ROLE OF THE LACTOFERRIN-BINDING PROTEIN IN PATHOGENESIS OF

STREPTOCOCCUS UBERIS

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ABSTRACT

The pathogenesis of the bovine environmental pathogen *Streptococcus uberis* has been extensively studied, but it remains incompletely understood. The organism produces a surface protein capable of binding lactoferrin (Lbp), originally identified as an M-like streptococcal protein. We hypothesized that Lbp may play a role as a virulence factor. Structural similarity and amino acid sequence homology of Lbp to M-related proteins of Group A streptococci suggested a possible functional similarity between Lbp and M-like proteins, which are involved in evasion of host antibacterial defenses, adhesion to host epithelial cells and intracellular invasion by the bacteria. Alternatively, high-affinity binding of the abundant iron-chelating component of the host milk (lactoferrin) suggests that Lbp of *S. uberis* might play role in iron acquisition by the bacterium. Finally, Lbp might serve as a receptor for signal transduction in the bacterial cell or alter host cell signalling during infection, when the bacteria with surface-bound lactoferrin adhere to or invade the host epithelial cells.

In order to test the hypothesis that Lbp is a virulence factor of *S. uberis*, a mutant strain of *S. uberis* unable to express the lactoferrin-binding protein was generated and the role of the protein was studied in comparative analyses of the mutant and the parent strains.

The results of our study indicated that unlike many streptococcal M-like proteins, the lactoferrin-binding protein of *S. uberis* did not appear to play a role in overcoming host innate and acquired immune antibacterial responses. Both *S. uberis* and its *lbp* mutant were ingested by bovine blood neutrophils and were similar in their
ability to survive in fresh bovine blood regardless of the presence either of lactoferrin or of anti-Lbp antibodies.

Lbp did not promote bacterial adhesion to host epithelial cells and it was not essential for the internalization of the bacteria by host epithelial cells, since both *S. uberis* and *lbp* mutant were found capable of adhering, invading, intracellular survival and intracellular growth when the bacteria were co-cultured with bovine mammary epithelial cells. No significant differences in numbers of adherent or internalized bacteria per host cell were found between wild type and *lbp* mutant cells.

*S. uberis* requirements for iron were determined to be low and Lbp was not essential for iron acquisition by the organism from iron-saturated lactoferrin.

To study the role of Lbp in bacterial virulence during infection of bovine mammary glands, dairy cows in the second half of their lactation periods were challenged with the wild type *S. uberis* and with the *lbp* mutant. The results of in vivo infection suggested that expression of Lbp by the bacteria was not essential for colonization of the host mammary gland and that expression of Lbp was not associated with differences in severity of mastitis or with different levels of shedding of the bacteria by infected animals.

To study the role of Lbp in signal transduction, differential bacterial cellular protein phosphorylation in the presence of bovine lactoferrin was analyzed. Since no differences in protein phosphorylation profiles were detected between *S. uberis* and the *lbp* mutant, it was concluded that Lbp is probably not a part of a classical bacterial two-component signalling pathway. However, we demonstrated that the expression of host genes potentially involved in cell morphogenesis, motility and signal transduction was
regulated depending on the expression of Lbp by *S. uberis*. Down-regulation of the expression of selected host genes was verified by quantitative reverse transcription PCR. Putative iron responsive elements were identified in mRNA of several of these genes. Down-regulation of these genes in the cells overloaded with ferric iron was demonstrated by RT PCR. These results indicate that Lbp of *S. uberis* may interfere with host cellular signalling pathways by inducing perturbations in the cell iron status. This suggests that Lbp of *S. uberis* may be a virulence factor, playing a role in signal transduction or in the regulation of gene expression in host cells.
AKNOWLEDGEMENTS

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosinetriphosphate</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain-heart infusion</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>bLf</td>
<td>Bovine lactoferrin</td>
</tr>
<tr>
<td>CAMP</td>
<td>Lytic factor named after Christie, Atkins, and Munch-Peterseon</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Cfuf</td>
<td>CAMP factor of <em>S. uberis</em></td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CPM</td>
<td>Count per minute</td>
</tr>
<tr>
<td>CR3</td>
<td>Complement receptor 3</td>
</tr>
<tr>
<td>C4BP</td>
<td>C4 binding protein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco modified Eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDDA</td>
<td>Ethylenediamine-di(o-hydroxyphenylacetic) acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-diamine tetra acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin-Radixin-Moezin family protein</td>
</tr>
<tr>
<td>FH</td>
<td>Factor H of serum complement</td>
</tr>
<tr>
<td>FHL-1</td>
<td>Factor H-like protein 1</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde phosphate dehydrogenase</td>
</tr>
<tr>
<td>GAS</td>
<td>Group A <em>Streptococcus</em></td>
</tr>
<tr>
<td>GBS</td>
<td>Group B <em>Streptococcus</em></td>
</tr>
<tr>
<td>HVR</td>
<td>Hypervariable region</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRE</td>
<td>Iron responsive element</td>
</tr>
<tr>
<td>IRP</td>
<td>Iron regulating protein</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus kinase/signal transducer and activator of transcription</td>
</tr>
<tr>
<td>kJ/M</td>
<td>Kilojoule per Mole</td>
</tr>
<tr>
<td>Lbp</td>
<td>Lactoferrin-binding protein</td>
</tr>
<tr>
<td>Lf</td>
<td>Lactoferrin</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>Mga</td>
<td>Multiple gene regulator of Group A <em>Streptococcus</em></td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Matrix ribonucleic acid</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium salt</td>
</tr>
<tr>
<td>NTA</td>
<td>Nitrilotriacetic acid</td>
</tr>
<tr>
<td>OF</td>
<td>Opacity factor</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI 3-K</td>
<td>Phosphoinositidil-3 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SCC</td>
<td>Somatic cell count</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SIC</td>
<td>Streptococcal inhibitor of complement</td>
</tr>
<tr>
<td>Stk</td>
<td>Serine/threonine protein kinase</td>
</tr>
<tr>
<td>Tbp</td>
<td>Transferrin binding protein</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TPK</td>
<td>Tyrosine protein kinase</td>
</tr>
<tr>
<td>SIg</td>
<td>Secretory immunoglobulin</td>
</tr>
<tr>
<td>SLO</td>
<td>Streptolysin O</td>
</tr>
<tr>
<td>SpeB</td>
<td>Streptococcal pyrogenic endotoxin B</td>
</tr>
<tr>
<td>VC</td>
<td>Viable count</td>
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</tbody>
</table>
1 LITERATURE REVIEW

1.1 Streptococcal Virulence and Mastitis Manifestation

The genus *Streptococcus* includes pathogens causing infections of various severities ranging from simple symptomless colonization to deadly necrotizing fasciitis and streptococcal toxic shock-like syndrome. For the purpose of classification, all known streptococci were organized into four divisions by their haemolytic reactions, serologic group carbohydrate antigens, and fermentation and tolerance tests. The divisions are known as pyogenic, viridans, lactic and enterococci. Within each division, Lancefield serological typing was used for further classification. For example, the pyogenic division includes the β-hemolytic strains with carbohydrate group antigens A, B, C, E, F, G and through to V. Within each group, further serologic division of clinically relevant isolates may take place. For example, group A streptococci are serologically separated into M protein types. This classification system underwent multiple revisions and the phylogenetic classification based on the 16S rRNA sequence similarities was offered (Facklam, 2002). However, the Lancefield serological classification (Group A, B, C, through to V) remains the most widely used system by clinical laboratories, researchers and taxonomists (Table 1.1.1).

