysis (Scatchard, 1949). When evaluating the inhibitory effect of bLf, apo-bLf, bTf, hLF and hTf on ^{125}I-bLf binding, the unlabelled proteins were used at concentration of 5.5 μM. All samples were tested in triplicate, and each experiment was repeated at least twice. The data presented are the means of two independent experiments (unless otherwise stated).

4.3.6 Proteolytic and heat treatment of *S. uberis*.

Bacteria (1 ml containing 10^{10} cells) were treated with proteases at 37°C for 2 h. Trypsin (Sigma) hydrolysis was performed in 100 mM Tris-HCl (pH 8.0), with a final enzyme concentration of 2,500 u/ml, and the reaction was stopped by addition of phenylmethylsulfonyl fluoride (500 μg/ml). Pepsin (Sigma) digestion was performed in 100 mM sodium acetate buffer (pH 4.5), with an enzyme concentration of 1,000 u/ml, and the pH of the reaction mixture was raised

to 7.4 to stop the hydrolysis. Proteinase K (Boehringer mannheim) treatment was carried out in 40 mM potassium phosphate buffer (pH 7.5), and the digestion was inhibited by the addition of phenylmethylsulfonyl fluoride (500 µg/ml). For heat treatment, the bacterial suspension (10^{10} cell/ml) was incubated in a water bath for 1 h at each of the following temperatures: 50°C, 80°C and 100°C. Both enzyme and heat-treated cells were washed once in PBS and resuspended in PBS-1% BSA prior to the binding experiments.

4.3.7 PAGE and Western blotting.

SDS-polyacrylamide gel electrophoresis (PAGE) of proteins was performed using the method described by Laemmli (Laemmli, 1970). Samples were solubilized in sample buffer at 37°C for 30 min in the absence of 2-mercaptoethanol (non-reducing conditions) or at 100°C for 5 min in the presence of 1% 2-mercaptoethanol (reducing conditions). Proteins were electrophoretically transferred to nitrocellulose membranes as recommended by the supplier (Bio-Rad) and blocked with TBS-1% BSA.

To identify the putative bLf-binding protein, Western blots were probed with ^{125}I-bLf as follows. ^{125}I-bLf was added to the membrane to a final concentration of 80 ng/ml in TBS-1% BSA and incubated at room temperature for 2 h. After three washes with TBS containing 0.05% Tween 20, the membrane was exposed to X-ray film for 24 h at room

temperature. To compete the ^{125}I-bLf binding, the transferred membrane was incubated with 35 µg/ml of unlabelled bLf for 2 h before incubation with ^{125}I-bLf.

4.4 Results

4.4.1 Time-dependent binding of bLF to *S. uberis*.

One *S. uberis* strain su-1 was tested for bLf binding in a ^{125}I-labelled protein binding assay. To study the kinetics of ^{125}I-bLf binding with *S. uberis*, binding was measured at different time intervals (Fig. 4-1). The time course showed that ^{125}I-bLf could bind to *S. uberis* in a time-dependent manner, with a requirement of approximately 90 min for 100% saturation. This binding saturation time was the basis for determining the incubation time in latter binding experiments.

4.4.2 BLf receptor saturability, affinity and copy number.

A competitive binding experiment using bLf (33% iron-saturated) as both radioligand and competitor was performed, and the specificity of bLf binding by *S. uberis* is demonstrated in Fig. 4-2. Unlabelled bLf effectively displaced the binding of ^{125}I-bLf to *S. uberis* in a dose-dependent manner. A concentration of approximately 270 nM of unlabelled bLf caused 50% blocking of ^{125}I-bLf uptake (indicated by arrows). Scatchard plot analysis (Scatchard, 1949) showed linearity, thus the demonstration of one bLf binding component is expected. The number of bLf molecules bound per *S. uberis* cell calculated from Scatchard plot was

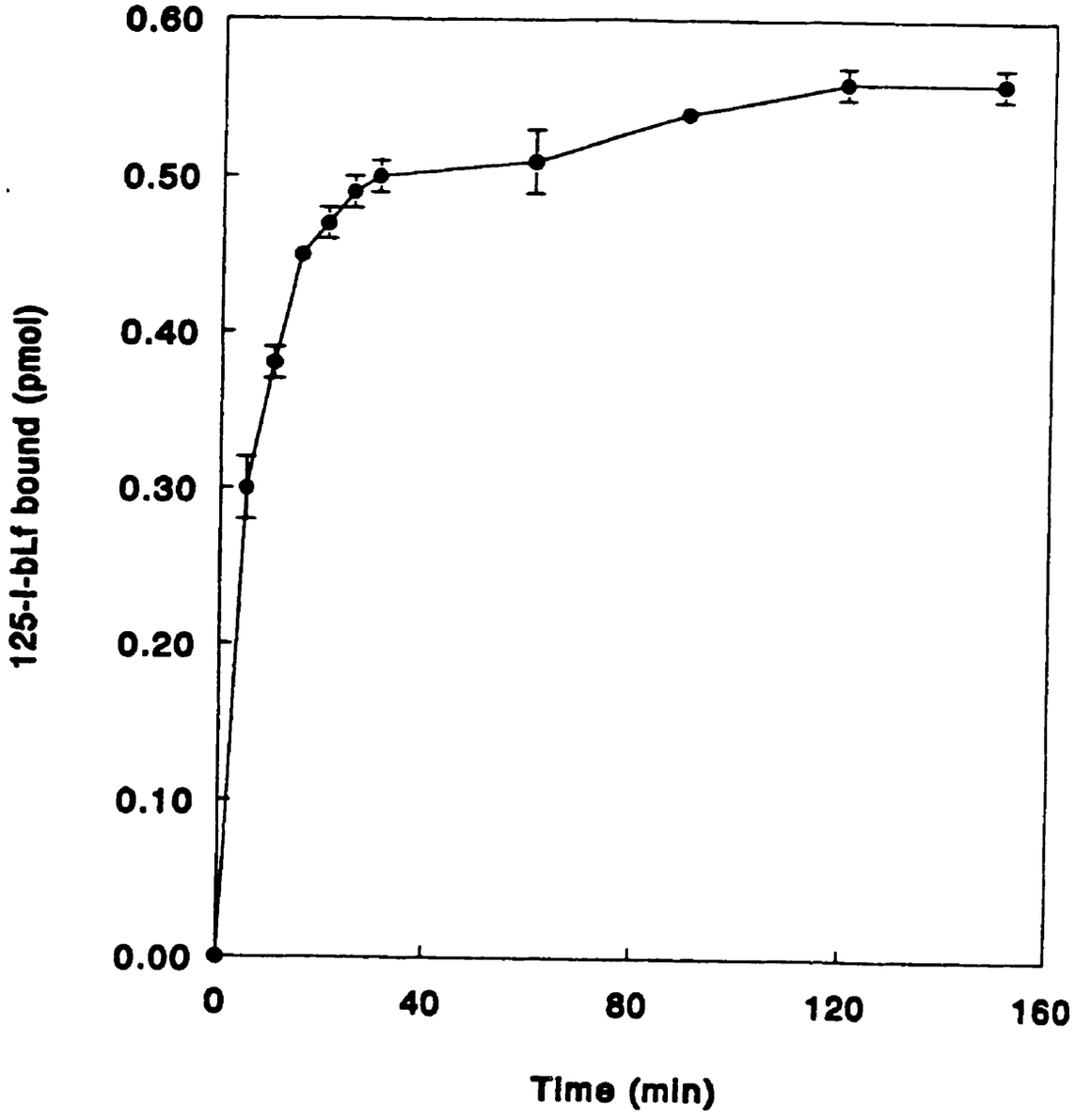


Figure 4-1. Time course of ^{125}I-bLf binding to *S. uberis*. 10^9 bacteria were incubated with 6.9 nm ^{125}I-bLf in 0.2 ml of PBS-1% BSA. At time intervals indicated, bacteria were pelleted and the amount of cell associated ^{125}I-bLf was determinated.

approximately 7800, with an affinity (Kd) of 1.0×10^{-7}M.

To determine whether bLf iron saturation could influence receptor binding, apo-bLf was used as a competitor in the ^{125}I-bLf binding assay. Result showed that apo-bLf could inhibit ^{125}I-bLF binding as effectively as the iron-saturated bLf (Table 4-1), indicating that both apo-bLf and iron-saturated bLf had the same binding receptor on the *S. uberis* cell.

To examine further the specificity of binding, the abilities of bTf, hLf and hTf to inhibit the binding of ^{125}I-bLf to *S. uberis* cells were also evaluated (Table 4-1). None of these proteins could interfered with the binding of ^{125}I-bLf to *S. uberis*, suggesting that the binding was bLf-specific.

4.4.3 Influence of iron-restricted conditions on bLf binding.

In an attempt to determine if the bLf-binding property of *S. uberis* was mediated by an iron-regulated bacterial component, EDDA, dipyridyl or desferrioxamine mesylate were incorporated in THB-YE broth to reduce the availability of iron. Cells from these iron-restricted conditions did not show higher ^{125}I-bLf binding than those from normal cultural condition (Fig. 4-3), indicating that iron-restricted condition did not notably modify the saturation of *S. uberis* by bLf.

Table 4-1. Inhibitory effects of unlabelled proteins and enzyme or heat treatment of bacteria on ^{125}I-bLf binding to *S. uberis*.

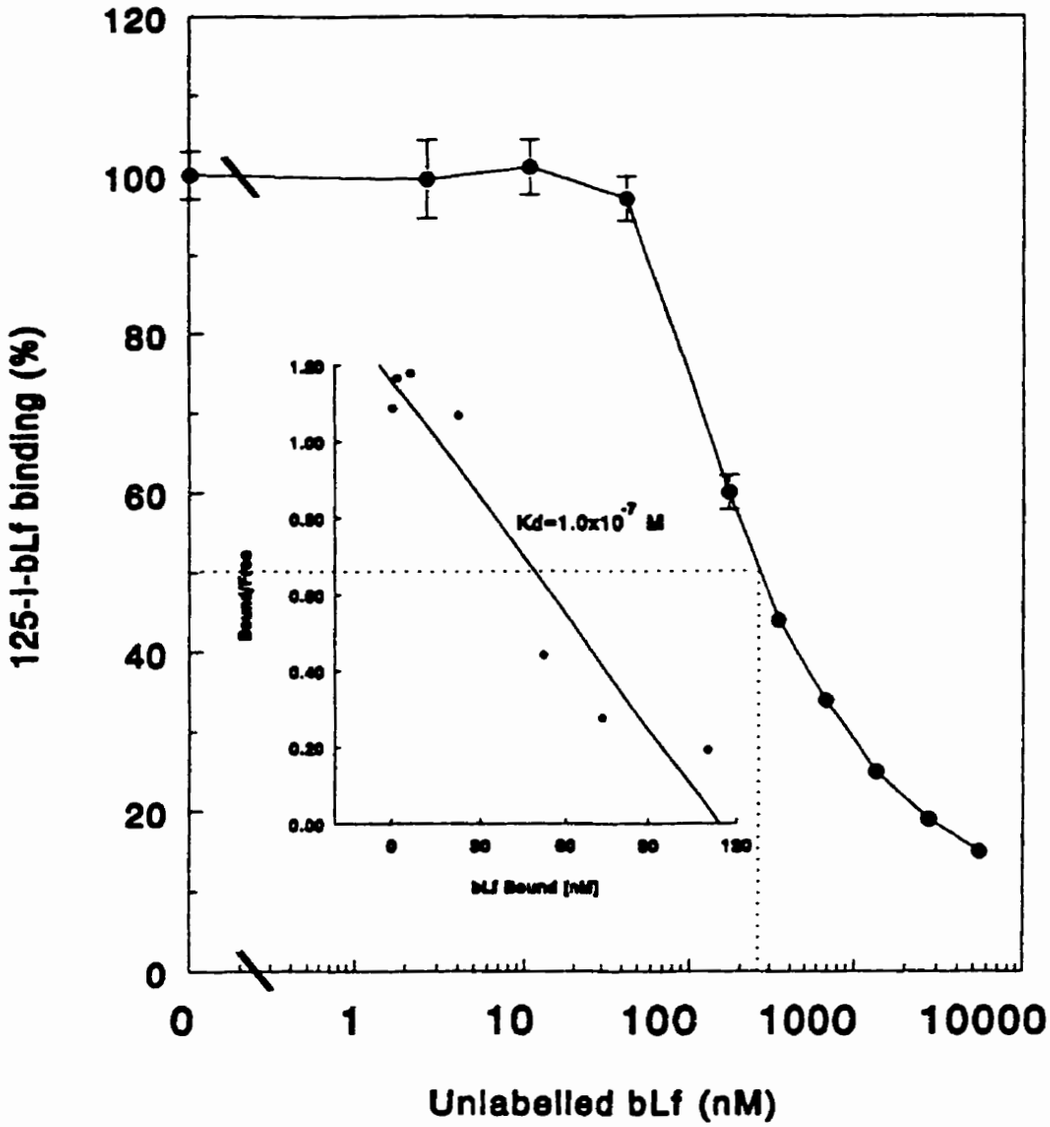
Inhibitor or cell treatment	Percentage inhibition[1] or decrease[2] in binding	
	mean	SD
Inhibitor		
bLf	94.6	0.1
apo-bLf	94.7	0.2
bTf	2.5	1.4
hLf	-14.0	2.4
hTf	1.9	1.3
Proteases		
Pepsin	85.7	3.7
Trypsin	89.1	4.4
Proteinase K	92.7	2.4
Heating		
50°C	-4.3	6.0
80°C	29.6	2.1
100°C	65.6	4.8

[1] Inhibition values were calculated as relative percentage of bLf binding to bacteria suspended in PBS in the absence of any inhibitor.
[2] Decrease in binding were calculated as relative percentage of bLf binding to bacteria without any treatment.

4.4.4 Sensitivity of the bLf binding component to protease hydrolysis and heat treatment.

Pepsin, trypsin and proteinase k treatment of *S. uberis* cells could abolish bLf-binding (Table 4-1), indicating the involvement of a surface-exposed cell wall protein(s) in the binding. This proteinaceous component was susceptible to temperature, since heat treatment of bacteria reduced binding to a certain degree (Table 4-1).

Figure 4-2. Competition binding assay using bLf (33% iron-saturated) as radiolabelled ligand and competitor. Percentage binding values were calculated as percentage of ^{125}I-bLf binding in the presence of increasing amounts of unlabelled bLf to bacteria suspended in PBS-1% BSA in the absence of unlabelled bLf. Inset: Scatchard plot (on the basis of one experiment; another experiment showed similar result) and affinity (Kd) of the binding of ^{125}I-bLf to *S. uberis* (Scatchard 1949). The line represents the best fit as determined by a linear regression analysis. A concentration of 270 nM of unlabelled bLf caused 50% displacement of ^{125}I-bLf binding (indicated by dotted lines).


120
100
80
60
40
20
0
125-I-bLf binding (%)
0
1
10
100
1000
10000
Unlabelled bLf (nM)
Kd=1.0x10^-7 M
Bound/Free
bLf Bound [nM]

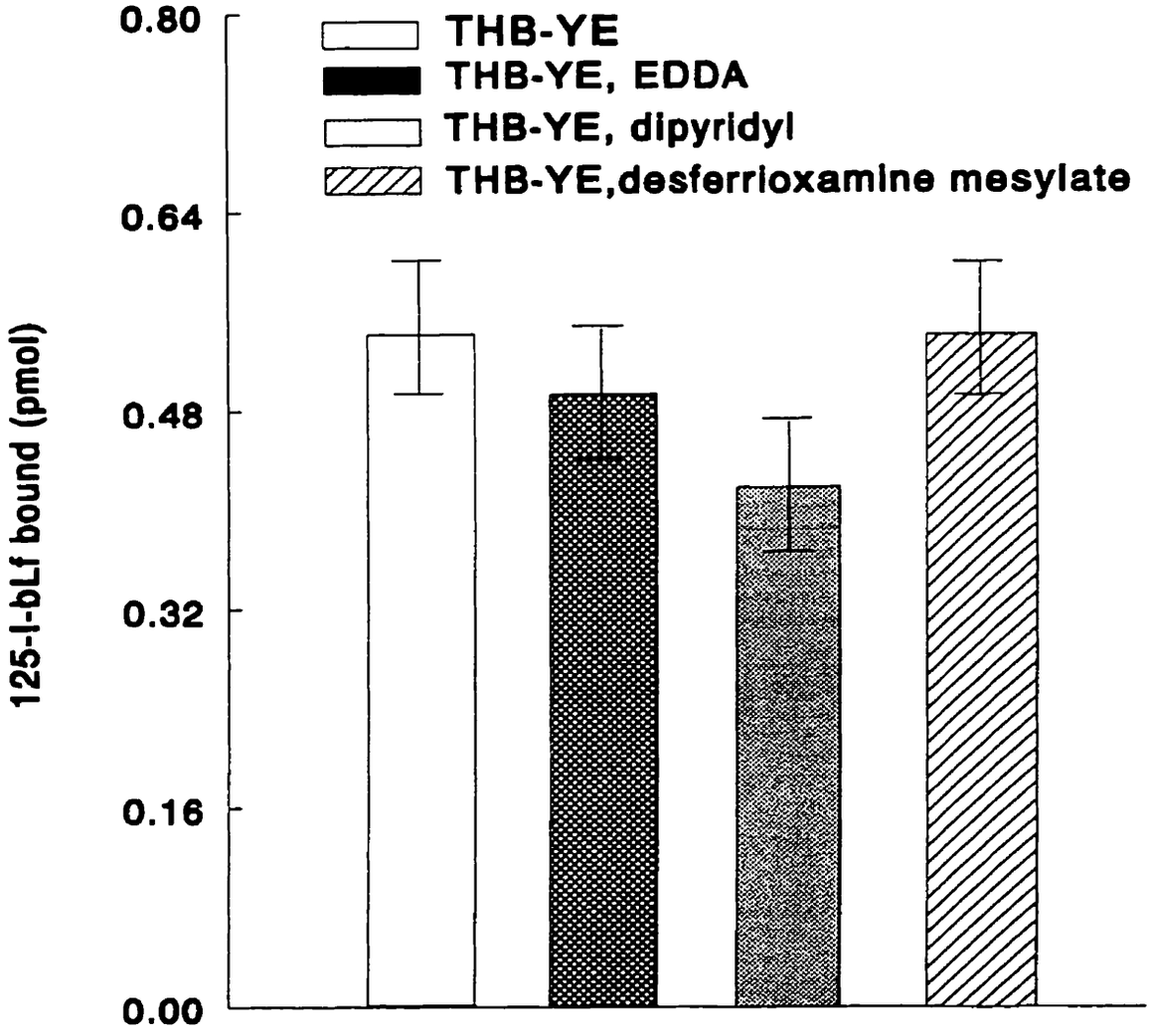


Figure 4-3. Influence of iron chelators on the expression of lactoferrin binding by *S. uberis*. Cells grown in THB-YE with or without EDDA, dipyridyl or desferrioxamine mesylate were incubated with 6.9 nM ^{125}I-bLf in 0.2 ml of PBS-1% BSA at room temperature for 2 h. After three washes, cell-bound radioactivity was determined.

4.4.5 Identification of a cell wall bLf-binding protein.

The presence of the functionally active bLf-binding protein (Lbp) in the cell wall preparation of *S. uberis* was detected by a Western blot probed with ^{125}I-bLf (Fig. 4-4). Under non-reducing condition, two components with apparent molecular weight of 165 and 76 kDa were identified as bLf-binding proteins of *S. uberis* (Fig. 4-4 A, lane 2). Proteins under reducing condition lost the bLf binding activity to a great extent (Fig. 4-4 A, lane 1). The protein bands in Fig. 4-4 A were demonstrated to be from specific binding to ^{125}I-bLf, since the presence of unlabelled bLf effectively blocked the binding (Fig. 4-4 B).

4.5 Discussion

Lf is a high-affinity iron-binding glycoprotein present abundantly in milk, the specific granules of PMNs and mucosal secretions (Masson *et al.*, 1966; 1969). It is related in structure and function to Tf, an abundant serum protein (Morgan, 1974). An important biological function of Lf is probably the sequestration of free iron from the environment of microorganisms, thus inhibiting their growth (Sanchez *et al.*, 1992). However, many bacteria such as *Neisseria* and *Haemophilus* species are able to acquire iron from host Lf through specific receptors (Lee and Schryvers, 1988; McKenna

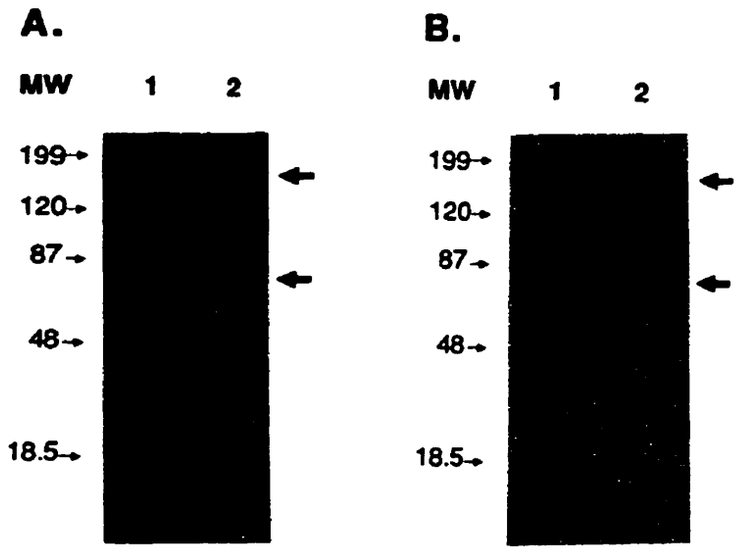


Figure 4-4. Demonstration of bLf-binding proteins in *S. uberis* by Western blotting. Samples were analysed in 7.5% SDS-PAGE and transblotted to a nitrocellulose membrane which was further incubated with ^{125}I-bLf in the absence (A) or presence (B) of unlabelled bLf. Lane 1. Cell wall of *S. uberis* under reducing conditions. Lane 2. Cell wall of *S. uberis* under non-reducing conditions. The molecular mass standards (kDa) are indicated by arrows to the left. Arrows to the right indicate the 165 kD and 76 kD protein bands.

et al., 1988; Schryvers, 1989; Schryvers and Morris, 1988a). The receptor-mediated interaction of Lf with some Gram positive bacteria has also been demonstrated (Naidu *et al.*, 1990; 1991a; 1991b; 1992; Rainard, 1992), although the involvement of iron uptake in this interaction needs to be elucidated.

To demonstrate whether *S. uberis* was able to express a specific receptor for bLf, we sought to determine whether the major prerequisites for a biological receptor could be fulfilled, namely, ligand specificity and concentration-dependent saturability. Bovine Lf binding to *S. uberis* was time and concentration dependent (demonstrated by the ability of the unlabelled ligand to compete for binding with ^{125}I-bLf), indicating the existence of a limited number of binding receptors on the cell surface. Scatchard plot analysis estimated that there were 7800 bLf-binding sites/cell. The affinity of the streptococcal receptor for bLf (1.0×10^{-7} M) is slightly lower than that described by Naidu *et al.* (1991b) for the bLf receptor of *S. aureus* (7.1×10^{-8} M).

S. uberis did not distinguish between bovine apo-Lf and iron-saturated Lf, since both forms were equally effective at blocking ^{125}I-bLf binding in the competitive binding assay. This finding is similar to that described for the *N. meningitidis* Lf receptor (Schryvers and Morris, 1988a) but contrasts with that for the Tf receptors. In competitive

binding experiments comparing iron-saturated Tf with apo-Tf, Schryvers and Morris (1988b) clearly showed that iron-saturated Tf was more effective in blocking the receptor than apo-Tf. However, Simonson *et al.* (1982) and Tsai *et al.* (1988) failed to demonstrate differences in recognition of iron-loaded or iron-free Tf by *N. meningitidis*. It was proposed that this discrepancy could be strain dependent or could be attributed to the methods used (Otto *et al.*, 1992). The former group (Schryvers and Morris, 1988b) used horseradish peroxidase (HRP)-labelled Tf, while the latter investigators (Tsai *et al.*, 1988) used radioactively labelled Tf (^{125}I-Tf) in competitive binding experiments. Tf may be modified by HRP in such a way that binding is less sensitive to competition by apo-Tf, or iron could be removed from ^{125}I-labelled Tf by cold Tf, thereby reducing its affinity for the receptor. Clearly, no discrepancy exists between our result and those described for the *N. meningitidis* lactoferrin receptor (Schryvers and Morris, 1988a) despite of the different bacterial strains and methods used.

A common feature which has emerged from studies of the transferrin-binding proteins of Gram negative bacteria is their remarkable specificity for the transferrin of their natural host (Morton and Williams, 1989; 1990; Schryvers and Lee, 1989; Williams and Griffiths. 1992). For example, humans are the only known natural reservoir for *H. influenzae*, which shows a marked preference for human transferrin and is unable

to bind or remove iron from porcine, rabbit, mouse, or dog transferrin (Morton and Williams, 1990). Similarly, the porcine pathogen *Actinobacillus pleuropneumoniae* is able to bind and acquire iron from porcine but not human transferrin (Gonzales *et al.*, 1990; Morton and Williams, 1989; 1990). In competition binding assays, the streptococcal receptor described here also demonstrated some lactoferrin species specificity in that human Lf could not effectively block the binding of bovine Lf. Interestingly, *S. aureus* Lf receptors could bind lactoferrins from both human and bovine sources (Naidu *et al.*, 1990; 1991a; 1991b; 1992). In this context, given that *S. uberis* is exclusively a bovine pathogen, while *S. aureus* causes infection in both humans and bovine, it is possible that the specificity for lactoferrins may also contribute to the host specificity of these bacteria. In addition, neither bovine nor human transferrin was able to block binding of ^{125}I-labelled bovine lactoferrin to the lactoferrin receptor of *S. uberis*. This observation is similar to that reported by Naidu *et al.* (1991b; 1992), in which human or bovine lactoferrin binding to lactoferrin receptors of *S. aureus* could not be blocked by human or bovine transferrin.

Although the expression of most of the transferrin and lactoferrin receptors of Gram negative bacteria such as *N. meningitidis* (Schryvers and Morris, 1988a; 1988b) and *H. influenza* (Schryvers, 1988; 1989) is iron regulated, *S.*

uberis receptor activity was not regulated by growth medium iron availability. Our results are consistent with the findings of Modun *et al.* (1994) and Rainard (1992) who showed that the binding of *S. aureus* to Tf and *S. agalactiae* to Lf was not regulated by iron.

Having demonstrated the existence of bovine lactoferrin receptor on *S. uberis*, we sought to identify the bacterial cell wall component(s) involved. Treatment of *S. uberis* cells with heat and proteolytic enzymes abolished lactoferrin binding, indicating the probable involvement of a protein(s) on the cell surface. We prepared a cell wall extract of *S. uberis* and solubilized it in SDS-PAGE sample buffer under non-reducing condition. Two lactoferrin binding proteins with molecular weights of 165 kDa and 76 kDa were identified by ^{125}I-bLf binding after SDS-PAGE and transfer to nitrocellulose. Since Scatchard plot analysis indicated that *S. uberis* bears only one bLf binding component, the 165 kDa protein was expected to be the dimer form of the 76 kDa. The lactoferrin binding protein of *S. uberis* differs from the transferrin receptors of *Haemophilus* and *Neisseria* spp., which consist of two distinct transferrin-binding proteins, termed Tbp1 and Tbp2, which range in molecular weight from 68 to 105 kDa depending on the strain (Williams and Griffiths, 1992). Interestingly, for both organisms, only Tbp2 was able to bind transferrin after SDS-PAGE and electroblotting (Stevenson *et al.*, 1992). The *S. uberis* Lbp is also different

from the lactoferrin receptors of the mentioned two species. To date, only one lactoferrin binding protein with a molecular weight of 98 to 105 kDa was identified from those species (Lee and Bryan, 1989; Pettersson *et al.*, 1994; Schryvers, 1989; Schryvers and Lee, 1989) and the gonococcal lactoferrin protein (Lbp1) was shown to share features with the functionally related gonococcal Tbp1 (Biswas and Sparling, 1995). If it is accepted that there is a difference between the *S. uberis* Lbp and the transferrin or lactoferrin receptors of Gram negative bacteria, it would be surprising to find that *S. uberis* Lbp differs from the bovine lactoferrin receptor of *S. aureus,* which consists of two distinct bLf-binding proteins with estimated molecular weights of 92 and 67 kDa (Naidu *et al.*, 1991b). Also, the streptococcal Lbp described here appears to be different from the *S. aureus* human lactoferrin-binding protein described by Naidu *et al.* (1992) as an approximately 450 kDa protein which under reducing SDS-PAGE gel condition was resolved into two components of 67 and 62 kDa.

5.0 CLONING AND CHARACTERIZATION OF *S. UBERIS* LBP GENE AND ITS UPSTREAM MGA

5.1 Summary

The gene for the bovine lactoferrin binding protein (*lbp*) has been cloned and sequenced from *S. uberis*. A single ORF encoding 561 amino acid residues was shown to generate two protein species in *E. coli*. These proteins were able to bind bovine lactoferrin and had molecular weights of 76 kDa and 165 kDa, similar to those of native *S. uberis* proteins. The 165 kDa protein was likely a dimer of the 76 kDa protein. Consistent with this, urea treatment resulted in a single band, and Northern blot analysis showed only one major transcript in both *S. uberis* and recombinant *E. coli*. A putative signal peptide was found at the N terminus of the deduced amino acid sequence and the C terminus had the features of a membrane anchor motif found in other surface proteins from Gram positive bacteria. Deletion analysis located the bLf binding domain to a 200 amino acid region at the N terminus of this protein. Analysis of the primary and secondary structure suggested that bLf binding protein of *S.*

uberis is a M-like protein. Consistent with this, a gene (*mga*) homologous to the group A streptococcal Mga, a positive regulator of M and M-like proteins was found in the adjacent region of the *lbp*. The transcript of *mga* was detected in recombinant *E. coli* but not in *S. uberis*. Southern blot analysis revealed that *mga* was distributed in all *S. uberis* strains that contained the *lbp*. However, the relationship of *mga* and *lbp* needs to be studied.

5.2 Introduction

S. uberis is an environmental pathogen responsible for a high proportion of cases of clinical and subclinical mastitis in both lactating and nonlactating dairy cattle (Bramley, 1984; Bramley and Dodd, 1984; Oliver, 1988). A few potential virulence factors have been identified from *S. uberis*, including hyaluronic acid capsule (Hill, 1988; Almeida and Oliver, 1993; Matthews *et al.*, 1994), hyaluronidase (Schaufuss *et al.*, 1989), uberis factor, believed to be similar to CAMP factor of *S. agalactiae* (Skalka and Smola, 1981; and our study in chaper 3), R-like protein (Groschup and Timoney, 1993), and plasminogen-activating protein (Leigh, 1993).

Since many Gram negative pathogens, notably *Haemophilus influenzae* and *Neisseria meningitidis*, have been shown to acquire Tf- or Lf-bound iron through bacterial surface receptors (Cornelissen and Sparling, 1994; Herrington and Sparling, 1985; Mickelsen *et al.*, 1982; Williams and Griffiths, 1992), and some Gram positive bacteria such as *Staphylococcus aureus*, coagulase-negative staphylococci and *Streptococcus agalactiae* have also been found to be able to bind Lf (Naidu *et al.*, 1990; 1991a; 1991b; 1992; Rainard, 1992), we characterized the bLf-streptococcal interaction in *S. uberis* strain su-1. The binding of ^{125}I-bLf to *S. uberis* was time-dependent, with a requirement of approximately 90

min for 100% saturation. Unlabelled bLf displaced the binding of ^{125}I-bLf to *S. uberis* cells in a dose-dependent manner. Apo-bLf could inhibit ^{125}I-bLf binding as effectively as iron-saturated bLf. Bovine transferrin, human lactoferrin and human transferrin did not interfere with bLf binding. The Scatchard plot was linear, implying an one-affinity (Kd=1.0 x 10^{-7} M) binding mechanism. We estimated that there were approximately 7800 bLf binding sites/cell. Reduced iron availability obtained by the presence of iron chelators did not notably modify the saturation of *S. uberis* by bLf. Heat- or protease treatment of bacterial cells reduced bLf binding to a great degree, indicating the involvement of a cell surface protein. Western blotting of a cell wall preparation probed with ^{125}I-bLf identified two components with apparent molecular weights of 165 and 76 kDa as the functionally active bLf binding proteins.

In this study, we sought molecular evidence for the identity of the streptococcal Lf receptor.

5.3 Materials and Methods

5.3.1 Bacterial strains, plasmids and media.

S. uberis strains used for study are listed in Table 5-1 (page 153). Bacteria were grown on base #2 sheep blood agar plates (PML Microbiologicals) at 37°C for 18 h, or in Todd-Hewitt broth supplemented with 0.3% yeast extract (THB-YE) at 37°C overnight. *E. coli* cells were grown in Luria broth or on Luria broth-agar plates. Ampicillin was used at 50 µg/ml for the growth of *E. coli* strains containing recombinant plasmids. The cloning vector used was pTZ18R (Mead *et al.*, 1986).

5.3.2 Preparation of bacterial cell wall, cell membranes, periplasma, whole cell lysate and culture supernatant.

Cell wall components of *S. uberis* were extracted as described in 4.3.2.

Outer and inner membranes were isolated from *E. coli* cells by sucrose density gradient centrifugation as described previously (Hancock and Nikaido, 1978). *E. coli* periplasmic proteins were prepared by the cold osmotic shock method described by Rioux and Kadner (1989).

To prepare whole cell lysates of *E. coli* transformants, bacteria were grown overnight, collected, washed once in 0.1 M phosphate-buffered saline (PBS), pH 7.2,

resuspended in water, exposed to one freeze-thaw cycle and sonicated for 2 min. After centrifugation at 6000 x g for 20 min, the supernatant (whole cell lysate) was collected, lyophilized and resuspended in 1/100 culture volume of water.

The 100 fold-concentrated cultural supernatant of the recombinant *E. coli* was obtained by precipitation with 10% trichloroacetic acid (TCA).

5.3.3 Preparation of iron-saturated bLf and ^{125}I-labelled bLf.

33% iron-saturated bovine lactoferrin was prepared as in 4.3.3. and iodinated as described in 4.3.4.

5.3.4 Lactoferrin binding assay.

Bacterial cells were harvested from culture media, washed once in 0.1 M PBS (pH 7.2), and resuspended in PBS containing 1% bovine serum albumin (PBS-1% BSA) to a density of 10^{10} bacteria/ml (c. OD_{600}=1.5). To assess the bLf-binding ability of the recombinant protein, increasing amounts of *E. coli* cell lysate plus supernatant were mixed with approximately 10^9 *S. uberis* and incubated with 1.0 x 10^4 cpm ^{125}I-bLf in a total volume of 200 µl. After 2 hours of incubation at room temperature, bacterial cells were pelleted and washed three times with 1 ml of ice-cold PBS containing 0.1 % Tween 20. Radioactivity present in the bacterial pellet was measured in a gamma-counter. All samples were tested in

triplicate, and the experiment was repeated twice. The data presented are the mean of two independent experiments ± standard deviation.

5.3.5 Preparation of antiserum.

Serum against the recombinant Lbp of *S. uberis* was raised in rabbits by subcutaneous injection of 0.5 ml of TCA-precipitated culture supernatant of recombinant *E. coli* in complete Freund's adjuvant. Two subcutaneous boosts with the same amount of sample in incomplete Freund's adjuvant were given to each animal.

5.3.6 PAGE and Western blotting.

SDS-polyacrylamide gel electrophoresis (PAGE) of proteins was performed as described by Laemmli (Laemmli, 1970). Samples under reducing and non-reducing conditions were prepared as in 4.3.7. Samples dissolved in sample buffer with 3 M urea were boiled for 30 min prior to loading. ^{125}I-bLf probed-Western blotting was performed as in 4.3.7. Immunoblotting probed with rabbit antiserum against the recombinant Lbp was as described in 3.3.7.

5.3.7 Recombinant DNA techniques.

Plasmid DNA was purified as described by Maniatis *et al.* (1989). When required, DNA fragments were isolated from agarose gels using a Gene Clean kit (Bio/can

Scientific).

To construct a gene library for *S. uberis*, chromosomal DNA was prepared as previously described (Caparon and Scott, 1991) and partially digested with *Sau*3AI. Fragments of 2,000 to 5,000 bp were recovered following sucrose density gradient centrifugation (Maniatis *et al.*, 1989). The ends of these fragments were partially filled in with dGTP and dATP and ligated into pTZ18R which was cut with *Sal*I and partially filled in with dTTP and dCTP. Transformation of *E. coli* DH5α competent cells was carried out as recommended by the supplier (GIBCO BRL, Gaithersburg, Md). To identify Lf-binding clones, transformants were replica plated onto nitrocellulose discs (Schleicher & Schuell, Keene, NH) and lysed in chloroform vapour. Nonspecific binding was blocked by incubation with TBS-1% BSA. Membranes were further incubated with ^{125}I-bLf as described in 4.3.7.

Restriction endonucleases, T4 DNA ligase, DNA polymerase I Klenow fragment and calf intestinal alkaline phosphatase were utilized according to the manufacturer's directions (Pharmacia Canada Ltd., Quebec, Canada).

DNA sequence was determined by the dideoxy-chain termination method of Sanger *et al.* (1977) on double-stranded plasmid templates by using a T7 Sequencing kit (Pharmacia Canada Ltd.).

5.3.8 RNA analyses.

RNA was isolated from *E. coli* and *S. uberis* as described in 3.3.9, and Northern blot analysis was carried out as described by Maniatis *et al.* (1989).

5.4 Results

5.4.1 Cloning and expression of the *lbp* gene.

A gene library was constructed in pTZ18R with chromosomal DNA from *S. uberis* (su-1). About 5000 transformants were initially screened for expression of bLf binding protein (Lbp) by colony blotting with ^{125}I-bLf. One colony with the strongest signal and six with weaker signals were selected and used to make whole cell lysates, which were further tested for their ability to bind ^{125}I-bLF under non-reducing conditions. The clone with the strongest signal, *E. coli* pLBP5, generated three major bands; two of them had molecular weights of 165 and 76 kDa (Fig. 5-1, lane 3) which are quite close in size to those of *S. uberis* (lane 1). The band slightly larger than 165 kDa could be the precursor form with the uncleaved signal peptide. No corresponding band was found from the whole cell lysate of *E. coli* pTZ18R, the host strain control (lane 2). The recombinant Lbp was also detected from the periplasm and supernatant of *E. coli* pLBP5 (lane 6 and 7, respectively), but not from outer or inner membranes (lane 4 and 5, respectively), indicating that the Lbp expressed in *E. coli* DH5α was not membrane-localized, but instead it could be secreted from the cell.

The presence of free and functionally active recombinant Lbp in the cell lysate and supernatant of *E. coli* pLBP5 was also detected by performing competitive inhibition

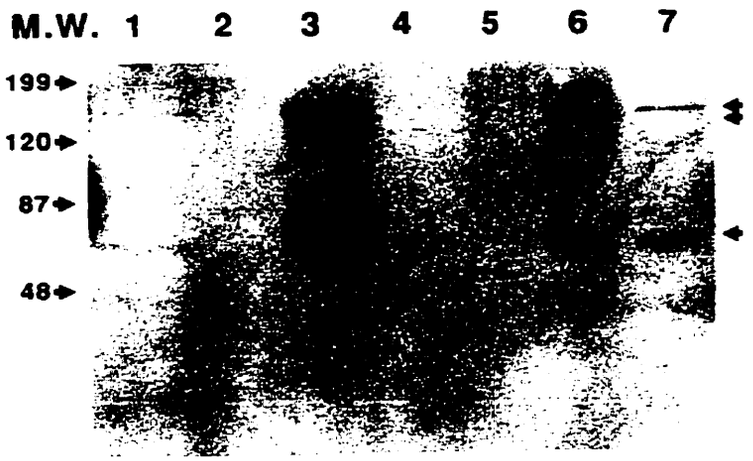


Figure 5-1. Expression of bLf-binding proteins in recombinant *E. coli* pLBP5. All samples were analyzed under non-reducing conditions in 7.5% SDS-PAGE and transblotted to a nitrocellulose membrane which was further probed with ^{125}I-labelled bLf. Lanes: 1, cell wall of *S. uberis;* 2, whole cell lysate of *E. coli* pTZ18R; 3, whole cell lysate of *E. coli* pLBP5; 4, outer membrane of *E. coli* pLBP5; 5, inner membrane of *E. coli* pLBP5; 6, periplasm of *E. coli* pLBP5; 7, supernatant of *E. coli* pLBP5. The molecular mass standards (kDa) are indicated by arrows to the left, and the ^{125}I-bLf-reactive bands are indicated by arrows to the right.

assays. The *E. coli* pLBP5 cell lysate and supernatant mixture effectively inhibited ^{125}I-bLf binding to *S. uberis* cells in a dose-dependent manner, while samples from *E. coli* pTZ18R did not (Fig. 5-2).