Among bacterial strains capable of causing disease in cattle, environmental streptococcal pathogens are becoming more prevalent, especially in herds that are free from contagious bacterial strains (Oliver, 1984). *S. uberis* is an important environmental
Table 1.1.1 Representatives of selected Lancefield serological groups of streptococci.

<table>
<thead>
<tr>
<th>Lancefield serogroup</th>
<th>Strains</th>
<th>Host species and disease (infection)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (GAS)</td>
<td>S. pyogenes</td>
<td>Human: respiratory tract, skin infection</td>
<td>Catalase-negative; β-haemolytic; CAMP-producing; Bacitracin sensitive</td>
</tr>
<tr>
<td>B (GBS)</td>
<td>S. agalactiae</td>
<td>Human: urogenital infections; Bovine: mastitis</td>
<td>CAMP-producing; β-haemolytic</td>
</tr>
<tr>
<td>C</td>
<td>S. equisimilis, S. equi</td>
<td>Swine, horse, dog: suppurative infections Human: pharyngitis Horse: strangles</td>
<td>α- or γ-hemolytic β-haemolytic</td>
</tr>
<tr>
<td>D</td>
<td>Enterococcus faecalis, E. faecium S. bovis, S. durans, S. avium</td>
<td>Human: appendicitis Human: meningitis Bovine: gastrointestinal infection;</td>
<td>Bile tolerant; high salt concentrations tolerant, α- or γ-haemolytic</td>
</tr>
<tr>
<td>A, C, D, F</td>
<td>S. anginosus</td>
<td>Human: abscess</td>
<td>β-haemolytic</td>
</tr>
<tr>
<td>G</td>
<td>S. canis</td>
<td>Dog: suppurative infection, necrotizing fasciitis</td>
<td>β-haemolytic</td>
</tr>
<tr>
<td>Not specified, Viridans</td>
<td>S. mutans; S. mitis; S. sanguis;</td>
<td>Human: tooth decay</td>
<td>α-haemolytic</td>
</tr>
<tr>
<td>K</td>
<td>S. salivarius;</td>
<td>Human: tooth decay</td>
<td>α- or γ-hemolytic</td>
</tr>
<tr>
<td>R</td>
<td>S. suis</td>
<td>Swine: meningitis</td>
<td>α-haemolytic</td>
</tr>
<tr>
<td>E, P, U, V</td>
<td>S. porcinus</td>
<td>Swine: lymph node infection</td>
<td>β-haemolytic</td>
</tr>
<tr>
<td>Not specified</td>
<td>S. pneumoniae</td>
<td>Human: meningitis, pneumonia</td>
<td>α-haemolytic;</td>
</tr>
<tr>
<td>Non-typable</td>
<td>S. uberis, S. parauberis</td>
<td>Bovine: mastitis</td>
<td>γ-haemolytic; CAMP-producing</td>
</tr>
</tbody>
</table>
veterinary pathogen responsible for a high proportion of bovine intramammary infections (Todhunter, 1995). Although the biology of this organism has been investigated due to its significant role as a veterinary pathogen, the reasons for its virulence are not completely understood. Generally, mastitis can be defined as an inflammatory response to intramammary infection. *S. uberis* may be capable of inducing an acute inflammation of the mammary gland (Vaarst, 1997), which may manifest itself as clinical mastitis where the milk is grossly abnormal and the affected quarter is inflamed, hot, swollen and painful. However, the majority of *S. uberis* intramammary infections are represented by the subclinical form of the disease (Zadoks, 2003), with no visual abnormalities in milk appearance or signs of inflammation, although subclinical mastitis is accompanied by decreased milk production, altered milk composition, increased number of neutrophils and the presence of viable bacteria in milk.

### 1.1.1 Inflammatory Response to Streptococcal Infection

The onset of a clinical mastitis takes place once the bacteria enter the mammary gland and an inflammatory reaction occurs (Pedersen, 2003) that is characterized by neutrophil recruitment into the mammary gland. A variety of inflammatory mediators are released into the milk and into the mammary tissue, attracting more neutrophils and causing their degranulation in an attempt to control the infection. This results in tissue damage and the release of more pro-inflammatory mediators, cytokines and antibacterial substances (Rambeaud, 2003). The severity of bovine mastitis was demonstrated to be associated with the stage of lactation at which the infection occurs (Cousins, 1980; Jones, 1989; Hill, 1988), the genetic background of the host (Weller,
1992) and the host’s general health, which is defined by the nutritional status (Barkema, 1999a) and the herd hygiene practices (Barkema, 1999b). Successful resolution of bacterial infection depends on the number of polymorphonuclear neutrophils (PMNs) (Sloth, 2003; Green, 2004) present both in milk and in circulation and on their functional activity i.e. the ability to ingest and kill the bacteria. Also, the course of infection and the severity of mastitis may be affected by previous exposure of the host to the pathogenic bacteria (Fang, 1998) and pathogen-specific immunity. For example, an elevated level of streptococcus-specific antibodies in vaccinated animals (Hill, 1994) was shown to be associated with reduced mastitis following challenge with *S. uberis*. Hence, both the host and bacterial factors (Casadevall, 1999) determine the course and the outcome of a bacterial infection. The dynamics of the intramammary production of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-8 (IL-8) in response to challenge with *S. uberis* has been studied (Rambeaud, 2003). However, the increase in somatic cell counts in milk was observed prior to the increased production of these cytokines. This suggests that the details of cytokine-regulated development and resolution of inflammation during streptococcal mastitis remain to be fully elucidated.

In subclinical mastitis, an elevated somatic cell count (SCC) was associated with decreased milk production (Banos, 1990; Simpson, 1995), indicating that the secretory epithelium may be partially damaged by the bacteria and/or recruited neutrophils. Compounds such as reactive oxygen species (ROS) (Bouhafs, 2000; Meli, 2003) and proteolytic enzymes (Prin-Mathieu, 2002) released from neutrophils can damage mammary secretory cells and reduce milk production. Some mechanisms of inflammation mediated by streptococci have been suggested from the study of GAS
infection in humans. For example, streptococcal M protein is able to form a complex with host fibrinogen and this complex activates host neutrophils upon binding to β-2-integrins. This results in a release of heparin-binding protein from the neutrophils. This protein is an inflammatory mediator that induces vascular permeability (Herwald, 2004), which is a key feature of inflammatory conditions.

1.1.2 Streptococcal Virulence Factors

Although the outcome of an infection is largely defined by host-specific factors (see above), the term “virulence” is usually attributed to the characteristics of a pathogen (Poulin, 1999). Streptococci express an array of virulence factors, usually cell surface or secreted components that may induce direct damage to the host, cause an adverse reaction or promote survival and dissemination of the organism.

Generally, streptococci are known for producing toxins. Extracellular pyrogenic exotoxins A, B, and C, and also the mitogenic factor exotoxin F of GAS play a role in toxic streptococcal shock syndrome by cross-linking MHC class II molecules (Scholl, 1989) with T-lymphocyte receptors (Tomai, 1992), resulting in non-specific activation of a massive number of T cells (Schlievert, 1979). T lymphocytes activated in an exotoxin-dependent manner produce large amounts of pro-inflammatory cytokines (Fast, 1989; Norrby-Teglund, 1994), which results in toxic shock. Exotoxin B of GAS is a cysteine protease (Kapur, 1993), which can exhibit pyrogenic properties by cleavage of the IL-1β precursor to release an active form of IL-1β – an inflammatory cytokine (Kapur, 1993). Superantigenic properties were also suggested for the M5 protein of GAS (Tomai, 1992). Streptolysins S and O represent other streptococcal toxins, both exhibiting proteolytic activity (Duncan, 1983). The cytolytic activity of
streptolysin S was implicated in the development of a soft tissue necrotizing infections (Betschel, 1998), while streptolysin O, in addition to its cytolytic activity, exhibits an immunomodulatory effect on various cell types (Bremm, 1985; Ruiz, 1998). GAS cell surface-associated C5a peptidase also can be classified as a proteolytic streptococcal toxin that reduces C5a-dependent chemotaxis by cleaving the C5a complement component (Cleary, 1992). Additionally, GAS produce a secreted co-haemolytic toxin CAMP factor that, together with staphylococcal β-toxin, synergistically lysed erythrocytes (Gase, 1999). CAMP activity was also demonstrated in products secreted by *S. uberis* (Skalka, 1980; Lopes, 1995) and the expression of a protein highly homologous to the *S. agalactiae* CAMP factor by *S. uberis* was demonstrated (Jiang, 1996a). It was suggested that a co-haemolytic toxin CAMP factor may be one of the *S. uberis* virulence factors (Jiang, 1996b).

Mimicry of the host antigens by components of the streptococcal cell may be important for pathogenesis and can cause autoimmune reactions. Such molecular mimicry can aid in the replication and dissemination of the organism throughout the host. Antibodies developed against streptococcal M proteins were demonstrated to be protective in a murine challenge model (Wittner, 1977), but it was suggested that antibodies to conformational epitopes of the M protein repeats in the coiled-coil region of the molecule can cross-react with host coiled-coil proteins such as laminin and myosin (Vashishtha, 1993), resulting in autoimmune reactions such as rheumatic fever and arthritis. Cross-reaction between α-helical coiled-coil tropomyosin of heart tissue and streptococcal M protein was experimentally demonstrated by ELISA and Western blotting (Fenderson, 1989). Protective anti-streptococcal antibodies against unique
epitopes of M proteins have been raised using synthetic oligopeptides as antigens (Beachey, 1984).

The survival of streptococci in non-immune fresh host blood is generally attributed to antiphagocytic properties of M or M-like proteins expressed on the surface of the streptococci. Inhibition of the internalization of the bacteria into host phagocytic cells has been studied and it was demonstrated that both strains expressing M protein and/or M-like protein H and mutant strains lacking expression of these proteins are effectively ingested by host neutrophils. Mutant strains lacking M protein were rapidly killed by host neutrophils, while the wild type strain was able to survive after being phagocytosed (Staali, 2003). It was suggested that bacterial evasion of host defenses may occur intracellularly and that survival inside human neutrophils may contribute to the pathogenesis of *S. pyogenes* and the recurrence of streptococcal infections.

*Streptococcus pyogenes* also produces a cysteine protease, which is able to cleave two fibrinogen-binding fragments of M1 protein and an IgG-binding NH₂-terminal fragment of protein H off the surface of the bacterial cell (Berge, 1995). Since M protein was implicated in adherence to host cells, it was suggested that such cleavage might promote bacterial dissemination, whereas the generation of soluble complexes between immunoglobulins and immunoglobulin-binding streptococcal surface proteins could play a role in the development of adverse immunological reactions.

It was also suggested that streptococcal C5a peptidase can digest a complement C5a component, blocking C5a-mediated migration of granulocytes to the site of streptococcal infection and in this way contributing to the streptococcal pathogenesis and virulence.
Although C5a peptidase may contribute to the streptococcal virulence, it was not required for exhibiting virulence by mucoid strains of GAS, as was demonstrated by studies of C5a knock-out mutants of a mucoid invasive strain in a murine infection model, where both acapsular and M protein-deficient mutants were partially attenuated (Ashbaugh, 1998).

It was demonstrated that both protein F1 and M protein of *S. pyogenes* promote efficient internalization of the bacteria by non-phagocytic cells in the presence of fibronectin (Jadoun, 1998). Streptococcal cysteine protease SpeB can effectively degrade cell-wall-attached fibronectin binding protein F1 complexed with host fibronectin in the presence of host serum, reducing bacterial internalization by host cells (Nyberg, 2004b), while M1 protein and protein H, two additional surface proteins of *S. pyogenes* that bind human plasma proteins, are resistant to this type of proteolytic degradation in the presence of their respective ligands.

The data described above indicate that it is streptococcal extracellular proteins that largely mediate virulence of streptococci. The present study is focused on the surface-associated lactoferrin-binding protein, which was suggested to be one of the virulence factors of *S. uberis* (Jiang, 1996b).

1.2 The Relationship Between Lbp of *S. uberis* and Related Proteins of Gram-positive and Gram-negative Bacteria

1.2.1 Cellular Localization and Membrane Topology of Lactoferrin-binding Proteins

Lactoferrin-binding proteins are produced by a variety of Gram-negative organisms (*Neisseria, Moraxella, Haemophilus*) and appear to play a central role in the
acquisition of iron from host lactoferrin. These proteins are surface-exposed, similar to the Lbp of *S. uberis* and M-like proteins of Gram-positive cocci. The differences between lactoferrin receptors of Gram-negative organisms and the Lbp of *S. uberis* may be attributed to membrane topology and protein structure. While lactoferrin receptors of Gram-negative bacteria are integral outer membrane proteins, Gram-positive cocci anchor their surface proteins in the cellular membrane since they lack outer cellular membrane and periplasm.