To demonstrate whether the protein with a molecular weight of 165 kDa was a dimer of the 76 kDa molecule, samples treated with 2-mercaptoethanol or urea were analyzed by Western blotting using rabbit antiserum against the supernatant of *E. coli* pLBP5 (Fig. 5-3). Mercaptoethanol treatment was shown to have no effect on the electrophoretic mobility of Lbp from either *S. uberis* or recombinant *E. coli* (lane 1, 2 and 4, respectively), while urea-treatment resulted in disappearance of the 165 and 76 kDa bands and appearance of a new band with an apparent molecular weight of 105 kDa (lane 6 and 7). This data indicates that the 165 kDa protein is likely a dimer of the 76 kDa subunit. The dimer could have been disassociated by urea to monomers which could be further denatured and unfolded, resulting in the apparent molecular weight increase from 76 kDa to 105 kDa. A significant increase in the apparent molecular weight of Tbp2 following urea-treatment has been observed in *Neisseria meningitidis* by other researchers (Vonder haar *et al.*, 1994). Our data also indicates that disulfide bonds are not essential for the formation of the oligomer.

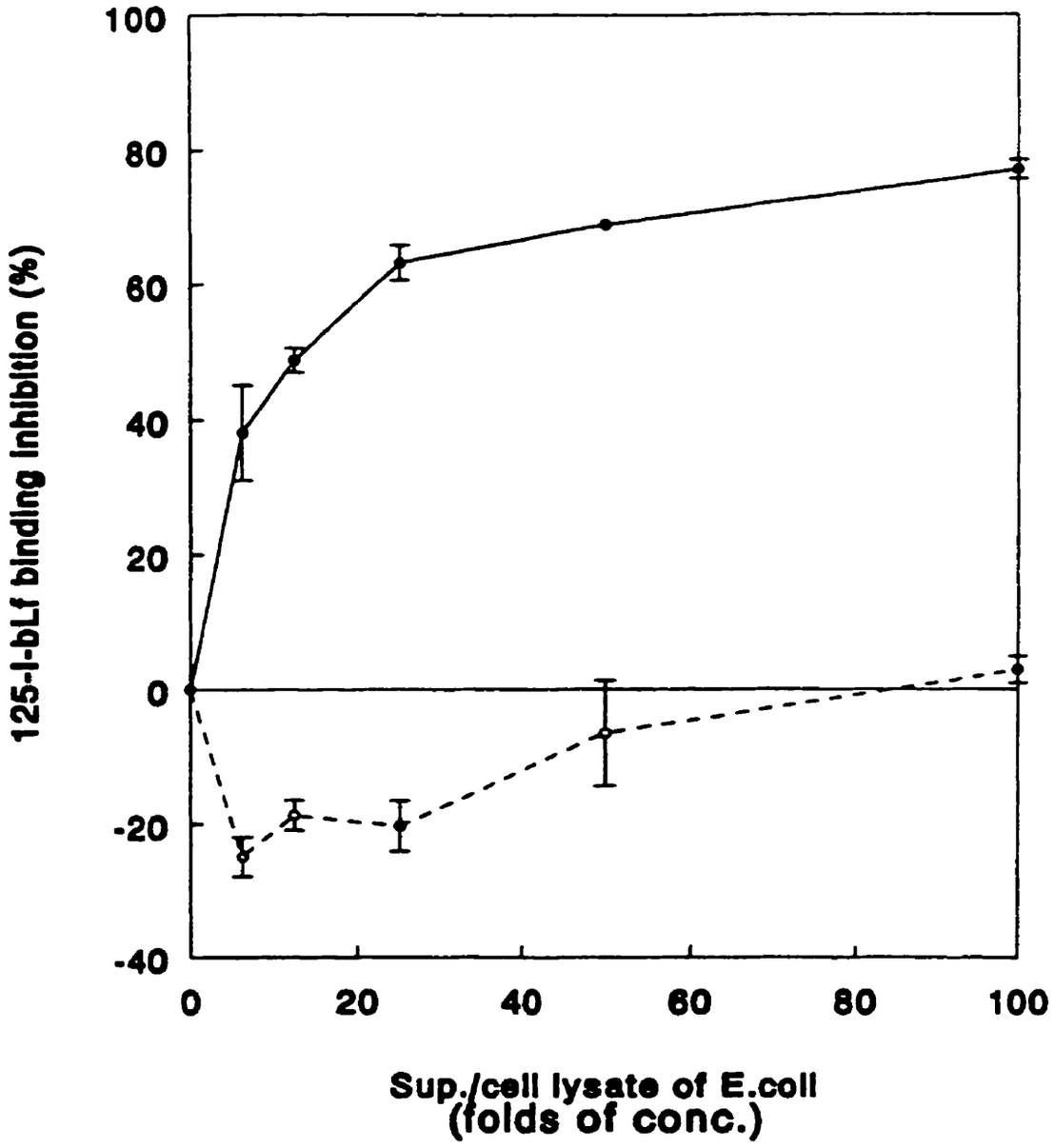


Figure 5-2. Inhibition of recombinant bLf-binding protein on ^{125}I-labelled bLf binding to *S. uberis*. Increasing amounts of a mixture of supernatant (sup.) and whole cell lysate (with equal volume) of *E. coli* pLBP5 (●) or *E. coli* pTZ18R (O) were mixed with 10^9 cells and incubated with 0.69 nM ^{125}I-bLf in 0.2 ml volumes. Inhibition values were calculated as relative percentage of bLf binding to bacteria suspended in PBS-1% BSA in the absence of any *E. coli* samples.

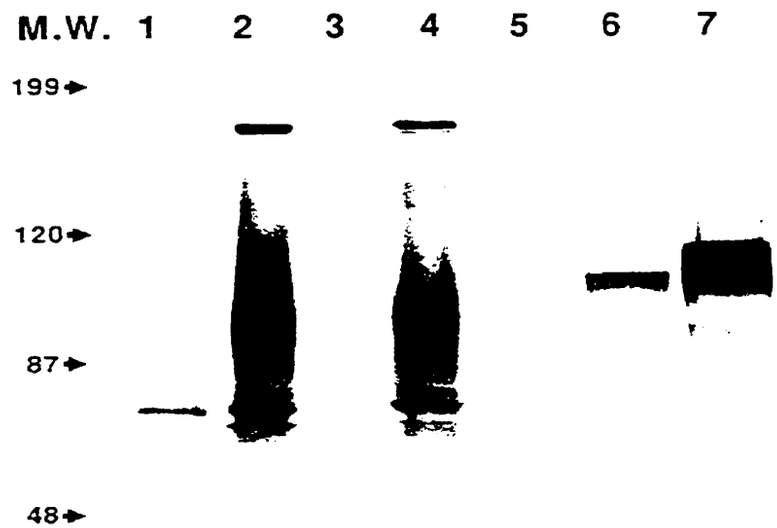


Figure 5-3. Immunoblot analysis of bLf-binding proteins in *S. uberis* and recombinant *E. coli*. Samples were analyzed in 7.5% SDS-PAGE and transferred to a nitrocellulose membrane which was further probed with rabbit antiserum against TCA-precipitated supernatant of *E. coli* pLBP5. Lanes: 1, cell wall of *S. uberis;* 2, supernatant of *E. coli* pLBP5; 3, supernatant of *E. coli* pTZ18R; 4, whole cell lysate of *E. coli* pLBP5; 5, whole cell lysate of *E. coli* pTZ18R. Samples for lane 1 to 5 were boiled for 5 min in a sample buffer containing 2-mercaptoethanol. Lane 6 and 7 are cell wall fraction of *S. uberis* and supernatant of *E. coli* pLBP5, respectively, which were boiled for 30 min in a sample buffer containing 3 M urea. The molecular mass standards (kDa) are indicated by arrows to the left.

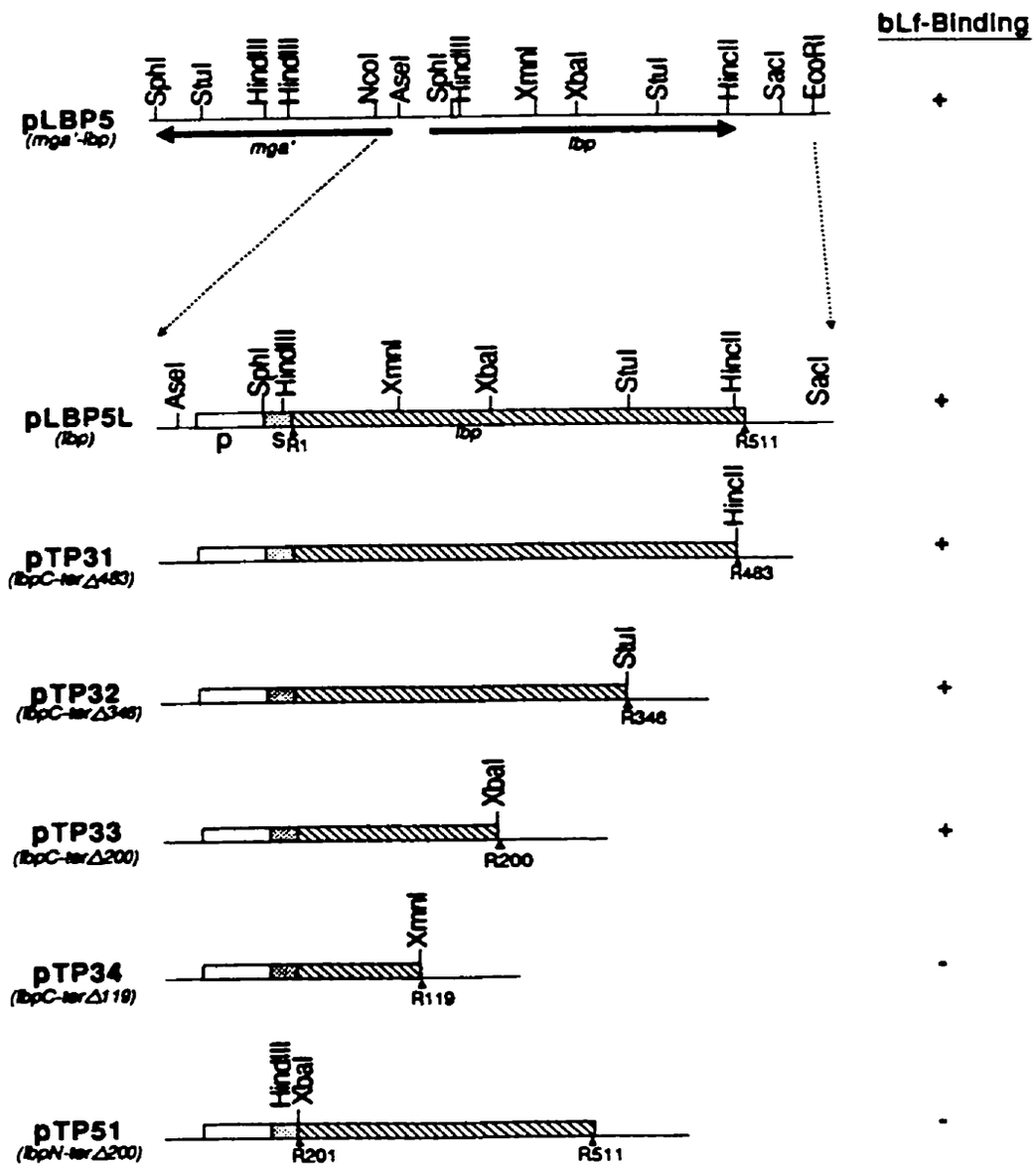
5.4.2 Nucleotide sequence determination and analysis.

To determine the nucleotide sequence of the Lbp gene, each of the *Hinc*II, *Hind*III, *Sac*I, *Sph*I and *Xba*I fragments of pLBP5 (Fig. 5-4) was individually cloned into the corresponding site of pTZ18R. By using universal and reverse primers, each fragment was sequenced in both orientations. Subsequent primers were synthesized on the basis of sequence information thereby obtained.

Two open reading frames were found from pLBP5 (Fig. 5-4). One was the Lbp-encoding gene *lbp*, whose presence in subclone pLBP5L resulted in a bLf-binding phenotype (Fig. 5-5, lane 1). The other ORF on the complementary DNA strand was incomplete. A GenBank database search showed that the presumed ORF gene product had significant homology to the VirR and Mry positive regulators in group A streptococci. Thereby, this ORF was named *mga'* (Fig. 5-4). The cloning and sequencing of the complete *mga* gene is presented in 5.4.4.

The *lbp* sequence contained two potential translation start codons (ATG); both of them, at positions 232 and 262 of the DNA sequence are associated with putative Shine-Dalgarno sequences (Fig. 5-6). These start points would give proteins with predicted size of 62.857 and 61.454, respectively. We have no evidence to indicate which ATG is used. Nevertheless, both of the predicted sizes are comparable to 76 KDa molecular weight shown on the blots

Figure 5-4. Restriction enzyme map of *lbp*, progressive deletions and summary of bLf binding data. Open boxes (p region) represent 5′ sequences containing promoter and ribosome binding site, shaded boxes (s region) represent lbp sequences encoding the signal peptides, and hatched boxes represent the *lbp* sequences coding for the mature or truncated proteins. R1 and R201 (R represents residue) indicate the first codon of the mature protein in pLBP5 and the truncated protein in pTP51, respectively. Other numbers indicate the last codon of each protein. Restriction sites are present on the top of the *lbp*. The bLf binding ability of each clone is shown on the right.


bLf-Binding
pLBP5
(mga'-lbp)
SphI
StuI
HindIII
HindIII
NcoI
AseI
SphI
HindIII
XmnI
XbaI
StuI
HincII
SacI
EcoRI
mga'
lbp
+
pLBP5L
(lbp)
AseI
SphI
HindIII
XmnI
XbaI
StuI
HincII
SacI
p
S
R1
lbp
R511
+
pTP31
(lbpC-ter Δ483)
HincII
R483
+
pTP32
(lbpC-ter Δ346)
StuI
R346
+
pTP33
(lbpC-ter Δ200)
XbaI
R200
+
pTP34
(lbpC-ter Δ119)
XmnI
R119
-
pTP51
(lbpN-ter Δ200)
HindIII
XbaI
R201
R511
-

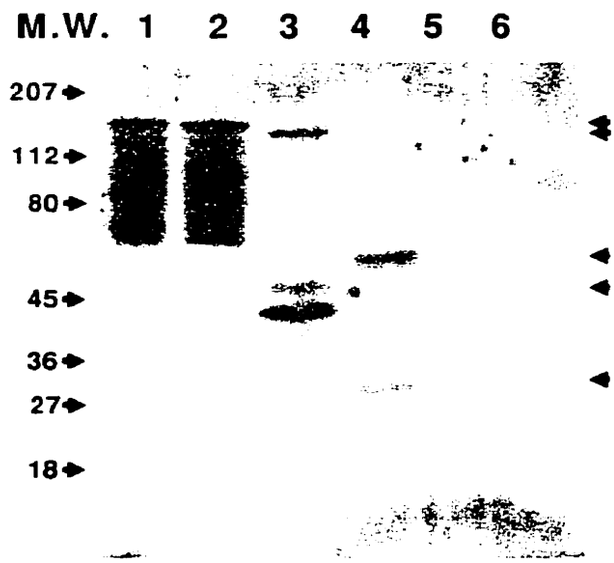


Figure 5-5. The analysis of bLf binding abilities of truncated Lbps produced in *E. coli*. Whole cell lysates (wcl) of *E. coli* clones producing mature or truncated Lbps were resolved in 10% SDS-PAGE under non-reducing condition as described in Materials and Methods, and transferred to nitrocellulose. The bLf binding ability of each protein was detected by probing the nitrocellulose membrane with ^{125}I-bLf. Lanes: 1, wcl of *E. coli* pLBP5L (complete *lbp* gene); 2 to 5, wcl of *E. coli* pTP31, pTP32, pTP33, pTP34 (3′ deletions); 6, wcl of *E. coli* pTP51 (5′ deletion). Numbers on the left of the figure indicate the positions of the molecular weight markers (in thousands), and the ^{125}I-bLf-reactive bands are indicated by arrows to the right.

```
TTATTTATTAATACGAGAAAACCTGTTTCCTTTTGGAGGAACCAGGAAATAATCTCAATC    60
                                                  -35
TAGCATTATATAGTGATAAAAACTCATTTATCATTCATTATTTTAAATAAAAATTAAAAA   120
   -35      -10            -10
GTTGAGCTTTATTATCACGATAAACTAATATATACTAATTTTGTAGTTTCGAGAAGCCCT   180
                                            SD
TTTAGCGTGTCTAAAACGTTATCATTTCAGCTTTTTGCAAAAACTAACAAAATGAGAAAA   240
                                                     M  R  K     3
TTCTATTATAAGGAGAAGAAAATGGAAATCAAACAAAAACATGGCAAGCATGCTTTACGC   300
  F  Y  Y  K  E  K  K  M  E  I  K  Q  K  H  G  K  H  A  L  R    23
AAAGCGGTCACCGCAGCGGTCTTAGCAGGGACAGCTTTCTCAAGCTTAGGAGGCTTCGCA   360
  K  A  V  T  A  A  V  L  A  G  T  A  F  S  S  L  G  G  F  A    43
GGAGCAGTAACAACAGTCAAGGCGGAAGAATTTACGGATAAATATTATCCTAAAATGAAT   420
  G  A  V  T  T  V  K  A  E  E  F  T  D  K  Y  Y  P  K  M  N    63
AAAATGGATGAAGAGGATCTTTTGCGATTAAGCGCTGAAAGCAAAAAGGACGTTCCTGCA   480
  K  M  D  E  E  D  L  L  R  L  S  A  E  S  K  K  D  V  P  A    83
ATACGAGAATTATTAAAAAGAATGACTACGGAATCAATACATGCTTTATTAGTGGGACTA   540
  I  R  E  L  L  K  R  M  T  T  E  S  I  H  A  L  L  V  G  L   103
GATTCTTCCCACATTTCTTATAGTGAAGAGAGTGGGTTCAATAATTTGCTTAGAAAATTT   600
  D  S  S  H  I  S  Y  S  E  E  S  G  F  N  N  L  L  R  K  F   123
GGGTCTATGAATAATGACGACCCTTCAGATTGGACTCATTATAAAAGTGGTGTTAGTTTA   660
  G  S  M  N  N  D  D  P  S  D  W  T  H  Y  K  S  G  V  S  L   143
GCTTCTATAATTGTCCAAGAAGTAAGAAGTAGATTGAATGAAAAAGATGAACTTGATGAA   720
  A  S  I  I  V  Q  E  V  R  S  R  L  N  E  K  D  E  L  D  E   163
                 A1
GAACTATCTAACAAGAAAGAAGAACTTCAGAAACTAACTGAAAAAATAGAAAAAACAATT   780
  E  L  S  N  K  K  E  E  L  Q  K  L  T  E  K  I  E  K  T  I   183
AAAGAAAAAGAGAATTTAAACAAAGAAATCACGGAAAAAAATTCTGAAATTTCTAAAATG   840
  K  E  K  E  N  L  N  K  E  I  T  E  K  N  S  E  I  S  K  M   203
                                            A1     B1
GAAGAGGAACTTAGTGAAAAAGAAAAAGAAATTGCAGAAAATAAAGAAGAACTAGCAGAT   900
  E  E  E  L  S  E  K  E  K  E  I  A  E  N  K  E  E  L  A  D   223
                       B1    C1
GCTTTAGGTGAACTTTTTGATGCTGAGGAAACAATTGATAAAAAAGAAGCTAAAGTTAAA   960
  A  L  G  E  L  F  D  A  E  E  T  I  D  K  K  E  A  K  V  K   243
GATTTAACTGAAAAATTAGATGCTTCTAGAAAAGAACATGAGGCACTTGCTAAAGAGTTT  1020
  D  L  T  E  K  L  D  A  S  R  K  E  H  E  A  L  A  K  E  F   263
GCAGAGTCTCAAAAAGGTTATGAAAAAGAGTTAGCTGATAAACACACTGCTTTAGGTGAA  1080
  A  E  S  Q  K  G  Y  E  K  E  L  A  D  K  H  T  A  L  G  E   283
                    C1                                    A2
GCTGAAAAACGTAATGCTGATTTAGAGGCTGGCAACAAAGAACTTAAAGAAAACTTAGAA  1140
  A  E  K  R  N  A  D  L  E  A  G  N  K  E  L  K  E  N  L  E   303
ATGGCTGAAGGTATCTCTGATGACTTGCAGAAGAAAGTCATGAAAGCAGAACAAGAGATG  1200
  M  A  E  G  I  S  D  D  L  Q  K  K  V  M  K  A  E  Q  E  M   323
AAAGAACTTTCTGCACAATTAGAAGAAGCAAAAGAAGAACTTGAAACTGAAAAAGCTAAG  1260
  K  E  L  S  A  Q  L  E  E  A  K  E  E  L  E  T  E  K  A  K   343
  A2     B2                                B2     C2
TTAGCAGAGTCAGAAAAAGAAAATGCAAAACTGACCGAAGAACGTGATGCTGCTAAGAAA  1320
  L  A  E  S  E  K  E  N  A  K  L  T  E  E  R  D  A  A  K  K   363
GAAGCTGAAAAAGTTCCTGAACTAGAAGAGCAAGTTGAAAAATTAGTTGAAGAAATCACT  1380
  E  A  E  K  V  P  E  L  E  E  Q  V  E  K  L  V  E  E  I  T   383
GCTGCTAAGAAAGAAGCAGAAGAGCTTCAAGCTAAAGCCGAAGGCCTTGAAAAAGACTTT  1440
  A  A  K  K  E  A  E  E  L  Q  A  K  A  E  G  L  E  K  D  F   403
                                      C2
GAAGCTGTTAAAGCAGAAAAAGAAGCCCTTGAAGCTGAAATTGCTAAATTGAAAGAAGAC  1500
  E  A  V  K  A  E  K  E  A  L  E  A  E  I  A  K  L  K  E  D   423
CACCAAAAAGAAGTGGACGCTCTCAATGCACTCCTTGCTGATAAAGAGAAAATGCTTAAG  1560
  H  Q  K  E  V  D  A  L  N  A  L  L  A  D  K  E  K  M  L  K   443
AACTTGCAAGACCAGCTTGACAAAGCTAAAGAAGAAGCTATGAAGAACGAGCAAATGAGC  1620
  N  L  Q  D  Q  L  D  K  A  K  E  E  A  M  K  N  E  Q  M  S   463
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```
CAAGAAGAAAAAGCTAAATTGCAAGCTGAATTGGATCAAGCTAAGAAAGAATTGGCAGAA 1680
  Q  E  E  K  A  K  L  Q  A  E  L  D  Q  A  K  K  E  L  A  E  483
AAAATCAAAGACATGCCAAACAAAGTGGCTCCTCAAGCCGAAGGCAAAGCCAATGCAGGT 1740
  K  I  K  D  M  P  N  K  V  A  P  Q  A  E  G  K  A  N  A  G  503
CAAGCAGCTCCAAACCAAAACCAAAACAACCAAGCACAAGCAAATCAAGCTAAGAACGGC 1800
  Q  A  A  P  N  Q  N  Q  N  N  Q  A  Q  A  N  Q  A  K  N  G  523
AACAACCTCCCATCAACAGGTGACAAACCAGTTAACCCACTCCTAGTGGCAAGTGGTCTC 1860
  N  N  L  P  S  T  G  D  K  P  V  N  P  L  L  V  A  S  G  L  543
TCCCTCATGATCGGAGCAGGTGCCTTCGTCTACGCCGGCAAACGCAAAAAAGGCTAAGTC 1920
  S  L  M  I  G  A  G  A  F  V  Y  A  G  K  R  K  K  G        561
ATAACAAAGGACTTCCAGAAACTTTCCTAAAAAATTATTTTCTATTCATACTAAGACAGA 1980

AAACTCCCCCTACCATGAGGTAAGGGGAGTTTTATTACACTTAAGGATAAATAGGCAACT 2040
       T                  T
CTGGGAAGAGGGCTTGTAAAATTTTAGGGGTGATGGCTTGGCCTGACCCTTCCTGACAGT 2100
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Figure 5-6. Nucleotide and deduced amino acid sequences of Lbp. Nucleotides and amino acids are numbered on the right of the sequences. The deduced amino acid sequence is shown in the single-letter code below the nucleotide sequence. Two possible ATG start codons at positions 232 and 262, and the TAA stop codon at 1915 are shown in bold. Two putative -35 and -10 promoter sequences and Shine-Dalgarno sequences (SD) are indicated. A putative rho-independent transcription terminator (T) is underlined. The double underline shows the presence of a putative signal peptide at the N-terminus of the ORF. The C-terminal hydrophobic trans-membrane domain is indicated by italics and the nearby surface anchor motif is shown by italics and double underline. The central repeated amino acid sequences are indicated by letter A, B, and C.

(Fig. 5-1 and 5-3). The reason for the discrepancy between the observed and calculated molecular weights of this protein is not clear. Posttranslational modification, such as lipid modification might have occurred and increased the apparent molecular mass in the SDS-PAGE determination. A similar difference observed in Gram negative bacteria has been demonstrated to be caused by protein lipid modification (Theisen *et al.*, 1992; 1993).

The DNA sequence shows two putative -10 and -35 promoter regions present at -88 and -102 from the first ATG. Downstream of *lbp* there is a potential rho-independent transcription terminator (Fig. 5-6).

Analysis of the N terminus of the predicted sequence of Lbp showed amino acids characteristic of signal sequences (Simonen and Palva, 1993) (Fig. 5-6). The features of the sequence are a positively charged N terminus rich in K and R residues, followed by a hydrophobic domain from amino acids 25 to 48 (Fig. 5-7) and the signal peptidase cleaving site, VKA, at positions 49 to 50, where cleavage occurs after the A residue. The presence of this putative signal sequence indicates that the Lbp is exported across the cytoplasmic membrane of *S. uberis*.

A search of the GenBank database revealed that the C terminus of the Lbp was highly homologous to the C terminal ends of streptococcal M proteins, plasminogen binding protein, fibrinogen and IgG-binding proteins. It shows all the general features well established for surface proteins of Gram positive cocci (Fischetti *et al.*, 1991). These features include a small cluster of four charged amino acids at the C terminus followed by a hydrophobic domain of 21 amino acids (Fig. 5-6 and 5-7). Adjoining the hydrophobic domain is the consensus membrane anchor motif LPSTGD. The next region of 50 amino acids is the cell wall-associated region characterized by a high proportion of proline and glycine (12%). GenBank

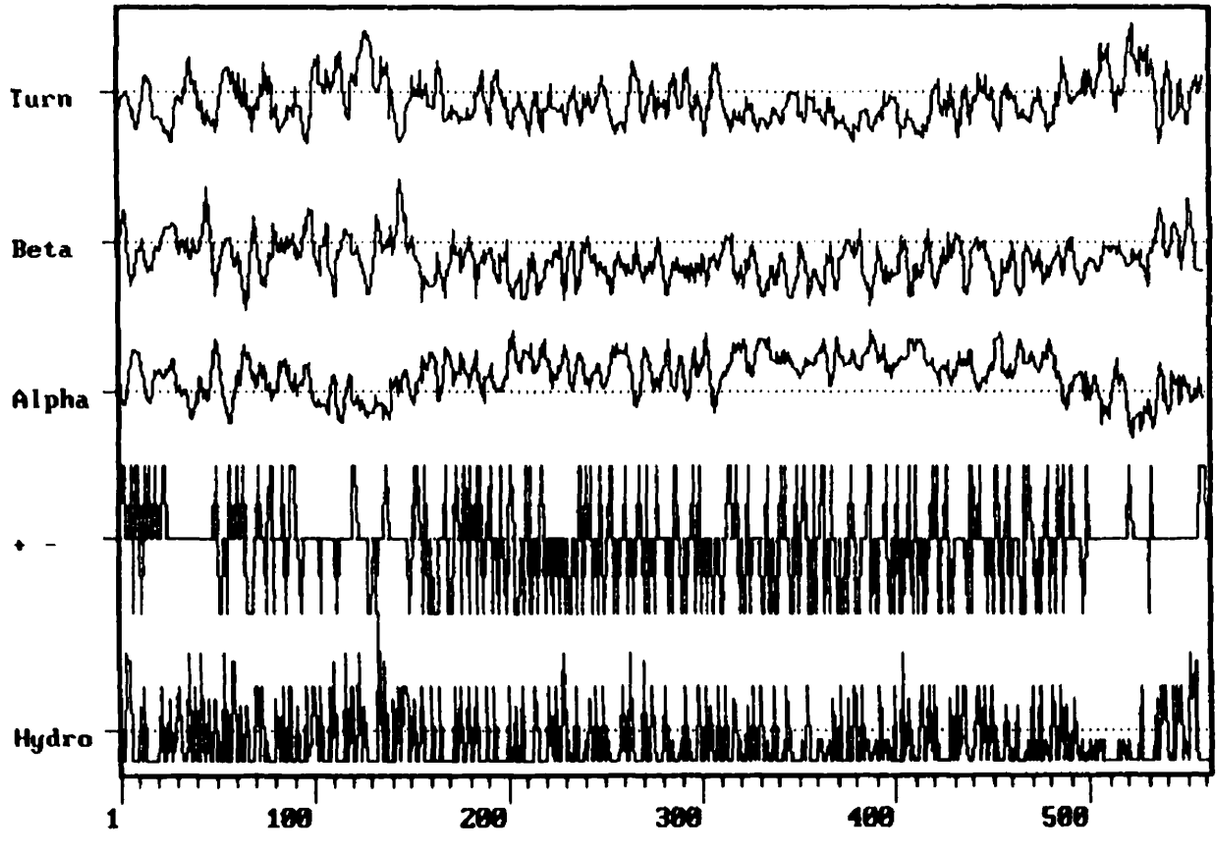

Figure 5-7. Profiles of the secondary structures, charged residues and hydrophobicity of Lbp. The deduced amino acid sequence of Lbp was analyzed with the Novotny-Auffray algorithm (Novotny, 1984). Plots marked Turn, Beta, and Alpha indicate the potential for beta turn-random coil, beta sheet, and alpha helix formation, respectively. The +/- plot shows regions of the molecule with net positive (upper) and negative (lower) charges. The hydrophobicity (Hydro) plot shows the hydrophobic regions of the protein. The positions of the amino acids are shown on the horizontal axis.

database searches also showed that Lbp is homologous to mammalian myosin heavy chain and kinesin heavy chain; it has 48% and 46% overall sequence homology with these two fibrillar proteins, respectively.

The region beyond the membrane anchor motif and proline/glycine rich region contains three blocks of amino acids which were found to have their corresponding internal homologies (Fig. 5-6). The A1 block contains 52 amino acids which is homologous to A2 block; B1 block containing 13 amino acids is homologous to B2 block; and the 59 amino acid-constituted C1 block is homologous to C2 block.

Analysis of the secondary structure of the translated protein showed an extensive α-helix region stretching from the proline/glycine-rich region to the β-sheet and turn region near the cleavage site of the signal sequence (Fig. 5-7).

5.4.3 Localization of the Lf-binding domain of Lbp.

To localize the Lf-binding domains of Lbp, gene deletions were constructed (Fig. 5-4). 3′ deletions pTP31, pTP32, pTP33 and pTP34 were generated at the restriction enzyme sites *Hinc*II, *Stu*I, *Xba*I and *Xmn*I of pLBP5L. A 5′ deletion pTP51 was constructed by removing the 643 bp *Hind*III-*Xba*I fragment of pLBP5L. All these deletions contained the *lbp* promoter. The resulting truncated Lbps were

produced in *E. coli* and were visualized after SDS-PAGE, transfer to nitrocellulose, and reaction with specific rabbit antiserum or ^{125}I-bLf. All the samples were tested under non-reducing conditions in these experiments. In Fig. 5-5, the analysis of truncated Lbps using ^{125}I-bLf is shown, and the bLf binding results are summarized in Fig. 5-4. Like full length Lbp (Fig. 5-5, lane 1), truncated proteins from *E. coli* pTP31, pTP32 and pTP33 could bind bLf in both monomeric and dimeric forms (lane 2, 3 and 4, respectively). Some degradation products from these clones also possessed binding ability. No band was found from *E. coli* pTP34 or pTP51 (lanes 5 and 6, respectively). This data indicates that the primary domain of Lbp involved in the binding of bLf resides in a 22-kDa N-terminal fragment.

5.4.4 Cloning and sequencing of the complete *mga* gene.

As described in 5.4.2, the plasmid pLBP5 contained an incomplete gene, *mga'*, in addition to *lbp*. The pLBP5 molecule was inverted to pLBP5i (Fig. 5-8) for convenience of description.

To obtain the complete *mga* gene, a 991 bp *Sty*I fragment from *mga'* was radioactively (^{32}P) labelled as a probe (VP1 in Fig. 5-8) and used to screen a *S. uberis* gene library. A positive *E. coli* DH5α clone was obtained and the restriction enzyme map of the plasmid pMGA14 is shown in Fig.

Figure 5-8. Construction of pMGA14F from pLBP5i and pMGA14. pMGA14F was generated by inserting the 1.5 kb *Sph*I-*Nhe*I fragment of pLBP5i into the *Sph*I and *Nhe*I sites of pMGA14. Lines indicate *S. uberis* DNA, while the box represents the multiple cloning sites of vector pTZ18R. The probe fragments used for Southern or Northern blot experiments are indicated by the hatched bars. The arrows indicate the locations of the open reading frames of *lbp*, *mga'* and *mga*.

KpnI
BamHI
HincII
dp
HindIII
SphI
NcoI
StyI
mga'
StyI
NheI
pLBP5I
HindIII
SphI
NcoI
StyI
HindIII
HindIII
BamHI
mga
StyI
NheI
HincII
HindIII
HincII
KpnI
HindIII
HincII
pMGA14
VP1
VP3
VP2
SphI
NcoI
StyI
HindIII
HindIII
BamHI
mga
StyI
NheI
HincII
HindIII
HincII
KpnI
HindIII
HincII
pMGA14F

5-8. To determine the nucleotide sequence of pMGA14, each of the *Bam*HI, *Hinc*II, *Hind*III and *Kpn*I restriction fragments of pMGA14 (Fig. 5-8) was individually cloned into the corresponding site of pTZ18R and sequenced from both orientations using universal and reverse primers. Subsequent primers were synthesized on the basis of sequence information thereby obtained. pMGA14 contained the complete ORF of Mga. However, the associated promoter region was not present. Since the 1.5 kb *Sph*I-*Nhe*I fragment of pLBP5i contained the majority of the 5′ region of the *mga′* and the complete promoter region, it was inserted into the *Sph*I and *Nhe*I sites of pMGA14 to generate pMGA14F which then contained the complete *mga* gene including the promoter (Fig. 5-8).

The sequence of a 3558 bp DNA fragment of pMGA14F is presented in Fig. 5-9. Starting at the ATG initiation codon at nucleotides 361-363 and terminating at a TAA codon at nucleotides 1858-1860, the deduced gene product, Mga, is comprised of 499 amino acid residues with a calculated molecular weight of 58,454 Da. The N terminus of Mga lacks the features of signal peptides as described (Simonen and Palva, 1993), suggesting that it is a cytoplasmic protein. Preceding the start codon of *mga* is a putative ribosome binding site AGGAGA. Sequences resembling the -35 and -10 promoter motifs have also been identified.

Figure 5-9. Nucleotide and deduced amino acid sequences of Mga and ORFs after Mga. Nucleotides and amino acids are numbered on the right of the sequences. The deduced amino acid sequence is shown in the single-letter code below the nucleotide sequence. Possible ATG start codons are shown in bold and the stop codons are indicated by "*". Putative -35 and -10 promoter sequence and Shine-Dalgarno sequence (SD) are indicated.

```
TTGCCATGTTTTTGTTTGATTTCCATTTTCTTCTCCTTATAATAGAATTTTCTCATTTTG   60
TTAGTTTTTGCAAAAAGCTGAAATGATAACGTTTTAGACACGCTAAAAGGGCTTCTCGAA  120
ACTACAAAATTAGTATATATTAGTTTATCGTGATAATAAAGCTCAACTTTTTAATTTTTA  180
TTTAAAATAATGAATGATAAATGAGTTTTTATCACTATATAATGCTAGATTGAGATTATT  240
                -35                         -10
TCCTGGTTCCTCCAAAAGGAAACAGGTTTTCTCGTATTAATAAATAATAGTAGAAAGTTG  300

AAGTACCCTATCACATCATAATCATTGCGACCGATTCTTGGTCCATATTAAGGAGATCCG  360
                                                   SD
ATGTATATCAAACAAAACCTTTTTTCCAATCAGCAATTACGCGGCCTCAAACTGGCCGTT  420
 M  Y  I  K  Q  N  L  F  S  N  Q  Q  L  R  G  L  K  L  A  V   20
CTCTTGAACCATGGTGAGGAACCTTGTGACTACACTCACATTTGCCGGCACTTGCACTGT  480
 L  L  N  H  G  E  E  P  C  D  Y  T  H  I  C  R  H  L  H  C   40
TCCTTTTTAACCTTGCAAACAGAGATTGCGCATTTGGCTTCCTTTGGAGAAGTTCGGGCT  540
 S  F  L  T  L  Q  T  E  I  A  H  L  A  S  F  G  E  V  R  A   60
CTCCCTTATGTGGAACCCCATTTGGAAGTTCAGTACCGACCGGAATTTGGTCCACAAAAA  600
 L  P  Y  V  E  P  H  L  E  V  Q  Y  R  P  E  F  G  P  Q  K   80
TTGTTTCAATCCATCTTAAATGAGACCCCCTCGATGCGCTTGGTGGAAGCCCTCTTTTAT  660
 L  F  Q  S  I  L  N  E  T  P  S  M  R  L  V  E  A  L  F  Y  100
AATGACTTTGACTCTTTAGAAGAACTGGCGGAAGCTCTTTTTACCAGCTTATCTACTTTA  720
 N  D  F  D  S  L  E  E  L  A  E  A  L  F  T  S  L  S  T  L  120
AAACGGCTGATTAAGCGTACCAATGTTTATTTACAGTTGGGGTTTAATTGCGTGATTAAC  780
 K  R  L  I  K  R  T  N  V  Y  L  Q  L  G  F  N  C  V  I  N  140
GTTCGATCGGTACGCATTGTTGGTCCTGAGCACTCAGTTCGCTTGCTCTATTTGAAATAC  840
 V  R  S  V  R  I  V  G  P  E  H  S  V  R  L  L  Y  L  K  Y  160
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 F  T  E  V  Y  D  Y  Y  A  W  P  F  L  D  S  I  N  E  I  A  180
CTGACAAGCTTGGTAGATGTGACAACCAGTAAGTTCTCTCATCGGGTGACGCACTCGCTT  960
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 F  A  Y  L  K  I  M  S  A  V  N  L  I  R  F  S  K  G  Y  R  220
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 V  N  E  V  Y  P  R  S  C  S  L  Y  E  K  L  K  E  D  P  T  240
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 F  L  H  L  S  E  L  F  W  R  D  F  G  K  P  L  D  Q  V  A  260
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 L  S  E  M  F  S  T  Y  F  R  Q  D  L  I  L  N  A  E  L  D  280
CGGCAAAACACGGATAAGGAAGTTGAGGGGGTGACCTATGAGGACATGCTTGAGACTTTG 1260
 R  Q  N  T  D  K  E  V  E  G  V  T  Y  E  D  M  L  E  T  L  300
GAGGGGATTGAAAATCGTTTCGGGATGGTCATTGCTAATAAGTCGGAAATCAGTCTCTTG 1320
 E  G  I  E  N  R  F  G  M  V  I  A  N  K  S  E  I  S  L  L  320
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 I  Y  N  A  L  I  L  K  E  H  D  I  Y  E  N  F  L  V  Y  D  340
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 Y  R  E  P  Y  I  A  Y  F  K  D  S  Y  P  R  L  L  E  A  F  360
CAGGAGGCTTTGTCAGCCCCTTTTAAAGCCAAGGGCGTGGAGCTAGCCAAAGAAAGGCAG 1500
 Q  E  A  L  S  A  P  F  K  A  K  G  V  E  L  A  K  E  R  Q  380
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 N  H  L  L  Y  I  L  L  V  N  W  D  N  L  F  F  H  I  S  R  400
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 A  I  D  K  Q  K  V  L  V  V  E  K  G  T  C  N  S  G  Q  F  420
TTAATTGCTTATGCCGGTCAGTACTTTGACATTGCTATTCATGAGCATCACCAGATTGAT 1680
 L  I  A  Y  A  G  Q  Y  F  D  I  A  I  H  E  H  H  Q  I  D  440
ATGCACAAGATCAAAGAGGAGTACGATGTGGTGTTGACGGACCAGACCTTAGAAGTGGTA 1740
 M  H  K  I  K  E  E  Y  D  V  V  L  T  D  Q  T  L  E  V  V  460
GATGGTGTCGACATCATCTACTTTAGCCAGCTAGTGCCAAGCATCGCCTTGGAAAAACTG 1800
 D  G  V  D  I  I  Y  F  S  Q  L  V  P  S  I  A  L  E  K  L  480
AACAAGTTCCTCAAGAAAAAAATTCAGAAGCATTTCTTAGAAGAACCGAAGGAAGAATAA 1860
 N  K  F  L  K  K  K  I  Q  K  H  F  L  E  E  P  K  E  E  *  499
ACCAAAGCTTTGTCAGTGACTAAATAACCCTTTACCTTAAGATTTTTTCCTGCCATTGGC 1920
GGTCGGATAAGAAAACCATTTCATAAAAAATTGAGGGCATCTCTTGGGTCAGAGGCTAGT 1980
CTTTATGCTATAATATTTGGTATGGATAAAATCATTAAATCAATCTCAGAAACAGCTCTT 2040
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TTAGAGCCTTTGTTCTTGACAGCACACAGACTGTGAAGACAGCTCAAGAAAAGCACCAAA 2100