Lactoferrin- and transferrin-binding proteins of Gram-negative bacteria (LbpA, TbpA) form several hydrophilic loops extending outward from the outer membrane (Fig. 1.2.1.1), while hydrophobic regions of the proteins are integrated into the membrane lipid bilayer (Pajon, 1997). The amino terminal regions of the LbpA and TbpA of *Neisseria meningitidis* are oriented into the periplasm and they interact with the TonB complex required for ATP-dependent iron transport against the ion concentration gradient (Moeck, 1998; Larson, 2002). The outward-directed loops form the lactoferrin receptor, whose affinity to the ligand is significantly enhanced by the presence of a second protein LbpB (Bonnah, 1998; Biswas, 1999). According to existing models, the LbpB and TbpB are anchored in the outer membrane and are exposed to the extracellular environment. Unlike the LbpA of Gram-negative organisms, only relatively short amino acid sequences of Gram-positive M-like proteins and Lbp of *S. uberis* anchor the respective M-like proteins in the cell membrane. The Lbp of *S. uberis* resembles M-like proteins of Gram-positive bacteria. The common feature of these proteins is their coiled-coil structure (Fischetti, 1990; Navarre, 1999) and anchoring in the cellular membrane via a specific C-terminal
Figure 1.2.1.1 Membrane topology and structure of lactoferrin- and transferrin-binding proteins.

I, Putative structure of the Lbp of *S. uberis* Su-1 as a cell surface-exposed M-like protein forming a coiled-coil dimer. The structure of M-like proteins as suggested by Fischetti, 1989. II, Transferrin receptor homologous to lactoferrin receptors of Gram negative bacteria: an integral outer membrane protein TbpA, as proposed for gonococcal TbpA (Yost-Daljev, 2004), and a surface-exposed accessory protein TbpB, as adapted from the review on organization of transferrin and lactoferrin receptors of Gram-negative bacteria (Gray-Owen, 1996). Putative numbered hydrophilic loops of TbpA are exposed outwards, while the N-terminal domain interacting with TonB is oriented into a periplasmic space.
sequence (Heden, 1993; Schnitzler, 1995b). Except for a short membrane-bound C-terminal region and the adjacent cell wall spanning portion of the protein, M-like proteins, including the Lbp of *S. uberis*, are oriented outward from the bacterial cell surface, forming fibril-like structures composed of protein dimers due to coiled-coil interaction between two molecules (Fig. 1.2.1.1).

### 1.2.2 The Role of Lactoferrin-binding Proteins in Iron Acquisition

Iron, the fourth most abundant chemical element in the Earth’s crust, is vital for almost all living organisms with very rare exceptions. The role of iron in intracellular chemistry is versatile (Lill, 1999) and includes being a co-factor for enzymes of the tricarboxylic acid cycle (Oexle, 1999), DNA biosynthesis (Jordan, 1998), gene regulation (McHugh, 2003), electron transfer, respiration (Yoon, 2003), oxygen transport (Barnes, 1973), nitrogen fixation, hydrogen production and consumption. This variety of functions is due to the ability of iron to easily accept its two main oxygenized states, ferrous (2+) and ferric (3+). The redox potentials of these two states range from +800 for ferrous form, easily oxidized by molecular oxygen, to -300 for aqueous ferric form, which can be reduced by common reductases such as pyridine dinucleotides. This wide range of redox potentials acquired at physiological conditions makes iron effective in electron transfer reactions and determines its role as a co-factor for an array of enzymes. At the same time, in the presence of molecular oxygen, oxidation of ferrous iron can trigger the formation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals, which are potentially dangerous for living cells (Dunning, 1998). The damage incurred by ROS to the cell is commonly known as oxidative stress. Prevention of oxidative stress requires effective and sensitive
regulatory mechanisms, allowing the cell to maintain the intracellular iron concentration at a level sufficient to support vital functions for which iron is important and, at the same time, preventing an intracellular iron overload, as well as maintaining an effective cellular repair system(s) to repair the damage resulting from oxidative stress. Of the two main oxygenized iron forms, ferrous iron is relatively soluble (1 mM) when its autoxidation is prevented by low oxygen tension while ferric iron has very low solubility ($10^{-17}$ to $10^{-12}$ M at pH 7) (Rose, 2003), and the presence of molecular oxygen favors the prevalence of ferric iron. In mammals, approximately 0.1% of the entire organism's iron circulates in the plasma as an exchangeable pool (Ponka, 1999). Essentially all circulating plasma iron normally is bound to transferrin. In mucosal secretions, iron is also bound by lactoferrin, a protein related to and homologous to transferrins. However, in milk only approximately 30% of iron is bound by lactoferrin, and the rest can be found within lipoid granules and chelated by low molecular weight compounds such as citrate. This chelation potentially can serve to render iron soluble under physiologic conditions, to prevent iron-mediated free radical toxicity, and to facilitate iron transport into cells. Pathogenic bacteria have developed effective iron-scavenging systems, regulated by an iron-responsive regulatory network that coordinates gene expression according to iron availability. These bacterial systems include synthesis, secretion and uptake of low molecular weight/high Fe$^{3+}$ affinity compounds (siderophores); utilization of host iron protoporphyrins; direct use of host transferrin and/or lactoferrin as a source of iron (Charland, 1995; Moeck, 1998); as well as gaining an access to intracellular host iron content (Larson, 2002).
Production and uptake of siderophores has been studied mostly in Gram-negative bacteria and there is no direct evidence of siderophore utilization by streptococci. Iron protoporphyrins can serve as a source of nutritional iron for *Streptococcus pyogenes* (Eichenbaum, 1996a), which expresses a haemolytic phenotype and is capable of intracellular invasion (both characteristics consistent with utilization of intracellular haemoproteins). Protoporphyrin-binding proteins of *S. pyogenes* were identified (Lei, 2002; Lei, 2003) and an operon involved in haem iron uptake was characterized (Bates, 2003). However, an additional route of iron acquisition by *S. pyogenes* was suggested, since the growth of the mutant strain lacking the surface haemoglobin receptor was not inhibited in the iron depleted medium supplemented with haemoglobin, whole blood, or ferric citrate. Utilization of haemin as an iron source was demonstrated for the human pathogen *S. pneumoniae* (Tai, 1993) and a mutant defective in haemin utilization was found to be attenuated in experimental animals.