CCTTATCATCTTCGACAGTAGCCCTTGGCCGCACCCTCATTGCCAATCAAATCTTAGCGG 2160

CCAACCAAAAAGGCGACAGCAAAATCACGGTTAAGGTCATTGGGGACTCCTCTTTTGGTC 2220

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                                                M  G  N  G     4
ATTTTGTCACAATTATTGACTATGGTACCGGCAATCCCTACACTTCAACAACACCATTAA 2400
 H  F  V  T  I  I  D  Y  G  T  G  N  P  Y  T  S  T  T  P  L    24
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 F  M  V  Q  A  L  P  G  A  S  E  E  E  I  A  R  Y  E  K  R    84
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 L  A  A  I  Y  G  D  E  G  Y  K  R  L  S  E  D  S  L  S  F   124
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 Q  C  D  C  S  K  D  R  F  E  S  A  L  M  S  L  P  N  T  D   144
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 L  Q  D  M  I  D  Q  D  H  G  A  E  I  I  C  Q  F  C  Q  T   164
GCTATCAGTTCTCAGAAGACGACTTGAAAGGGATTATCAATGACAAAGCTTAATTCTTCC 2880
 R  Y  Q  F  S  E  D  D  L  K  G  I  I  N  D  K  A  *         181
                                          M  T  K  L  N  S  S     7
TTTATGATTGGGAACGTGGAGATTCCCCATCGTACTGTTTTAGCCCCTATGGCCGGGGTA 2940
  F  M  I  G  N  V  E  I  P  H  R  T  V  L  A  P  M  A  G  V   27
ACCAATTCGGCCTTCCGGACCATTGCTAAGGAGTTTGGCGCTGGACTGGTGGTCATGGAA 3000
  T  N  S  A  F  R  T  I  A  K  E  F  G  A  G  L  V  V  M  E   47
ATGATTTCCGAAAAAGGCCTCCTTTACAACAATGAAAAAACCTTACACATGCTCCACATT 3060
  M  I  S  E  K  G  L  L  Y  N  N  E  K  T  L  H  M  L  H  I   67
                                   M  K  K  P  Y  T  C  S  T     9
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  D  E  N  E  H  P  M  S  I  Q  L  F  G  G  D  A  E  G  *      85
L  M  K  T  N  T  Q  C  L  F  S  C  L  G  E  M  P  K  V  K     29
GAGCGGCAGACTTCCATTCAAACCAACACCAAGGCAGATATTGTCGATATTAATATGGGG 3180
T  S  G  R  L  P  F  K  P  T  P  R  Q  I  L  S  I  L  I  W     49
                                                      M  G     2
TGCCCGGTCAATAAAGTGGTTAAAAATGAAGCTGGGGCCAAATGGCTTCGTGACCCAGAT 3240
G  A  R  S  I  K  W  L  K  M  K  L  G  P  N  G  F  V  T  Q     69
  C  P  V  N  K  V  V  K  N  E  A  G  A  K  W  L  R  D  P  D   22
AAAATCTACCACATTGTTTCGGAAGTGACTTCTGTTTTAGACATTCCCTTGACCGTTAAG 3300
I  K  S  T  T  L  F  R  K  *                                   78
  K  I  Y  H  I  V  S  E  V  T  S  V  L  D  I  P  L  T  V  K   42
ATGCGTACAGGGTGGGCAGATAGTTCACTGGCTGTTGAGAACGCCTTGGCTGCGGAGTCG 3360
  M  R  T  G  W  A  D  S  S  L  A  V  E  N  A  L  A  A  E  S   62
GCTGGGGTGTCAGCCCTTGCCATGCATGGGCGTACCCGTGAACAAATGTACACAGGAACC 3420
  A  G  V  S  A  L  A  M  H  G  R  T  R  E  Q  M  Y  T  G  T   82
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  C  D  H  E  T  L  A  R  V  S  K  A  I  T  K  I  P  F  I  G  102
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  N  G  D  V  R  T  V  Q  D  A  K  F  M  I  E  E  I  G  V  D  122
GCTGTCATGATCGACTCT                                            3558
  A  V  M  I  D  S                                             128
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A search of the GenBank database revealed that the deduced protein of *mga* has 34% overall sequence identity to the VirR and Mry proteins (Chen *et al.*, 1993; Perez-Casal *et al.*, 1991). Studies have showed that these positive regulators of group A streptococcal M proteins contain helix-turn-helix DNA binding domains, and it is believed that via these domains, the regulators may interact directly with specific DNA sequences to influence transcriptional activity (Chen *et al.*, 1993; Perez-Casal *et al.*, 1991). Attempts to identify a similar DNA-binding domain with visual inspection and analysis with PCGENE software failed to detect such a motif in Mga of *S. uberis*. However, a region at amino acid residues 106 to 125 (Fig. 5-9) showed 90% identity to the sequence of the DNA-binding domain of VirR49 (Podbielski *et al.*, 1995).

Following *mga*, there are four more ORFs with 181, 85, 78 and 128 amino acid residues, respectively (Fig. 5-9). Since no significant sequence alignments were found from GenBank, no more discussion will be given on these ORFs.

5.4.5 Northern blot analysis of the *lbp* and *mga* transcripts.

To analyze the *lbp* and *mga* transcripts, RNA was prepared from *S. uberis* (su-1), *E. coli* DH5α(pLBP5), *E. coli* DH5α(pLBP5L), *E. coli* DH5α(pMGA14F) and *E. coli* DH5α(pTZ18R), and used for two Northern blots (Fig. 5-10 A and B). Blot A

(Fig. 5-10 A) was probed with the 1.5 kb *Hind*III-*Hpa*I internal fragment of *lbp* (LP1 in Fig. 5-11). A 2.0 kb band was seen in lanes containing RNA from *S. uberis*, *E. coli* DH5α(pLBP5) and *E. coli* DH5α(pLBP5L) (Fig. 5-10 A, lane 1, 2 and 3, respectively), but was absent in lane 4 which contained RNA from *E. coli* DH5α(pTZ18R). This data indicates that only one major transcript was generated by *lbp* in recombinant *E. coli* as well as in native *S. uberis*. Blot B (Fig. 5-10 B) was probed with the 1.0 kb *Nco*I-*Nhe*I internal fragment of *mga* (VP2 in Fig. 5-8). A 1.8 kb band was seen in lane 2 and 3 which contained RNA from *E. coli* DH5α(pLBP5) and *E. coli* DH5α(pMGA14F), respectively. No band was found from *S. uberis* and *E. coli* DH5α(pTZ18R) RNA samples (lane 1 and 4, respectively). The *mga* and *mga'* genes were transcribed in recombinant *E. coli*, and the stop codon of the vector must have been utilized during the transcription of *mga'* gene. However, there is no experimental evidence to prove whether the absence of a visible hybridization band from *S. uberis* was due to the low quantity of the gene transcript or the inactivity of the *mga* gene. Clearly, there was no doubt about the quality of *S. uberis* RNA, since the same batch of RNA sample worked well when probed with *lbp*.

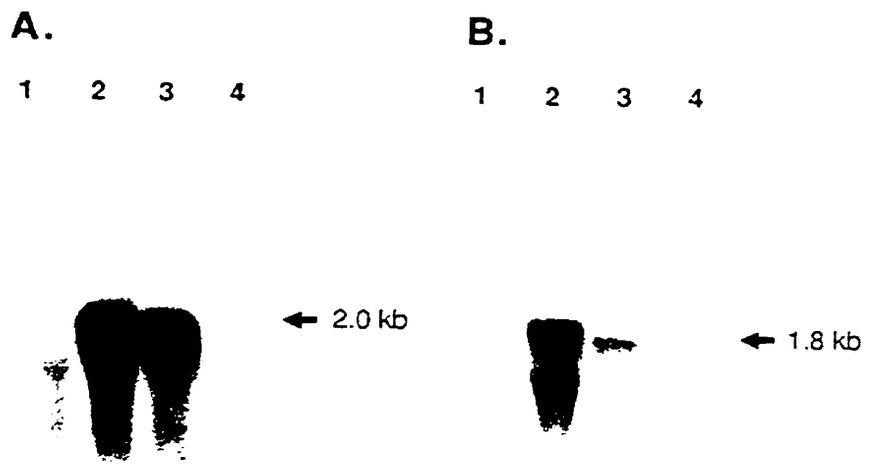


Figure 5-10. Northern blot analysis of RNA from *S. uberis* and recombinant *E. coli* clones. A, the origin of the probe (LP1) is indicated in Fig. 5-11. Lane 1, 2, 3 and 4 are RNA samples from su-1, *E. coli* DH5α(pLBP5), *E. coli* DH5α(pLBP5L) and *E. coli* DH5α(pTZ18R), respectively. B, the origin of the probe (VP2) is indicated in Fig. 5-8. Lane 1, 2, 3 and 4 are RNA samples from su-1, *E. coli* DH5α(pLBP5), *E. coli* DH5α(pMGA14F) and *E. coli* DH5α(pTZ18R), respectively. The arrowheads indicate the position and size of the hybridizing bands.

5.4.6 Southern blot analysis of the *lbp* and *mga* distribution in *S. uberis* strains.

A collection of *S. uberis* including 5 ATCC strains and 42 field isolates (Table. 5-1) was used in Southern hybridization experiments. Chromosomal DNA was prepared and digested with the restriction endonuclease *Hin*dIII and separated on agarose gels. To analyze the *lbp* gene distribution, a 1.5 kb *Hin*dIII-*Hpa*I fragment which contained the most DNA sequence from the region encoding Lbp (LP1 in Fig. 5-11) was used as a probe. This probe hybridized with DNA from 42/47 strains (Fig. 5-12 and Table 5-1). This result meant either that *lbp* shared a region of homology with most of the *S. uberis* strains or that among these strains, there were different regions of the *lbp* gene homologous to the *lbp*

Table 5-1. *S. uberis* strains and DNA hybridization with LP and VP probes

Strain	Source[a]	Probe				
		LP1	LP2	LP3	LP4	VP3
su-1	ATCC9927	3.40[b]	3.40	3.40	3.40	0.87
su-2	ATCC13386	-[c]	-	-	-	-
su-3	ATCC13387	3.60	-	-	3.60	3.60
su-4	ATCC19436	2.70	-	-	2.70	1.70
su-5	Chirino 93-1869	-	-	-	-	-
su-6	Chirino 93-2017	-	-	-	-	-
su-7	Chirino 93-8678-2	4.40	-	-	4.40	4.40
su-8	Chirino-S. uberis	5.00	-	-	5.00	0.91
su-9	ATCC27958	3.80	-	-	3.80	0.87
su-10	Greenfield 93-4997	3.20	-	-	3.20	1.50
su-11	Greenfield 93-4997	3.20	-	-	3.20	1.50

Table 5-1. (continued)

Strain	Source[a]	Probe				
		LP1	LP2	LP3	LP4	VP3
su-12	Leduc	3.80	-	-	3.80	0.87
su-13	Leduc	3.80	-	-	3.80	0.87
su-14	Alix	5.00	-	-	5.00	5.00
su-15	Magrath	4.10	-	-	4.10	0.90
su-16	Ohaton	5.70	-	-	5.70	0.90
su-17	Winfield	2.90	-	-	2.90	1.40
su-18	Not known	4.10	-	-	4.10	1.00
su-19	Gibbons	3.60	-	-	3.60	1.20
su-20	Ardrossan	4.40	-	-	4.40	4.40
su-21	Not known	3.40	-	-	3.40	1.00
su-22	Dapp	2.90	-	-	2.90	1.42
su-23	Thorsby	3.20	-	-	3.20	1.40
su-24	Ponoka	3.80	-	-	3.80	0.87
su-26	Warburg	4.40	-	-	4.40	0.90
su-27	Leduc	-	-	-	-	-
su-28	Fort MacLeod	3.00	-	-	3.00	1.80
su-29	Sherwood Park	5.00	-	-	5.00	5.00
su-30	Barrhead	5.00	-	-	5.00	5.00
su-31	Wainwright	5.00	-	-	5.00	5.00
su-32	Tofield	3.60	-	-	3.60	1.03
su-33	Barrhead	5.00	-	-	5.00	5.00
su-34	Barrhead	5.00	-	-	5.00	5.00
su-35	Medicine Hat	5.00	-	-	5.00	5.00
su-36	Ponoka	5.00	-	-	5.00	5.00
su-37	Ponoka	5.00	-	-	5.00	5.00
su-38	Ponoka	5.00	-	-	5.00	5.00
su-39	Ponoka	5.00	-	-	5.00	5.00
su-40	Millet	3.60	-	-	3.60	1.03
su-41	Lacombe	3.00	-	-	3.00	1.40
su-42	Daysland	5.00	-	-	5.00	5.00
su-43	Ohaton	5.30	-	-	5.30	0.97
su-44	Ohaton	5.00	-	-	5.00	5.00
su-45	Didsbury	-	-	-	-	-
su-46	Didsbury	4.70	-	-	4.70	4.70
su-47	Didsbury	5.00	-	-	5.00	5.00
su-48	Didsbury	4.70	-	-	4.70	4.70

[a] All strains are field isolates except five ATCC (American Type Culture Collection) strains.
[b] Size of hybridizing fragment (in kilobase).
[c] Lack of hybridization.

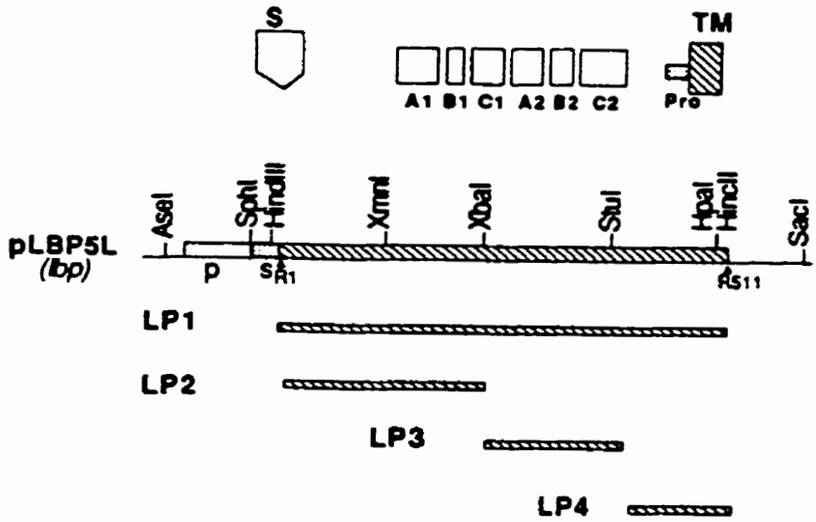


Figure 5-11. Probes used in hybridization analysis and structure of Lbp. The structure of Lbp (on the top) is constructed from the DNA sequence analysis. The signal peptide, proline rich region and transmembrane domain are indicated by S, Pro and TM respectively. The A, B and C repeat regions are also shown. Probes used in hybridization are indicated by the hatched bars below the map.

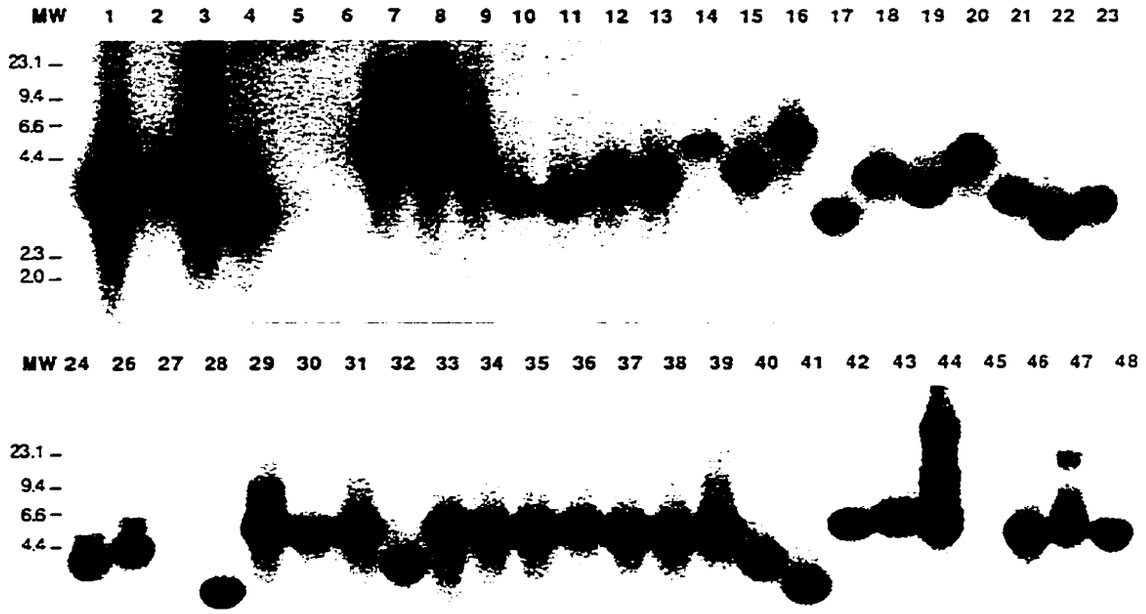


Figure 5-12. Southern blot analysis of the *lbp* distribution in *S. uberis* strains. The origin of the probe (LP1) is indicated in Fig. 5-11. Chromosomal DNA from each *S. uberis* strain was cut with *Hin*dIII. Numbers on the top are *S. uberis* strain numbers. Molecular weight standard (in kb) is labelled on the left. The size of each hybridizing band is listed in Table 5-1.

probe. To locate the homologous region(s) more specifically, this *lbp* probe was subdivided into smaller segments (Fig. 5-11). Two probes, the 643 bp *Hin*dIII-*Xba*I fragment (LP2) and the 437 bp *Xba*I-*Stu*I fragment (LP3) covering the N-terminal and central portion of the coding region in the *lbp* gene respectively hybridized only with su-1, the strain from which the *lbp* gene was originally cloned, whereas the 409 bp *Stu*I-*Hap*I fragment from the C-terminal region (LP4) hybridized with all the strains that were hybridized by probe LP1 (Table 5-1). It appears to be clear that the region of the *lbp* gene encompassing the coding sequences for part of the C repeat, the proposed wall attachment region and the membrane anchor is conserved among *S. uberis* strains, while the region encoding the N-terminal portion is of great variability. The diversity in sizes of chromosomal restriction fragments from the different strains that hybridized with the *lbp* probes would indicate some degree of restriction site heterogeneity (a different location for *Hin*dIII site) among these strains. The appearance of multiple bands of strain 44 on the blot (Fig. 5-12) was caused by incomplete DNA digestion, since further purified DNA showed only one hybridization band in each strain (data not shown).

In order to study whether there was a *mga*-related gene in other *S. uberis* strains, the previous blot (Fig. 5-12) was stripped and reprobed with a 572 bp *Hin*dIII fragment that encompassed the 5′ region of the *mga* gene (VP3 in Fig.

5-8). Hybridization was detectable in all *S. uberis* strains that showed homology with the *lbp* gene (Fig. 5-13 and Table 5-1). Because of the presence of background bands caused by incomplete stripping (Fig. 5-13), the specific band which reacted with the *mga* probe in each strain was listed in Table 5-1.

5.6 Discussion

The Lbp gene of *S. uberis* was cloned in *E. coli* after screening a gene library by colony blotting with ^{125}I-bLf. *E. coli* transformants produced functionally active bLf-binding proteins with molecular weights of 76 kDa and 165 kDa, similar to those of native *S. uberis*. Considering the one-affinity binding phenomenon of bLf with *S. uberis* (chapter 4), we wondered whether the 165 kDa protein was a dimer of the 76 kDa molecule. Treatment with the reducing reagent β-mercaptoethanol did not have any effect on the mobilities of these proteins, indicating the absence of disulphide bridges in the protein structure. This was confirmed after the sequencing data was available; there are no appropriate cysteine residues within this protein. After boiling for 30 min in the presence of 3 M urea, proteins were completely denatured. This treatment resulted in a significant molecular weight decrease of the 165 kDa protein, probably resulting from the dissociation of the dimer, and an

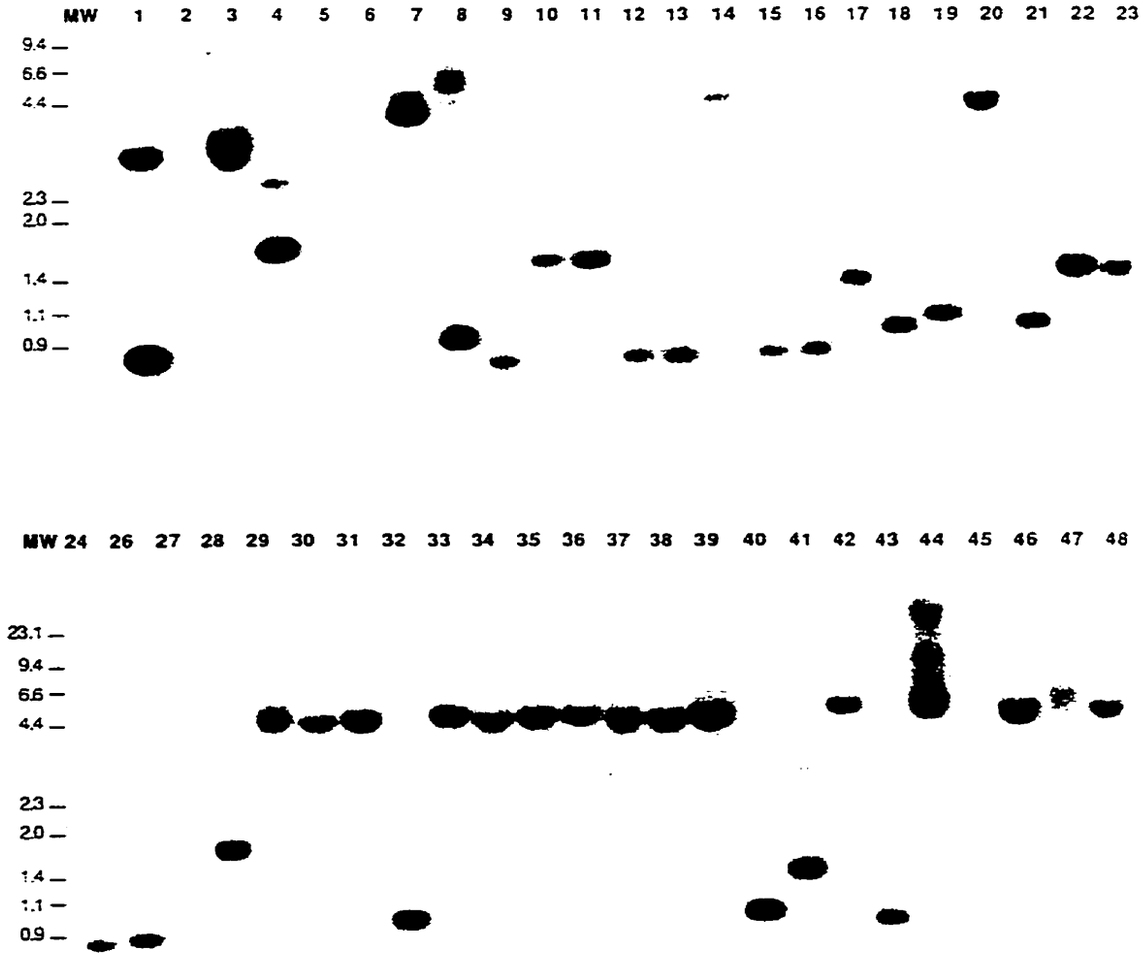


Figure 5-13. Southern blot analysis of the *mga* distribution in *S. uberis* strains. The origin of the probe (VP3) is indicated in Fig. 5-8. Chromosomal DNA from each *S. uberis* strain was cut with *Hin*dIII. Numbers on the top are *S. uberis* strain numbers. Molecular weight standard (in kb) is labelled on the left. The size of each hybridizing band is listed in Table 5-1.

increase in molecular weight of the 76 kDa resulting from protein unfolding. The dimer structure seems to be very stable, since treatment with lower concentration of urea or shorter boiling time could not alter the mobility of the 165 kDa band (data not shown). The presence of both monomeric and dimeric Lbp in *S. uberis* cell wall preparations (Fig. 5-1, lane 1) may imply the existence of both forms on the bacterial surface. However, it is possible that only the dimeric form exists on the bacterial cell; the monomer might come from the cytoplasm due to partial cell lysis caused by glass bead treatment. The dissociation of the dimer to a monomer during sample preparation seems unlikely because of the stability of the dimer.

The complex nature of the *S. uberis* Lbp was further confirmed from the nucleotide sequence analysis. As expected, the two Lbps were encoded by a single ORF of 1,683 bp. It is quite possible that two copies of the translated product interact with each other to form a homodimer. Interactions between subunits could be extensive and extend throughout the length of the molecule due to the existence of the high α-helical content. We have no evidence to prove whether α-helices adopt a coiled coil structure similar to those of group A streptococcal M proteins (Fischetti, 1989). However, like M proteins (Fischetti, 1989), Lbp shares significant sequence homology with a number of α-helical coiled structure-containing mammalian fibrillar proteins such as

human myosin heavy chain and kinesin heavy chain.

The deduced amino acid sequence of Lbp indicated the existence of a signal peptide of 50 amino acids (if translation started at the first ATG start codon) at the N terminus, as expected for a protein that appears on the outside of a bacterial cell. The structure of this signal peptide is comparable to the consensus structures for signal peptides in proteins from Gram positive bacteria described previously (Simonen and Palva, 1993; Goward *et al.*, 1993; Perlman and Halvorson, 1983; von-Heijne, 1983; 1986).

Analysis of the C terminus of Lbp revealed the presence of an amino acid sequence typical of membrane anchor motifs found in many other proteins from Gram positive bacteria (Fischetti *et al.*, 1991). Such regions have been shown to play a role in the anchoring of proteins in the cell wall (Pancholi and Fischetti, 1988; Schneewind *et al.*, 1992; 1993). It is probably via the C-terminal amino acids that Lbp becomes anchored on the cell surface. The membrane anchor motifs described here appear to have no function in *E. coli* since the Lbp expressed in *E. coli* was mainly secreted, similar to the streptococcal M6.1 protein which was found predominantly in the periplasm (Fischetti *et al.*, 1984).

The 5′ and progressive 3′ deletions performed in the *lbp* gene allowed the definition of a large domain (about 200 codons) at the N terminus which could bind bLf. A comparison of this region with the transferrin binding domain

of Tbp2 from *Neisseria* (Vonder Haar *et al.*, 1994) showed no significant homology. Also, the bLf binding region reported here did not contain any of the domains with transferrin binding activity found in the transferrin-binding protein (TfbA) of *Actinobacillus pleuropneumoniae* (Strutzberg *et al.*, 1995). This is not surprising since our previous study showed that bovine or human transferrin could not block the binding of bovine lactoferrin to *S. uberis* (chapter 4), which indicates a difference between the bLf-binding domain and the Tf-binding domain. To date, there is no information on Lf-binding domains available for comparison. To locate the bLf binding domain more precisely, more progressive deletions will be needed. An alternative approach is to synthesize oligopeptides based on the amino acid sequence of the large domain we have localized and use them to inhibit the binding of bLf to *S. uberis*. The one's capable of blocking binding should represent the Lf-binding region. This work is in progress.

While sequencing the Lbp gene, we found a second ORF, *mga*, in the adjacent region. The putative product of this gene (containing 499 amino acid residues) has comparable molecular size and sequence to VirR12 (499 residues) and Mry (530 residues), the positive regulators of the M protein genes of group A streptococci (Chen *et al.*, 1993; Perez-Casal *et al.*, 1991). However, Mry and VirR12 had 98% homology (Chen *et al.*, 1993), whereas Mga showed only 34% homology with

them. This may be attributed to the difference of species. Similarly, VirR49 of an OF$^-$ GAS showed less homology (76%) to Mry or VirR of OF$^-$ GAS (Podbielski *et al.*, 1995).

The cytoplasmic location of Mga was suggested by the absence of a signal peptide at the N terminus of the deduced protein. A potential -10 and -35 promoter was found. However due to the high A and T content in this region, identification of such a promoter through analysis of the sequence alone may be of limited value. Thereby, experimental determination of the *S. uberis mga* promoter appears to be necessary for further comparison of this promoter with those of Mry and VirR.

Studies have shown that *mry* is autoregulated and environmentally regulated in response to the level of CO_2 (Okada *et al.*, 1993). Expression of *mry* was stimulated by increased concentrations of CO_2. In our experiments, *S. uberis* cells were cultured under conditions without an additional supplementation of CO_2. The absence of a stimulating environment could have resulted in a very low level of *mga* expression. This could be the reason that Northern blot analysis did not detect any *mga* transcript from *S. uberis*. It would be expected that in recombinant *E. coli*, *mga* would not likely be regulated by environmental signals due to the absence of other regulatory components such as the sensing protein.

It would be interesting to know whether *mga*

regulates the expression of *lbp*. No potential VirR-binding boxes that are present in the promoters of M and M-like protein genes (Podbielski *et al.*, 1992; 1995) were found in the immediate upstream portion of the -35 region of *lbp* by homology comparisons. We then blotted 47 *S. uberis* strains with *mga* and *lbp* specific probes, intending to see the distribution of *mga* and its relatedness to *lbp* in this streptococcal group. Results showed that all the strains that were *lbp* positive contained *mga*. However, neither sequence comparisons nor blotting results lead to any conclusions regarding the relationship of *mga* and *lbp*. Generating a *mga*-negative *S. uberis* strain will allow us to evaluate whether *mga* is required for *lbp* expression by comparing the Lf binding activity and *lbp* mRNA level of *mga*-negative mutant and the wildtype strain. To further define and quantify the environmental impact such as culture media, CO_2 or O_2 levels, and different phases of infection *in vivo*, on the regulatory circuit, a shuttle vector with a reporter gene such as the CAT (staphylococcal chloramphenicol transacetylase) gene, which is capable of replicating in *S. uberis* needs to be constructed. After inserting the *lbp* promoter next to the promoterless CAT gene and introducing this construct into *S. uberis*, the cells should be grown *in vitro* and *in vivo* for comparison of the *mga* mRNA level and CAT activity, which would reflect the *lbp* expression in response to particular environmental stimuli.

Southern blot analysis of *lbp* in *S. uberis* strains using subgenomic probes revealed that the C-terminal sequence is conserved among strains, whereas the N-terminal region shows greater variation. This phenomenon resembles that described for M protein genes in group A streptococci. By using DNA hybridization with probes from the structural gene for the M6 protein, Scott *et al.* (1986) showed that the C-terminal region is highly conserved among strains of different M serotypes and the N-terminal region is highly variable. Consistent with this, detailed amino acid sequence comparisons and antibody reactivities revealed limited differences within the conserved C-terminal regions and much more extensive variability at the N termini (Bessen and Fischetti, 1990b; Bessen *et al.*, 1989; Kehoe, 1991). In the case of Lf binding proteins of *S. uberis*, however, limited sequence and serological data are available for comparisons. In M proteins, the N-terminal region is distal to the streptococcal cell surface, and thus would be expected to be the region of the molecule most exposed to immunological selective pressure. Therefore it is understandable to find that the N-terminal region varys in sequence among different serological types of M protein. In contrast, the sequence of the C-terminal region of the molecule should be evolutionarily conserved to assure attachment to the streptococcal surface. Since Lbp has a structure similar to that of M protein, it is not surprising to find that its C-

terminal sequence is conserved among strains, whereas the N-terminal region shows variation.

Since the N-terminal part of the Lbp is variable among *S. uberis* strains, our finding that this part of the molecule is responsible for Lf binding seems surprising. However, similar observations have been reported in *Actinobacillus pleuropneumoniae*. Three isoforms of transferrin binding proteins (Tbp2) from different *A. pleuropneumoniae* serotypes contain a variable N-terminal half and a conserved C-terminal half (Bunka *et al.*, 1995; Gerlach *et al.*, 1992); the N-terminal half of the molecule is responsible for transferrin binding (Strutzberg *et al.*, 1995).

6.0 EVALUATION OF THE PROTECTIVE CAPACITY OF THE RECOMBINANT CAMP FACTOR AND LACTOFERRIN BINDING PROTEIN AGAINST CHALLENGE BY *S. UBERIS*

6.1 Summary

The CAMP factor and lactoferrin-binding protein genes of *S. uberis* were overexpressed under the control of the *tac* promoter in plasmid pGH433 in *E. coli*. CAMP factor and Lbp were prepared as inclusion bodies, solubilized and used to immunize lactating dairy cows. Animals vaccinated with CAMP factor generated specific antibody in serum and milk, and possessed low somatic cell counts in milk after challenge. In contrast, Lbp did not induce the production of specific antibody in the immunized animals, and the mean somatic cell count in milk of these animals was similar to that of animals which received a placebo.

6.2 Introduction

Mastitis is an inflammatory reaction of udder tissue to bacteria, chemical, thermal, or mechanical injury (Schultz, *et al.*, 1978). The inflammatory response is characterized by an increased number of somatic cells in milk, including neutrophils, macrophages and epithelial cells sloughed from milk-producing tissue during lactation as a result of cell death. The threshold value for somatic cell counts (SCC) was set at 500,000 cells/ml by the International Dairy Federation (Bramley, 1991), above which, animals would have clinical mastitis. Inflammatory changes affect the process of milk synthesis both quantitatively and qualitatively. The level of the main constituents such as fat, protein and lactose declines and the pH value is shifted to a more alkaline level (Kitchen, 1981).

S. uberis is one of the chief bacteria involved in bovine mastitis. At least 20% of all cases of clinical mastitis are now due to this organism (Wilesmith, *et al.*, 1986). Since *S. uberis* is widely distributed in the environments such as bedding, pastures and many sites of the cow (Bramley, 1982; Kruze and Bramley, 1982), the conventional control measures based on post-milking teat disinfection, dry-cow antibiotic therapy and other hygienic milking techniques reducing the residual contamination of the teat skin by pathogens are of limited effectiveness against

mastitis caused by this bacterial species. Vaccination would appear to be a promising approach for the control of *S. uberis* infection. Studies have shown that a primary infection with *S. uberis* can considerably reduce the rate of infection following a second challenge with the same strain (Hill, 1988b). Local vaccination with killed *S. uberis* protected the mammary gland against experimental intramammary challenge with the homologous strain (Finch *et al.*, 1994). Also, subcutaneous vaccination with live *S. uberis* caused a dramatic modification of the pathogenesis of mastitis with the same strain (Hill *et al.*, 1994).

Attempts to develop mastitis vaccine(s) should be based on knowledge of the virulence determinants and protective antigen(s) of the bacterial species. It is known that *S. uberis* produces hyaluronic acid capsule (Hill, 1988), hyaluronidase (Schaufuss *et al.*, 1989), R-like protein (Groschup and Timoney, 1993), and a cohemolysin, the CAMP factor (Skalka and Smola, 1981). However, very little is known of their roles in inducing protection against *S. uberis* mastitis.

In our laboratory, we cloned and sequenced the gene coding for the CAMP factor, a 28 kD extracellular protein (Jiang *et al.*, 1996). We also identified a bovine lactoferrin-binding protein (Lbp) on the bacterial surface, which has a structure similar to M protein of group A streptococci (chapter 4 and 5). On the basis of previous

work, the CAMP factor and Lbp have been expressed in an IPTG-inducible expression system. To determine whether these proteins could induce protection against *S. uberis* mastitis, a preliminary experiment was conducted and some of the results are presented here.

6.3 Materials and Methods

6.3.1 Bacterial strains, vectors and media.

S. uberis strain su-1 (ATCC 9927) was obtained from a clinical case of bovine mastitis. Bacterial cells were grown in tryptic soy broth, aliquoted and stored at -70°C on blood beads until needed. The *E. coli* strain DH5α was used in all transformation experiments. *E. coli* cells were cultured in Luria medium, and the medium for the growth of transformants was supplemented with 50 μg/ml of ampicillin. The plasmid pGH433 (Anderson *et al.*, 1991) was used to express the recombinant proteins under the control of an IPTG (isopropyl β-D-thiogalacto-pyranoside)-inducible promoter.

6.3.2 PAGE and Western blotting.

SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) was used for analysis of the CAMP factor. Lbp inclusion bodies were dissolved in sample buffer in the presence of 4 M urea and run on a SDS-PAGE gel containing 4 M urea. Western blotting using convalescent serum from an su-1-infected cow was as described in 3.3.7 except that a 1:75 dilution of cow convalescent serum and a 1:5000 dilution of goat anti-bovine IgG coupled to alkaline phosphatase were used.

6.3.3 Protein purification.

A culture of *E. coli* transformants (1 L) was grown

to an absorbance at 660 nm of 0.5 and induced with 2 mM IPTG. After 2 h of continuous, vigorous shaking at 37°C, the cells were harvested and the protein inclusion bodies were prepared as described in 3.3.5. Antigens for ELISA were purified from SDS-PAGE gels or 4 M urea SDS-PAGE gels by elution.

6.3.4 Determination of protein purity and concentration.

Protein purity was estimated by SDS-PAGE and subsequent Coomassie blue staining. The protein concentration was determined using Bio-Rad DC protein assay as described by the supplier. Bovine serum albumin (Pierce Chemical Co., Rockford, IL) was used as a standard. The amount of target protein vs. total protein was determined after scanning the Coomassie blue stained SDS-PAGE with a Bio-Rad 620 Densitometer.

6.3.5 Vaccine preparation and vaccination.

The vaccines consisted of proteins emulsified in the adjuvant VSA3 which had been diluted with 0.1 M PBS, pH 7.2. Each 2 ml dose of vaccine contained VSA (0.67 ml), PBS (1.33 ml), and 50 µg of protein antigen dissolved in 5-10 µl of 4 M guanidine hydrochloride. Fifteen healthy lactating dairy cows from the Pennsylvania State University Mastitis Research Herd were vaccinated intramuscularly at drying off and again 28 days later. Animals were assigned to three

groups of five cows. Group 1 was given CAMP factor; group 2 was given Lbp; and group 3 (placebo) was given adjuvant only.

6.3.6 Challenge.

The bacterial challenge culture was prepared by rolling the stock bead cultures onto esculin blood agar plates containing 5% whole blood. After 24 hours incubation at 37°C, a single colony was used to inoculate 100 ml of Ultra High Temperature pasteurized (UHT) milk and incubated for 12 hours at 37°C. The 24 hour culture was mixed well and a 100 µl aliquot was removed to inoculate a second 100 ml of UHT milk. After a second 9 hour incubation at 37°C, the culture was serially diluted in 10-fold increments using sterile saline. The colony forming units (CFU) per ml of each dilution was determined by absorbance on a spectrophotometer and confirmed by plating onto blood agar. Animals were challenged by intramammary infusion of 200 CFU of *S. uberis* in 1 ml of saline in the teat canal of one quarter on day four of lactation.

6.3.7 Sampling.

Milk and blood samples were obtained at the times outlined in Table 6-1.

Table 6-1. Immunization, challenge and Sampling schedule.

TIME	SAMPLE
dry off, D-0	serum, milk, immunization
14 days dry, D+14	serum
28 days dry, D+28	serum, immunization
52 days dry, D+52	serum
calving, C-0	serum, milk
4 days lactation, CH-0	serum, milk, challenge
7 days lactation, CH+3	serum, milk
14 days lactation, CH+10	serum, milk
21 days lactation, CH+17	serum, milk

6.3.8 Antibody Titers.

Total IgG titers for each experimental antigen were determined by an indirect ELISA. Nunc Immunlon-2 plates were coated with antigen in carbonate buffer. Prior to use, the plates were blocked with TBST (100 mM Tris-Cl, pH 8.0; 150 mM NaCl; 0.05% Tween-20) and 3% BSA for 1 hour. After blocking, the plates were washed with distilled water. Serum and milk samples were serially diluted in 3-fold increments using TBST containing 1% BSA. Rabbit antisera for each antigen was also diluted and served as a positive control. Negative control samples contained TBST with 1% BSA. The diluted samples and controls were transferred to the coated plates and were incubated for 1 hour at room temperature. The plates were washed thoroughly with distilled water and all wells were

incubated with a horse radish peroxidase conjugate of goat anti-IgG diluted 1:2000 in TBST containing 1% BSA. Following a 1 hour incubation at room temperature, the plates were washed with distilled water. The amount of antibody present in samples was visualized using ABTS substrate. The titers of each sample were based on the absorbance reading at 405 nm with a reference wavelength of 495 nm. A positive reading for samples was one in which the absorbance was two times the absorbance of the blank (negative control). Titers were determined by taking the reciprocal of the last dilution giving a positive reading. Consistency among assay plates was monitored by the absorbance reading of positive controls.