Although iron-saturated transferrin, lactoferrin or cytochrome c did not support the growth of *S. pyogenes* (Eichenbaum, 1996a) or *S. pneumoniae* (Tai, 1993) in an iron-depleted medium, host lactoferrin was found to be bound by human strain *S. pneumoniae* surface protein A (Hakansson, 2001). Binding of host lactoferrin by distinct protein(s) of bovine pathogen *S. uberis* strains UT888, UT366, UT754, UT102, ATCC 13387 (Fang, 1999) and Su-1 (Jiang, 1996b; Moshynskyy, 2003) has been demonstrated, although the role of these proteins in iron acquisition has not been studied.

### 1.2.3 Secondary Structure of M-like Proteins

The M-related streptococcal proteins share a number of structural
characteristics. Typically, these proteins contain a charged N-terminal domain, followed by a variable region composed of several repeats (Gubbe, 1997; Meehan, 2002). The repeat regions form an α-helix, and the α-helix regions assume a coiled-coil structure in a homodimer.

One of the suggested classifications of M-related proteins, distinguishing true M proteins from M-like proteins, is based on the type of repeats present in the molecule (Cedervall, 1997). The M protein family can be divided into two classes, A and C, according to the type of repeat region found. The types of known repeats are A, B, C and D (Fig. 1.2.3.1), which in some cases are separated by inter-repeat regions. Within these repeats, smaller 7 amino acid (heptad) repeats can be observed (Fischetti, 1988). It is thought that this heptad organization contributes to the α-helix formation and, on a higher level, to organization of coiled-coil dimers. If the heptad pattern is characterized by an amino acid distribution not optimal for coiled-coil formation, the M-like protein dimer may be unstable at elevated temperatures (Nilson, 1995). The stability of coiled-coil dimers formed by M-related proteins depends on the type of repeat (A or C) in the central repeated region of the molecule. The differences in temperature stabilities of coiled-coil structures, as well as in their abilities to bind respective ligands, were demonstrated for Mrp4 (class A) and Arp4 (class C) proteins, both of which are expressed by Group A *Streptococcus* (Cedervall, 1997). The coiled-coil conformation may also be stabilized by the bound ligand (Gubbe, 1997).
Coiled-coil structure formed by two $\alpha$-helical molecules (I), a corresponding primary protein structure (II), as described for M6 protein of GAS (Fischetti, 1989); III, a predicted structure of the lactoferrin-binding protein of *S. uberis* (Jiang, 1996b).

The C-terminal conserved region of M-related proteins consists of a membrane-anchoring region and a cell wall spanning domain. Cell wall-associated region may form a complex structure with the glycolipids of the cell wall, responsible for bacterial surface hydrophobicity and possibly mediating streptococcal adherence to the host epithelial cells (Okada, 1995). A, B, C and D types of repeats are determined as at [http://blocks.fhcrc.org/blocks-bin/getblock.sh?IPB003345](http://blocks.fhcrc.org/blocks-bin/getblock.sh?IPB003345).
1.2.4 Genetic Organization and Regulation of the Gene Expression

Regulation of M protein expression in GAS is attributed to the open reading frame located 72 base pairs upstream of its gene. As was observed, the region more than 1000 base pairs upstream of the promoter region of the gene coding for M protein in group A streptococcus is required for full expression of M protein (Caparon, 1987; Robbins, 1987). This region was demonstrated to contain a gene designated mga, formerly known as mry (Perez-Casal, 1991) or vir. Later it was established that Mga is a common positive regulator of a range of streptococcal genes and is required for expression of several streptococcal virulence factors including C5a peptidase (McIver, 1995; Kihlberg, 1995), M protein (Caparon, 1987; Robbins, 1987; McIver, 1995), M-like proteins (Kihlberg, 1995), streptococcal inhibitor of complement (SIC) (Kihlberg, 1995) and itself (McIver, 1995). The set of genes regulated by Mga is commonly referred to as the Mga regulon. Several helix-turn-helix motifs normally associated with interactions with gene promoter regions were identified in the Mga protein (McIver, 2002). A study of the mga mutants with altered helix-turn-helix motifs demonstrated that expression of the gene coding for M protein correlated directly to the DNA-binding capability of Mga. Expression of genes in the regulon is regulated in response to environmental stimuli (Caparon, 1992; McIver, 1995) and possibly depends on the growth stage of the bacterial culture (McIver, 1997). Consistent with this, M protein expression was upregulated in response to elevated CO$_2$ concentration (Caparon, 1992) and both Mga and M protein underwent phase variation in S. pyogenes (Bormann, 1997).

The M proteins family of Streptococcus pyogenes contains three related
proteins: Emm (class I and II), Mrp (FcrA), and Enn. The genes coding for these proteins are located in a locus of the *S. pyogenes* chromosome between the genes *mga* and *scpA*. It was also noted, that the composition of these gene clusters generally parallels the ability of strains to express the serum opacity factor by human pathogens of Group A streptococci (Whatmore, 1994). Serum opacity factor-positive (OF+) strains of *S. pyogenes* usually contain all three genes in their Mga regulons, while OF- strains can contain from 1 to 3 M-like protein genes.

The presence of an open reading frame homologous to *mga* of GAS has been demonstrated in the region upstream of the gene coding for the Lbp of *S. uberis* (Jiang, 1996b). Such genetic organization is consistent with Lbp being a streptococcal M-like protein.

### 1.3 Functional Studies of Streptococcal M-related Proteins

Lbp of *S. uberis*, as well as lactoferrin-binding proteins of Gram-negative bacteria, bind their respective host lactoferrins in a species-specific manner. The difference seems to be in the biological function of these proteins. While LbpA and LbpB of Gram-negative bacteria are involved in the acquisition of ionic iron from ferrated host lactoferrin (Schryvers, 1998), the role of Lbp of *S. uberis* remains to be determined. Lbps of Gram-negative bacteria do not discriminate between apo- and holo-forms of the host lactoferrin (Schryvers 1988). Therefore, binding of both forms of bovine lactoferrin with similar efficiency (Moshynskyy, 2003) by the Lbp of *S. uberis* does not exclude a possible role of the Lbp of *S. uberis* in iron acquisition.

The streptococcal M and M-like proteins related to Lbp of *S. uberis* seem to perform multiple functions and are able to bind a variety of ligands. Bindings of these
Table 1.3.1. Ligands and suggested functions of some streptococcal M-like proteins.