6.3.9 Milk compositional analysis.

Milk pH values, total somatic cell counts, fat, protein, and lactose were determined using a Fossomatic Cell and Milk Analyzer (A/S Foss Electric, Hillerød, Denmark).

6.3.10 DNA manipulations.

All molecular techniques were as recommend by the supplier (Pharmacia Canada Ltd.) or Maniatis *et al.* (1989). When required, plasmid DNA fragments were isolated from agarose gels using a Gene Clean kit (Bio/can Scientific).

6.4 Results

6.4.1 Expression of the CAMP factor and Lbp under the control of the *tac* promoter.

A recombinant plasmid containing the carboxy-terminal 98% of the CAMP factor gene (*cfu*) was constructed by cloning a 1.7 kb *Eco*RI-*Bam*HI fragment from pJLD21-2 into the *Bam*HI site of pGH433 which provides a 12 amino acid leader peptide and an IPTG-inducible *tac* promoter. The ends of the plasmid fragments were filled in by the Klenow fragment before ligation. The resultant construct, designated as pGH-CAMP (Fig. 6-1), produced a positive CAMP reaction on blood agar plates (data not shown).

Plasmid pGH-LBP was constructed by inserting the 1.8 kb *Sph*I-*Rsa*I fragment from pLBP5 into the *Bam*HI site of pGH433 (Fig. 6-1). Before ligation, the insert was treated with mung bean nuclease to remove the 3′ overhang from the end generated by *Sph*I, and the *Bam*HI-cut vector was filled in by the Klenow fragment to produce blunt ends. The pGH-LBP contained the carboxy-terminal 96% of the Lbp gene (*lbp*), which was preceded by a 12 amino acid leader peptide and the *tac* promoter provided by vector pGH433. Analysis of the nucleotide sequence at the fusion site revealed identity to the sequence of the *lbp* presented in chapter 5.

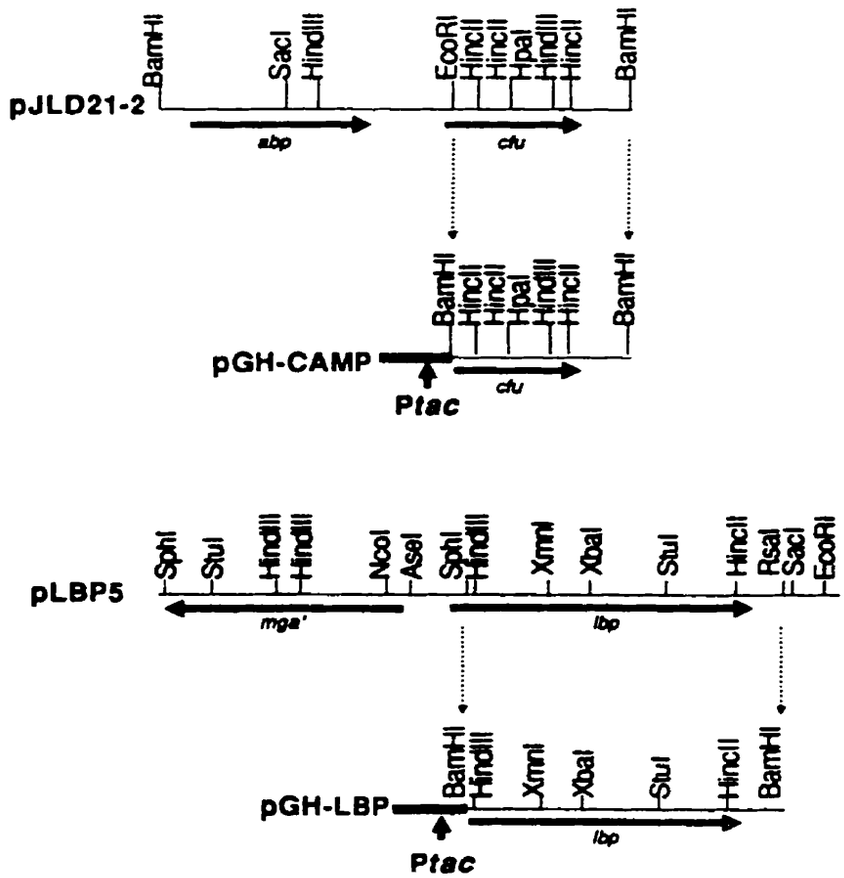


Figure 6-1. Physical maps of the recombinant plasmids used to express the aggregated CAMP factor and Lbp. The plasmid pJLD21-2 and pLBP5 contain 2.5 and 3.7 kb of *S. uberis*-derived DNA respectively, in the vector pTZ18R. The plasmid pGH-CAMP and pGH-LBP were constructed by subcloning an *Eco*RI-*Bam*HI fragment from pJLD21-2 and a *Sph*I-*Rsa*I fragment from pLBP5 into vector pGH433, respectively. P_{tac} indicates the location of the *tac* promoter. The CAMP factor and Lbp genes are shown by arrows labelled as *cfu* and *lbp* respectively.

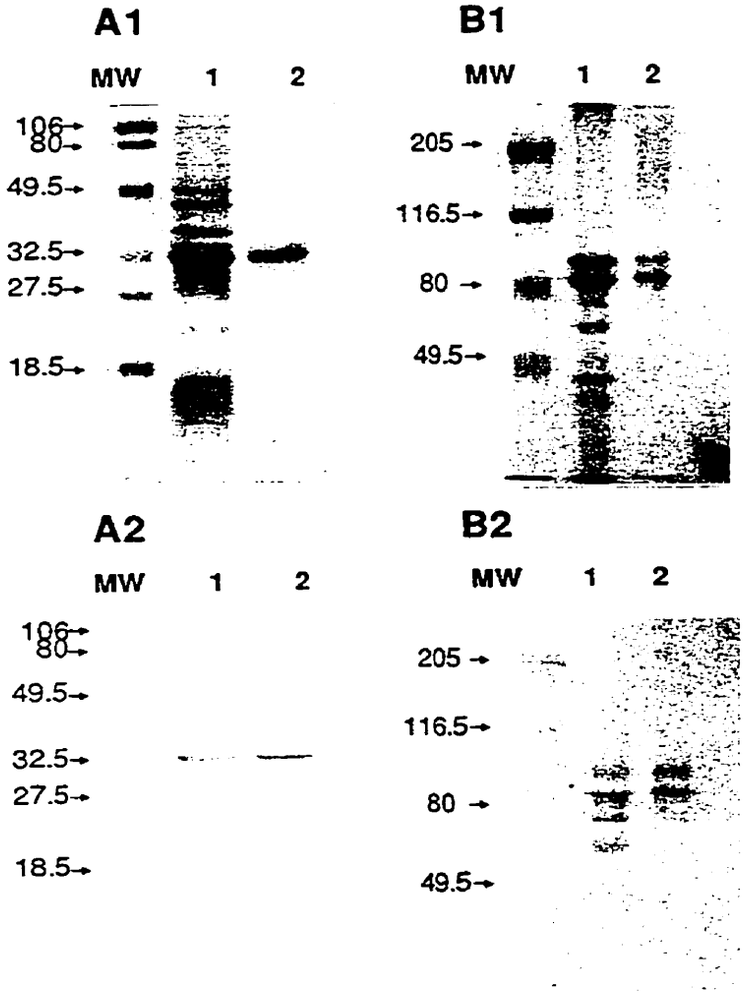
6.4.2 Purification of recombinant proteins.

The CAMP factor and Lbp of *S. uberis* were expressed in *E. coli* DH5α using pGH-CAMP and pGH-LBP respectively. In this system, expression of the recombinant protein was repressed under normal growth conditions. Upon IPTG-induction, the recombinant protein was produced in an aggregated form; the 28 kD CAMP factor made up 17% of the total protein (Fig. 6-2 A1, lane 1) and the 82 and 90 kD Lbp made up 36% of the total protein (Fig. 6-2 B1, lane 1). Isolated protein aggregates were dissolved in 4 M guanidine hydrochloride and used for vaccine formulation. The CAMP factor and Lbp were purified from SDS-PAGE gel and 4 M urea SDS-PAGE gel respectively (Fig. 6-2 A1, lane 2 and Fig. 6-2 B1, lane 2, respectively) and used as antigens for ELISA. Both aggregated and purified CAMP or Lbp were demonstrated to be antigenically active by Western blotting using convalescent serum from *S. uberis* infected cow (Fig. 6-2 A2 and B2).

6.4.3 Somatic cell counts in milk following experimental bacterial challenge.

The mean somatic cell count in milk from challenged quarters of nonvaccinated control animals increased up to 3,000,000 cell/ml 3 days following challenge (Fig. 6-3). In contrast, the animals which had received CAMP factor vaccinations did not show any elevation in milk SCC after

Figure 6-2. SDS-PAGE and immunoblot analysis of the recombinant CAMP factor and Lbp. A 1, coomassie blue-stained 10% polyacrylamide-SDS gel. Lane 1, CAMP factor aggregate protein; lane 2, purified CAMP factor. A 2, immunoblot of the gel presented in panel A 1 using convalescent serum from *S. uberis* infected cow. B 1, coomassie blue-stained 7.5 % polyacrylamide-SDS gel containing 4 M urea. Lane 1, Lbp aggregate protein; lane 2, purified Lbp. B 2, immunoblot of the gel presented in panel B 1 using convalescent serum from *S. uberis* infected cow. The molecular weight (MW) standards (kD) are represented by arrows to the left.


A1
MW
1
2
106
80
49.5
32.5
27.5
18.5
B1
MW
1
2
205
116.5
80
49.5
A2
MW
1
2
106
80
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27.5
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B2
MW
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2
205
116.5
80
49.5

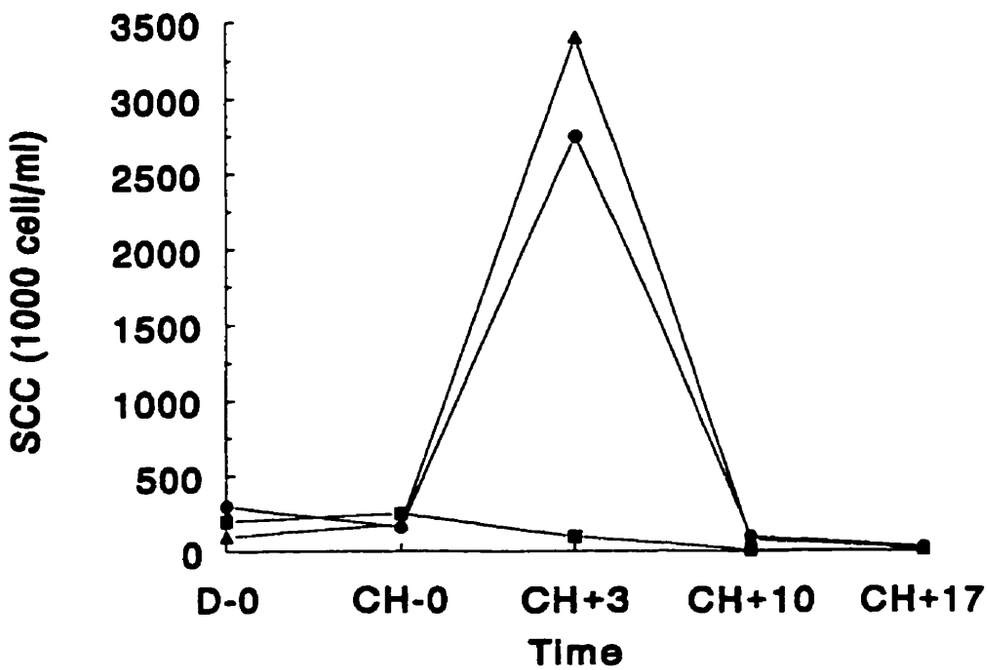

Figure 6-3. Effect of vaccination with CAMP factor and Lbp on somatic cell counts in milk. Somatic cell counts (SCC) per milliliter of milk are means of 5 cows. D-0, day of dry off; CH-0, day of challenge (4 days lactation); CH+3, 7 days lactation; CH+10, 14 days lactation; CH+17, 21 days lactation. Symbols: ■, CAMP factor; ▲, Lbp; ●, placebo.

challenge. However, the challenged quarters of animals vaccinated with Lbp showed a rapid increase in the mean SCC which was close to that in the control group (Fig. 6-3).

6.4.4 Effect of vaccination of CAMP factor and Lbp -specific antibody titers.

The mean levels of antibodies specific to CAMP factor and Lbp in the serum and milk of the control animals were low prior to experimental challenge (Fig. 6-4). In contrast, the mean levels of CAMP factor-specific antibodies were significantly elevated in the serum and milk of CAMP factor-vaccinated animals prior to challenge compared with the levels before vaccination or with levels in the control cows (Fig. 6-4). However, Lbp-specific antibody levels in the serum and milk of animals vaccinated with Lbp were not significantly increased following vaccination when compared with prevaccination levels or levels in the control animals (Fig. 6-4).

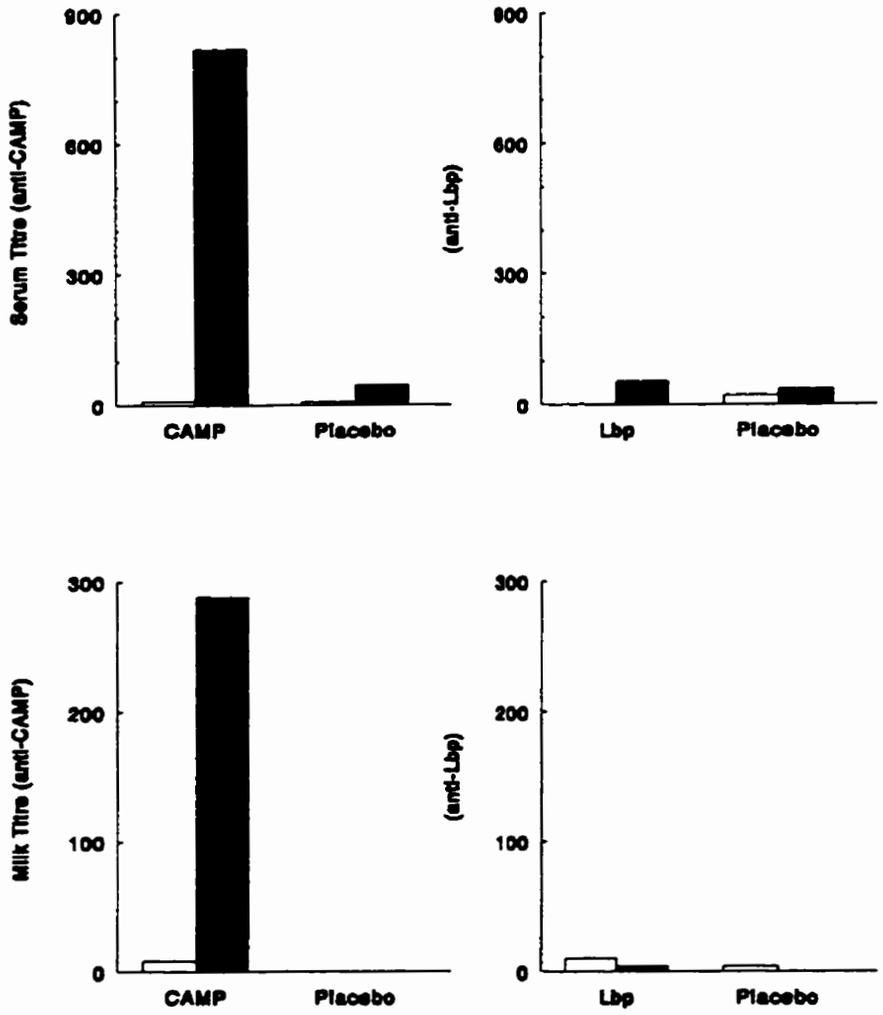

Figure 6-4. Serum and milk antibody titers to CAMP factor and Lbp in immunized and unimmunized control animals. Antigens for immunization are shown at the bottom of each panel. Symbols: □, prevaccination (D-0); ■, postvaccination (CH-0).

6.4.5 Changes of pH value and main constituent of milk after challenge.

The mean pH values of milk from animals vaccinated with placebo, CAMP factor and Lbp changed in a similar pattern after challenge (Fig. 6-5). The percentage of fat, lactose and protein in milk from immunized and unimmunized animals also changed in a similar pattern after challenge. No obvious decrease of these values occurred. The changes of milk fat, lactose and protein are shown in Fig. 6-6, Fig. 6-7, and Fig. 6-8.

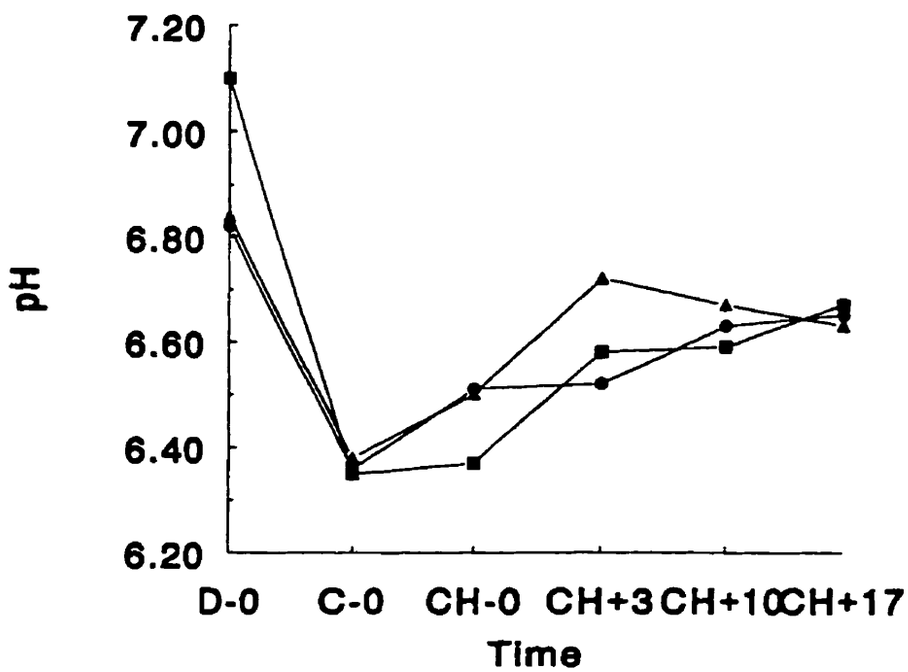

Figure 6-5. Changes in pH of milk following intramammary challenge. D-0, day of dry off; C-0, day of calving; CH-0, day of challenge (4 days lactation); CH+3, 7 days lactation; CH+10, 14 days lactation; CH+17, 21 days lactation. Symbols: ■, CAMP factor; ▲, Lbp; ●, placebo.

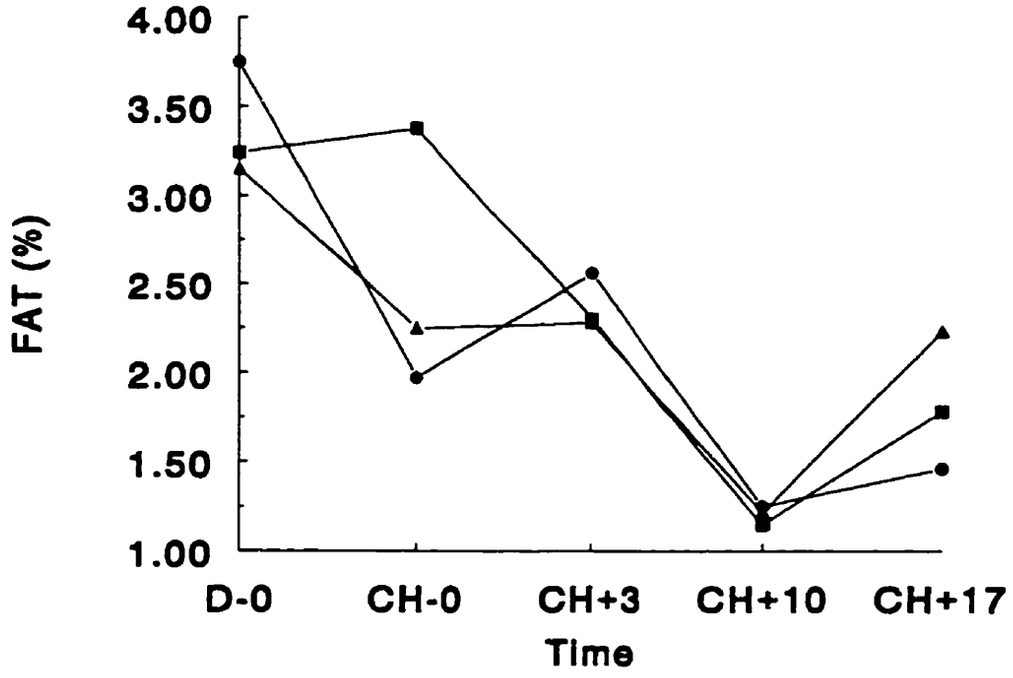

Figure 6-6. Changes of the percentage of milk fat following intramammary challenge. D-0, day of dry off; CH-0, day of challenge (4 days lactation); CH+3, 7 days lactation; CH+10, 14 days lactation; CH+17, 21 days lactation. Symbols: ■, CAMP factor; ▲, Lbp; ●, placebo.

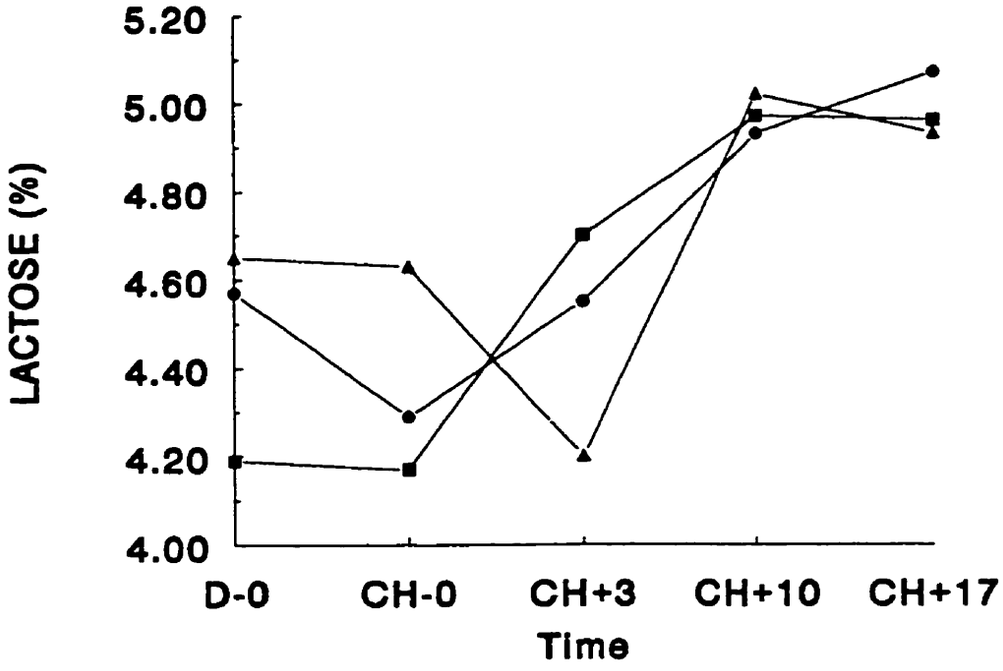

Figure 6-7. Changes of the percentage of milk lactose following intramammary challenge. D-0, day of dry off; CH-0, day of challenge (4 days lactation); CH+3, 7 days lactation; CH+10, 14 days lactation; CH+17, 21 days lactation. Symbols: ■, CAMP factor; ▲, Lbp; ●, placebo.

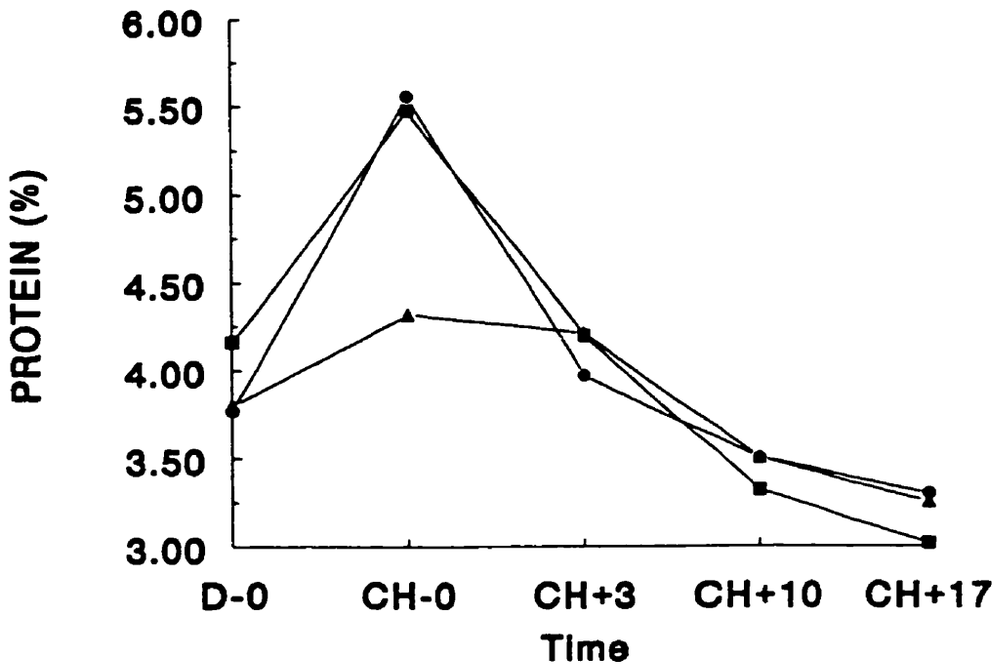

Figure 6-8. Changes of the percentage of milk protein following intramammary challenge. D-0, day of dry off; CH-0, day of challenge (4 days lactation); CH+3, 7 days lactation; CH+10, 14 days lactation; CH+17, 21 days lactation. Symbols: ■, CAMP factor; ▲, Lbp; ●, placebo.

6.5 Discussion

The failure of existing control measures to significantly reduce new intramammary infections by *S. uberis* has emphasized the need to investigate an alternative approach such as vaccination. The development of effective vaccines must be based on an understanding of the defence mechanisms responsible for resistance to infection. The infiltration of polymorphonuclear leukocytes (PMN) into the mammary gland following bacterial infection has long been recognized as a primary protective mechanism against mastitis caused by coliforms (Hill *et al.*, 1978; Jain *et al.*, 1971) and staphylococci (Schalm *et al.*, 1976). Anti-toxin immunity mediated by antibodies directed at bacterial toxins is another key mechanism of ameliorating the pathological changes in staphylococcal mastitis (Anderson, 1978).

CAMP factor is a cohemolysin which causes synergistic hemolysis of sheep or bovine erythrocytes in the presence of the β-toxin of *Staphylococcus aureus* (Christie *et al.*, 1944; Phillips *et al.*, 1980). The lethal effect of partially purified CAMP factor to rabbits (Skalka and Smola, 1981) suggests that this protein may play a pathogenic role in some diseases, including bovine mastitis. Therefore, immunization with this protein may reduce the severity or even prevent the occurrence of mastitis. The lactoferrin-binding protein of *S. uberis* has been identified as an M-like protein (chapter 4 and 5). Although the iron acquisition

function of this protein has not been demonstrated, its surface exposure would still make it a potential vaccine candidate by increasing the levels of opsonic antibody in milk and potentiating the speed of PMN recruitment into the mammary gland. Furthermore, our experiment showed that both CAMP factor and Lbp could be recognized by bovine serum from an animal which recovered from an *S. uberis* infection, indicating that both proteins are expressed *in vivo* and cows respond to them immunologically. Based on all these considerations, we tested the protective capacity of the recombinant CAMP factor and Lbp in lactating dairy cows against challenge by *S. uberis*.

Both CAMP factor and Lbp were produced as inclusion bodies in the expression system used, facilitating the purification of large quantities of pure recombinant proteins. We do not know why aggregated Lbp showed two major bands following PAGE (Fig. 6-2 B1) with molecular weights lower than the Lbp (105 kD) from the culture supernatant of recombinant *E. coli* (Fig. 5-3, lane 7 in Chapter 5). It is possible that the 82 kD protein is a degradation product of the 90 kD protein, or the 90 kD protein is a precursor with the uncleaved signal peptide. Amino-terminal sequence analysis of these two products should be able to clarify this question. When we analysed the aggregated Lbp on a 4 M urea gel (Fig. 6-2 B1), we did not boil the sample. Therefore the protein structure might not be completely unfolded as in the

case of Lbp from the supernatant (Fig. 5-3, lane 7 in Chapter 5) which was boiled for 30 min in the presence of 3 M urea. Hence, the unboiled Lbp aggregate might migrate faster and show a lower apparent molecular weight.

Our data clearly show that the milk somatic cell counts of cows vaccinated with CAMP factor were significantly lower than those of control animals following bacterial challenge. Immunized animals possessed high levels of CAMP factor-specific antibodies in both serum and milk. It may be that anti-CAMP factor antibodies could neutralize CAMP factor produced by the invading bacteria and therefore prevent the cell infiltration in the challenged quarters, although there is no direct evidence for this. It is tempting to suggest that CAMP factor may have direct chemotactic activity which primes the recruitment of neutrophils or may, by acting on enzyme systems or complement, cause the release of chemotactic substances.

Unlike CAMP factor, Lbp did not induce high levels of specific antibodies in the immunized cows. In these animals, bacterial challenge resulted in high somatic cell counts, indicative of mastitis. Therefore, vaccination with Lbp did not prevent the occurrence of mastitis. However, the serological response to vaccination was very poor and therefore it is not possible to draw any conclusions regarding the protective capacity of Lbp. The importance of IgA in mucosal secretions has been well documented (Norcross,

1991; Nordhaug *et al.*, 1994), and we have no data available at this time on the isotype of the antibodies which were present. Therefore, it may be possible to utilize a different means of antigen delivery to specifically stimulate an IgA or IgG_2 response (Nordhaug *et al.*, 1994). The potential involvement of mammary gland lymphocytes in the protection against *S. uberis* mastitis following vaccination with killed *S. uberis* via the intramammary or subcutaneous route has been suggested elsewhere (Finch *et al.*, 1994). Recent observations have indicated that the protection against mastitis caused by *S. uberis* does not appear to be related to levels of specific antibody. Intramammary or subcutaneous administration of *S. uberis* at drying off was shown to dramatically reduce both the incidence of clinical mastitis and numbers of bacteria recovered from the milk following challenge with the same strain during the next lactation (Finch *et al.*, 1994; Hill *et al.*, 1994). Although there was a significant increase in the levels of *S. uberis*-specific immunoglobulin following vaccination, there was no increase in the opsonic activity of serum and milk. Furthermore, it has been demonstrated that *S. uberis* can resist the bactericidal activity of neutrophils despite the presence of Ig bound to the surface of the bacteria (Leigh and Field, 1994). Therefore, assessment of cellular immune response should be of equal importance to that of antibody response when evaluating a vaccination regime against *S. uberis* mastitis.

The lactoferrin binding property of Lbp raises the question of whether Lbp-specific antibody could inhibit or limit bacterial growth by interfering with iron acquisition. When evaluating Tbp2 within the Tbp1-Tbp2 complex as a potential antigen for future meningococcal vaccines, it was found that Tbp2 can elicit antibodies that are not only bactericidal but also inhibitory for meningococcal growth (Lissolo *et al.*, 1995). To raise the level of Lbp-specific antibody, an appropriate vaccine formulation and immunization schedule should be developed by studying the effect of route of administration, dose of antigen, adjuvant, time of administration in relation to lactation stages and number of booster injections.

During our vaccine trial, no obvious clinical signs of mastitis were observed after challenge in immunized or unimmunized animals. Therefore, it is not surprising that the pH value and main constituent of milk were not influenced.

The data obtained from our preliminary animal experiments is very limited. However, it is very clear that CAMP factor can induce an antibody response and prevent cell infiltration in the mammary gland of the challenged animals. To further demonstrate the roles of CAMP factor and Lbp in the induction of protective immunity, the trial is currently being repeated at a higher challenge dose.

7.0 GENERAL DISCUSSION AND CONCLUSIONS

The objective of this thesis was to identify potential virulence factors and protective antigens of *S. uberis*. The work accomplished during this study includes: characterization of the *S. uberis* CAMP factor gene, identification of a bovine lactoferrin binding protein in *S. uberis*, cloning and characterization of the *S. uberis* lactoferrin binding protein gene, and evaluation of the protective capacity of the recombinant CAMP factor and lactoferrin binding protein against challenge by *S. uberis*. In addition, two gene loci upstream from the CAMP factor and lactoferrin binding protein genes were also identified, which are, respectively, homologous to the polar amino acid binding protein of Gram negative bacteria and the positive regulator Mga of group A streptococci. This chapter will briefly interpret the results obtained and present some general conclusions.

CAMP factor, originally identified in group B streptococci, is a cohemolysin which can cause complete hemolysis of sheep or cow erythrocytes in the presence of β-toxin of *Staphylococcus aureus* (Bernheimer *et al.*, 1976; Christie *et al.*, 1944). The production of CAMP factor by *S.*

uberis was demonstrated decades ago (Heeschen *et al.*, 1967; Skalka and Smola, 1981). However, although a role for CAMP factor in pathogenicity has been suggested by some researchers (Fehrenbach *et al.*, 1988; Jürgens *et al.*, 1987; Skalka and Smola, 1981), its role in bovine mastitis and inducing protective immunity has not been investigated.

In our experiments, CAMP factor was purified from the culture supernatant of a CAMP reaction positive *S. uberis* strain. The CAMP factor gene (*cfu*) was cloned and expressed in *E. coli*. The recombinant protein possessed CAMP activity and had a molecular weight similar to that of native *S. uberis* CAMP factor. It was recognized by mouse antiserum directed against the purified *S. uberis* protein. All these data indicate that the recombinant CAMP factor generated from our expression system is similar to native *S. uberis* CAMP factor with respect to its physical and immunological properties, which should enable us to further study its mode of action *in vitro* and *in vivo* in the absence of other factors from its original host.

We have fully characterized the *S. uberis* CAMP factor gene and its promoter region. This will provide a sound basis for further study of the role of CAMP factor in virulence of *S. uberis*. A promising approach is to construct *cfu* mutants and test their ability to establish an infection after an experimental challenge. The most direct method for constructing *cfu* mutants is an insertional inactivation

strategy where an interior fragment of *cfu* can be subcloned into a shuttle vector and delivered into *S. uberis* to facilitate homologous recombination into the chromosome. If the mutant is unable to establish an infection or if the severity of the infection is significantly reduced relative to the wild-type strain, then we would conclude that the CAMP factor contributes to the virulence of this bacterium.

Amino acid sequence alignment showed that the *S. uberis* CAMP factor has 66% identity to the sequence of *S. agalactiae* CAMP factor. Also, monospecific antibodies raised against *S. uberis* CAMP factor cross-reacted with *S. agalactiae* CAMP factor. All these data indicate that there is significant similarity between the CAMP factors from these two organisms. Therefore, research on *S. uberis* CAMP factor may contribute to the study of *S. agalactiae*, another important pathogen of bovine mastitis.

The distribution of the CAMP factor gene in eight *S. uberis* strains was examined by Southern blotting with a *cfu*-specific probe. Three CAMP reaction positive *S. uberis* strains were shown to contain the *cfu* gene. Therefore, provided that CAMP factor is a protective antigen, it will only result in strain-specific immune protection as opposed to species-specific protection. In this case, it would be advantageous to generate a chimeric vaccine containing CAMP factor and other strain- or species-specific protective antigen(s). An even more attractive approach is the

possibility of developing a chimeric protein by constructing a chimeric gene containing the protective proteins from different strains.

During the course of our sequencing work on the CAMP factor gene, an *abp* gene capable of coding for a 277-residue polypeptide was identified. The deduced protein sequence has similarity to a number of polar amino acid and opine binding proteins of Gram negative bacteria. To assign a specific function to this protein, an *abp* mutant should be constructed and grown in minimal media containing different amino acids as the sole source of nitrogen. Differences in the growth curves of the mutant strain with respect to the wild-type strain would indicate that the *abp* gene product plays a role in the transport of a particular amino acid. However, no difference does not necessarily mean that this protein is not involved in amino acid transport since, as is the case for other organisms (Townsend and Wilkinson, 1992), where each amino acid might have more than one transport system.

The *abp* gene product was recognized by convalescent serum of *S. uberis*-infected cattle (data not shown), indicating that it is expressed during disease and cows respond to it immunologically. Since more transport systems besides the Abp-dependent one might exist for a particular amino acid, the interaction of Abp and its specific antibody might not be able to interfere with the transport of the

amino acid and thereby modulate bacterial growth. Therefore blockage of Abp's biological function by antibody does not appear to be able to confer protection to *S. uberis* infection. However Abp is surface-located and may be able to induce production of opsonizing antibodies and therefore, may still have vaccine potential.

It is well documented that many bacteria, especially Gram negative ones, are capable of obtaining iron from transferrin and lactoferrin (Herrington and Sparling, 1985; Mickelsen *et al.*, 1982). The acquisition of iron from these glycoproteins is essential for the establishment and dissemination of most bacterial infections. It is known that bacterial surface receptors mediate the transferrin- and lactoferrin-mediated processes (Cornelissen and Sparling, 1994; Williams and Griffiths, 1992), and thereafter are recognized as important virulence factors.

Our study demonstrated that *S. uberis* possesses a receptor for bovine lactoferrin, the major iron-binding glycoprotein in milk. The *S. uberis* Lbp gene is the first and only one that has been identified and characterized in Gram positive bacteria to date. Sequence analysis of this receptor gene did not show any structural similarity between *S. uberis* Lbp and the Lf or Tf receptors of Gram negative bacteria. Unlike Gram negative Lf or Tf receptors, which contain membrane-spanning regions functioning as gated channels for iron (Pettersson *et al.*, 1994b; Postle, 1993; Rutz *et al.*,

1992), *S. uberis* Lbp does not possess any regions with potential for the transit of free iron through the membrane. Provided that *S. uberis* can use lactoferrin as an iron source, a transport system containing more than one component may be responsible for iron uptake, in which Lbp binds lactoferrin from the surrounding medium and mediates the contact of lactoferrin with other component(s) functioning as an iron channel. The isolation and study of *lbp* mutants will demonstrate whether Lbp is essential for the acquisition of iron from lactoferrin. The availability of the cloned receptor gene will allow the construction of defined mutants that are deficient in Lbp.

If *S. uberis* does not use lactoferrin as an iron source, the existence of the lactoferrin binding protein on the bacterial surface must have other biological function(s). It has been shown that lactoferrin is capable of a direct bactericidal effect on a variety of microorganisms (Arnold *et al.*, 1980; 1981). The mechanism of this Lf-mediated bactericidal activity is not clear yet. A total shutdown of all cell functions with lactoferrin treatment, including the incorporation of deoxyribonucleic acid and ribonucleic acid precursors and amino acids, as well as a shutdown in the uptake or metabolism, or both, of carbohydrates has been observed (Arnold *et al.*, 1982). Therefore, it is tempting to speculate that the interaction between Lf and the target sites, rather than the specific Lf receptor, could trigger a

shutdown of cell function and ultimately lead to cell death; binding of lactoferrin with its specific receptor on the bacterial surface might shelter the target sites from lactoferrin binding, thereafter prevent lactoferrin-mediated bacterial killing. The resistance of a Lf receptor-containing strain and the sensitivity of the corresponding Lf receptor-negative mutant to Lf-mediated bactericidal effect will prove the above hypothesis.

The sequence and structural similarity of *S. uberis* Lbp to M proteins of group A streptococci makes this protein a new member of the M-like protein family. We have demonstrated that *S. uberis* does not produce Fc-receptors (data not shown). However, we do not know whether Lbp, like other M-like proteins, has fibrinogen- binding property. Also, whether *S. uberis* Lbp possesses antiphagocytic properties, a characteristic of M protein, needs to be investigated. The phagocytic resistance of *S. uberis* has been demonstrated *in vitro* and this resistance has been shown to be mediated by the capsule (Thomson and Leigh, 1993).

The presence of a *mga*-like gene adjacent to the gene for a potential virulence factor (the lactoferrin-binding protein) raises a number of interesting questions: (1) whether the *S. uberis mga* gene product, like its homolog in group A streptococci, is required for virulence of *S. uberis;* (2) whether *mga* regulates expression of *lbp;* (3) are there other virulence determinants coordinately-regulated by

mga; (4) what is the sensor component of the *mga* two-component regulatory system. Answers to these questions will contribute to our understanding of the virulence and pathogenicity of *S. uberis*.

Our preliminary animal experiments indicate that CAMP factor probably is a protective antigen which can induce an antibody response and prevent cell infiltration in the mammary gland of the challenged animals. The trial is currently being repeated at a higher challenge dose and this will further demonstrate the roles of both CAMP factor and LBP in the induction of protective immunity.

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