<table>
<thead>
<tr>
<th>Streptococcal M- or M-like protein identification</th>
<th>Identified in strain</th>
<th>Ligand</th>
<th>Suggested role in pathogenesis</th>
<th>Host species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>S. pyogenes (GAS)</td>
<td>Not specified</td>
<td>Invasion</td>
<td>Human</td>
<td>(Purushothaman, 2003)</td>
</tr>
<tr>
<td>M1</td>
<td>S. pyogenes (GAS)</td>
<td>Fibronectin</td>
<td>Internalization</td>
<td>Human</td>
<td>(Cue, 2001)</td>
</tr>
<tr>
<td>M6</td>
<td>S. pyogenes (GAS)</td>
<td>Undetermined host cell surface protein</td>
<td>Adherence</td>
<td>Human</td>
<td>(Wang, 1994)</td>
</tr>
<tr>
<td>M6</td>
<td>S. pyogenes (GAS)</td>
<td>CD46</td>
<td>Inhibition of complement activation</td>
<td>Human</td>
<td>(Giannakis, 2002)</td>
</tr>
<tr>
<td>M6</td>
<td>S. pyogenes (GAS)</td>
<td>Not specified</td>
<td>Resistance to phagocytosis; autoimmunity</td>
<td>Human</td>
<td>(Perez-Casal, 1992; Quinn, 2001)</td>
</tr>
<tr>
<td>M5, M6, M19, M24, M28</td>
<td>S. pyogenes (GAS)</td>
<td>Serum factor H</td>
<td>Inhibition of complement activation</td>
<td>Human</td>
<td>(Horstmann, 1988)</td>
</tr>
<tr>
<td>M5</td>
<td>S. pyogenes (GAS)</td>
<td>Not specified</td>
<td>Molecular mimicry</td>
<td>Human</td>
<td>(Dale, 1985)</td>
</tr>
<tr>
<td>M24</td>
<td>S. pyogenes (GAS)</td>
<td>Unknown</td>
<td>Adherence; Resistance to bactericidal effect of host blood</td>
<td>Human</td>
<td>(Courtney, 1994)</td>
</tr>
<tr>
<td>M3</td>
<td>S. pyogenes (GAS)</td>
<td>Fibrinogen, albumin and fibronectin</td>
<td>Resistance to phagocytosis; adherence; internalization</td>
<td>Human</td>
<td>(Schmidt, 1993; Eyal, 2003)</td>
</tr>
<tr>
<td>M3</td>
<td>S. pyogenes (GAS)</td>
<td>Not specified</td>
<td>Adherence; internalization</td>
<td>Human</td>
<td>(Ellen, 1972; Eyal, 2003)</td>
</tr>
<tr>
<td>M protein</td>
<td><em>S. pyogenes</em> (GAS)</td>
<td>Fibrinogen</td>
<td>Activation of neutrophils</td>
<td>Human</td>
<td>(Herwald, 2004)</td>
</tr>
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<td>---</td>
</tr>
<tr>
<td>M protein</td>
<td><em>S. pyogenes</em> (GAS)</td>
<td>Glycosaminoglycan</td>
<td>Adhesion</td>
<td>Human</td>
<td>(Frick, 2003)</td>
</tr>
<tr>
<td>M protein</td>
<td><em>S. pyogenes</em> (GAS)</td>
<td>Mucin</td>
<td>Adhesion</td>
<td>Human</td>
<td>(Ryan, 2001)</td>
</tr>
<tr>
<td>Unknown</td>
<td><em>S. uberis</em></td>
<td>Glycosaminoglycan</td>
<td>Adhesion; invasion</td>
<td>Bovine</td>
<td>(Almeida, 1999a)</td>
</tr>
<tr>
<td>Unknown</td>
<td><em>S. uberis</em></td>
<td>Lactoferrin</td>
<td>Adhesion</td>
<td>Bovine</td>
<td>(Fang, 2000)</td>
</tr>
<tr>
<td>Lbp</td>
<td><em>S. uberis</em></td>
<td>Lactoferrin</td>
<td>Signal transduction</td>
<td>Bovine</td>
<td>This study</td>
</tr>
<tr>
<td>MIG</td>
<td><em>S. dysgalactiae</em></td>
<td>IgG, α-2-macroglobulin, IgA</td>
<td>Resistance to phagocytosis; internalization</td>
<td>Bovine</td>
<td>(Song, 2001; Song, 2004)</td>
</tr>
<tr>
<td>Arp4</td>
<td><em>S. pyogenes</em> (GAS)</td>
<td>IgA</td>
<td>Unknown</td>
<td>Human</td>
<td>(Husmann, 1995)</td>
</tr>
<tr>
<td>Mrp</td>
<td><em>S. pyogenes</em> (GAS)</td>
<td>IgG, IgA</td>
<td>Resistance to phagocytosis</td>
<td>Human</td>
<td>(Podbielski, 1996b)</td>
</tr>
<tr>
<td>M22</td>
<td><em>S. pyogenes</em> (GAS)</td>
<td>C4b-binding protein (C4BP), IgA</td>
<td>Resistance to phagocytosis</td>
<td>Human</td>
<td>(Carlsson, 2003)</td>
</tr>
<tr>
<td>C3 binding protein</td>
<td><em>S. pneumoniae</em></td>
<td>C3 complement component</td>
<td>Resistance to complement-mediated immunity</td>
<td>Human</td>
<td>(Cheng, 2000)</td>
</tr>
</tbody>
</table>
ligands were implicated in resistance to host immunity, molecular mimicry, adhesion and invasion of host cells (Table 1.3.1).

Historically, streptococcal surface antigens conferring resistance of the bacteria to phagocytosis by peripheral blood polymorphonuclear leukocytes were defined as streptococcal M proteins (Bessen, 1992). Later, when surface molecules sharing structural characteristics and amino acid sequence homology with M proteins, but not directly responsible for antiphagocytic properties, were identified and characterized, they were designated as M-like proteins. Finally, some M-like proteins were described as components allowing streptococci to withstand complement attack or to survive within phagocytic cells after being ingested. In contrast, M proteins also were shown to play roles in other functions during streptococcal infection: they were implicated in adherence of streptococci to host cells (Courtney, 1992), in invasion of the host cells (Eyal, 2003) and in regulation of gene expression by the host cells in response to streptococcal infection (Herwald, 2004). Selected streptococcal M and M-like proteins, their respective ligands and suggested functions are summarized in Table 1.3.1.

1.3.1 Resistance to Host Innate Immunity.

Before a pathogen can establish infection in a nonimmune host, it must evade the host’s innate defenses. For extracellular bacterial pathogens it is often important to evade phagocytosis by neutrophils, which are rapidly recruited to the site of initial infection. In the case of the bovine mastitis pathogen *S. uberis*, the ability of a strain to resist host antimicrobial defense factors was correlated to ability to colonize the host and to cause clinical manifestation of mastitis (Leigh, 1990). Both ingestion and killing of the bacteria by phagocytic cells are promoted by activation of complement via either
the classical or the alternative pathway. Modulation of the process of ingestion of the bacteria by host phagocytes, resistance to killing after being internalized, as well as regulation of the serum complement represent important characteristics of streptococcal pathogenesis.

1.3.2 Phagocytosis and Phagocytic Killing

The antiphagocytic M protein of *Streptococcus pyogenes* was considered to be a key factor in its resistance to phagocytosis by leukocytes of the host peripheral blood (Morris, 1955). It was demonstrated by direct bactericidal assays of GAS *emm* and *mrp* mutants that, if present, both *mrp* and *emm* gene products contribute to resistance to phagocytosis of GAS by decreasing bacterial binding to granulocytes (Podbielski, 1996b), while reintroduction of the gene coding for the wild type protein into a mutant strain could restore the ability of *S. pyogenes* to resist phagocytosis by host polymorphonuclear leukocytes (Perez-Casal, 1992).

Antibodies directed against the hyper variable region (HVR) of M protein block its antiphagocytic property (Beachey, 1987), which means that this region may be important in conferring resistance to phagocytosis.

Capsular polysaccharide provides resistance to opsonophagocytic killing of bacteria, as was demonstrated for *Enterococcus faecalis* (Hancock, 2002). Resistance to phagocytic killing *in vitro* of a mucoid strain of Group A *Streptococcus* associated with an outbreak of rheumatic fever depended on the ability to produce hyaluronic acid capsule if the level of expression of M protein remained unaltered (Wessels, 1991). Also, it was demonstrated that GAS could use M protein and hyaluronic acid capsule in evasion of opsonophagocytotic killing *in vivo* in a murine subcutaneous infection model.
(Ashbaugh, 1998). However, an ability to produce hyaluronic acid capsule did not affect infectivity or virulence of *S. uberis* (Field, 2003). For this reason, the role of M- or M-like protein(s) of *S. uberis* in resistance to the host antimicrobial defenses may be an interesting aspect of pathogenesis of this organism.

Due to the presence of a thick cell wall, the bacterial membrane is poorly accessible to the membrane attack complex formed as a result of complement activation. However, deposition of complement on bacterial cells promotes their ingestion by professional host phagocytes. For this reason, inhibition of complement activation on the surface of the bacterial cell potentially might be beneficial for the bacteria during infection. As was demonstrated by mutagenesis of the gene coding for M6 protein of *S. pyogenes*, the C repeat region takes part in binding of human complement factor H to the streptococcal surface, although a factor distinct from the C-repeat region of M6 protein was implicated in *S. pyogenes* ability to resist phagocytosis (Perez-Casal, 1995). Two distinct regions of M22 protein of *S. pyogenes* were implicated in binding of C4BP regulator of complement activation and host immunoglobulins, cooperating in providing resistance to opsonization by both immune and naïve host sera (Carlsson, 2003). C3-Binding protein from *Streptococcus pneumoniae* has been reported as a component of the pneumococcal cell wall, important for inhibition of host complement activation and enhancing resistance of the bacteria to the complement-mediated killing (Cheng, 2000).

Tissue phagocytes represent an important component of the host innate immunity and link the innate and adaptive immune systems (Underhill, 2002). They ingest a broad range of microbial pathogens through recognition of specific structures
such as pathogen-associated molecular patterns (Janeway, 2002) and also receptors that recognize bacteria coated with serum opsonins. Phagocytosis of bacteria activates various cell-surface receptors on phagocytes and triggers corresponding signal transduction pathways (Greenberg, 1999). These pathways differ by activation of downstream events which include killing of the ingested microorganism(s) (Takahashi, 1995), cytokine production (Albanyan, 2000), antigen presentation (von Delwig, 2002), and induction of apoptosis (Ali, 2003). Bacterial pathogens have evolved diverse mechanisms of defense to combat the innate immune system including resistance to the killing by phagocytic cells, which is an important component of the defense against bacterial infections in a nonimmune host. Although the details of the antiphagocytic resistance of Gram-positive bacteria remain to be elucidated, certain bacterial components related to such resistance have been identified. Production of streptolysin O (SLO) by GAS results in a cytotoxic effect and lysis of human keratinocytes and polymorphonuclear cells, which greatly impairs polymorphonuclear leukocyte killing of GAS in vitro, especially when SLO is expressed by an acapsular GAS mutant (Sierig, 2003). Although tissue resident macrophages ingest opsonized GBS, intracellular bacteria are killed only if macrophages are activated by interferon γ (IFN-γ) or by granulocyte-macrophage colony-stimulating factor (Marodi, 2000). It was also suggested that GBS might impair microbiocidal systems in macrophages by inhibiting protein kinase C (PKC)-dependent signal transduction pathways, preventing IFN-γ- and LPS-dependent macrophage activation (Cornacchione, 1998). Comparison of the fate of opsonized and non-opsonized GBS ingested by a murine macrophage-like cell line J774 demonstrated that human serum containing anti-GBS antibodies does not affect
bacterial entry but significantly reduces the intracellular survival of GBS, suggesting that GBS are able to enter and persist efficiently in macrophages by evading intracellular antibacterial activities commonly associated with opsonin-mediated uptake (Valenti-Weigand, 1996).

For *S. uberis*, it was established that although resistance to phagocytosis and to killing by peripheral blood polymorphonuclear leukocytes can vary between strains (Leigh, 1990), this does not always correlate with resistance of *S. uberis* to phagocytosis by macrophages (Grant, 1997).

A distinct strategy for avoidance of phagocytic killing was identified in Group B streptococcus (GBS): a product of the *ponA* gene, encoding the penicillin binding protein PBP1a, promotes resistance to phagocytic killing independent of capsular polysaccharide and does not affect C3 deposition on GBS (Jones, 2003).

These diverse mechanisms of resistance to killing by host phagocytes represent an important aspect of streptococcal colonization of the host.

### 1.3.3 Role of M Protein in Molecular Mimicry

Molecular mimicry between a pathogen and its host has been proposed as a mechanism that may influence development of autoimmunity as well as host unresponsiveness to a bacterial infection.

Immunologic cross-reactivity between streptococcal M protein and heart muscle myosin (Dale, 1985) was implicated in the development of myosin-specific antibodies in individuals with streptococcal throat infection (Cunningham, 1988). Seven amino acid residue repeats, extending through the M protein, are largely responsible for the formation of the α-helical coiled-coil structure (Fischetti, 1988) that resembles the
structure of the heart myosin. The latter protein also contains heptapeptide repeats and forms \( \alpha \)-helical coiled-coils (McLachlan, 1983). Being an \( \alpha \)-helical coiled-coil protein, streptococcal M protein structurally and immunologically mimics the rod region of myosin (Cunningham, 1989). During streptococcal infection, such mimicry may result in production of anti-streptococcal antibodies that cross-react with host cardiac tissues (Cunningham, 1988; Dale and Beachey, 1986). The structural similarity between M protein and cardiac myosin is significant enough to produce inflammatory disease due to autoimmune mechanisms activated during streptococcal infection (Quinn, 2001). The cross-reacting antibodies may react to autoantigens such as cardiac myosin, inducing rheumatic-like inflammatory heart disease (Kodama, 1990). These and similar observations (Quinn, 1998) lead to the formulating of the hypothesis that a bacterial antigen can break immune tolerance \textit{in vivo} and cause a disease of an autoimmune origin. This makes streptococcal M proteins central to the mimicking of host antigens and responsible for a significant proportion of adverse immunopathologic reactions to streptococcal infection.

1.3.4 Role of M Proteins in Adhesion and Invasion of Host Epithelial Cells

Bacterial adherence to host tissues is considered a key step in the establishment of infection by a successful pathogen (Frost, 1975; Reynolds, 1987; Rikitomi, 1997). Infection of a mammary gland by bacteria during lactation implies that the bacteria must be able either to effectively adhere to the gland tissue or to maintain a reproduction rate allowing the organism to perpetuate itself at the site of infection.

Invasion of the host cells is often considered to be beneficial for a bacterial pathogen because it allows better access to the intracellular sources of nutrients and an
opportunity to evade host immune surveillance. The question of whether streptococcal adherence is a process of two consecutive steps or of several independent adhesion events remains open. The adherence of *Streptococcus faecium* to the avian intestinal epithelium depends on two bacterial surface components (Fuller, 1981), suggesting that the adherence may occur in more than one step: first an initial contact between the bacterium and the host cell is made, resulting in weak adherence. Following that, a secondary, stronger attachment occurs, resulting in higher affinity adherence of the bacterium to the respective host cell (Hasty, 1996). The competitive inhibition of adhesion of *S. pyogenes* by lipoteichoic acid (weak inhibition) and by recombinant fibronectin binding protein (strong inhibition) is consistent with a two-step adherence process (Talay, 1992). The consensus model is that streptococci use multiple adhesins to attach to host cells and the types of adhesins expressed by a particular strain determines its tissue specificity (Courtney, 2002). It is now widely accepted that streptococci adhere to epithelial cells and colonize mucosal surfaces in a highly specific manner. However, in many cases the details of adhesion and invasion of many streptococcal strains, as well as of bacterial adhesins and their ligands, remain to be elucidated. Examples of streptococcal and host components implicated in adhesion and intracellular invasion are listed in Table 1.3.4.1.

Bovine lactoferrin added to the culture medium was demonstrated to promote adhesion of three *S. uberis* strains to cultured bovine epithelial cells, while pre-treatment of the bacteria with lactoferrin enhanced adherence of two strains and retarded adherence of the third (Fang, 2000). This suggests that although bovine lactoferrin can potentially promote the adherence of *S. uberis* to host epithelial cells,
Table 1.3.4.1. Streptococcal factors implicated in adhesion to and invasion of host epithelial cells.

<table>
<thead>
<tr>
<th>Species</th>
<th>Streptococcal factor</th>
<th>Host’s component(s)</th>
<th>Results of interaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pyogenes</em></td>
<td>Lipoteichoic acid</td>
<td>Not specified</td>
<td>Adhesion, invasion</td>
<td>(Sela, 2000)</td>
</tr>
<tr>
<td><em>S. pyogenes</em></td>
<td>M protein</td>
<td>Glycosaminoglycans CD46</td>
<td>Adhesion</td>
<td>(Frick, 2003; Okada, 1995)</td>
</tr>
<tr>
<td><em>S. pyogenes</em></td>
<td>R28 protein</td>
<td>Not specified</td>
<td>Adhesion, invasion</td>
<td>(Stalhammar-Carlemalm, 1999)</td>
</tr>
<tr>
<td><em>S. pyogenes</em></td>
<td>Fibronectin-binding protein; protein F1</td>
<td>Fibronectin</td>
<td>Adhesion, invasion</td>
<td>(Courtney, 1996; Molinari, 1997)</td>
</tr>
<tr>
<td><em>S. pyogenes</em></td>
<td>Not specified</td>
<td>Vitronectin</td>
<td>Adhesion</td>
<td>(Valentin-Weigand, 1988)</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>Choline-binding protein</td>
<td>Polymeric immunoglobulin receptor</td>
<td>Adhesion, invasion</td>
<td>(Lu, 2003)</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>PspA</td>
<td>Serum complement, secretory IgA, polymeric immunoglobulin receptor</td>
<td>Adhesion, invasion</td>
<td>(Rosenow, 1997; Brock, 2002)</td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>Laminin-binding protein</td>
<td>Laminin</td>
<td>Adhesion</td>
<td>(Spellerberg, 1999)</td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>Surface protein of group B <em>Streptococcus</em> 1 (spb1)</td>
<td>Not specified</td>
<td>Invasion</td>
<td>(Adderson, 2003)</td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>C5a peptidase</td>
<td>Fibronectin</td>
<td>Adhesion, invasion</td>
<td>(Cheng, 2002; Molinari, 1997)</td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>FbsA (fibrinogen receptor)</td>
<td>Not specified, possibly fibrinogen</td>
<td>Adhesion</td>
<td>(Schubert, 2004)</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>Not specified</td>
<td>Lactoferrin</td>
<td>Adhesion</td>
<td>(Fang, 2000)</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>Not specified</td>
<td>Glycosaminoglycans (heparin sulfate), milk proteins</td>
<td>Adhesion</td>
<td>(Almeida, 2003)</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>Not specified</td>
<td>Laminin, fibrinogen, fibronectin, collagen</td>
<td>Adhesion, invasion</td>
<td>(Almeida, 1999b)</td>
</tr>
<tr>
<td><em>S. dysgalactiae</em></td>
<td>MIG</td>
<td>Not specified</td>
<td>Decreased internalization</td>
<td>(Song, 2004)</td>
</tr>
</tbody>
</table>
other strain-specific bacterial factors may also be involved. The hypothesis that lactoferrin may serve as a bridging molecule between a host cell and a bacterial cell also implies that they both express their respective surface lactoferrin receptors. Therefore, effective streptococcal adhesion to the host epithelial cells depends not only on the expression of the required adhesin(s) by the bacterium but also on the expression of a proper receptor by the host cells. Affinity of the host cell receptor to the bacterial adhesin is likely to determine the host specificity and tissue tropism of the bacteria.

The ability of bacteria to sense their surroundings and to rapidly respond to changes is crucial for bacterial survival and adaptation within the host (Finlay, 1997). Correspondingly, expression of the bacterial adhesins can be modulated in response to the environmental changes and regulated by bacterial two-component regulatory systems. For example, expression of the fibronectin-binding protein F2 in S. pyogenes is elevated in aerobic conditions (VanHeyningen, 1993) and regulated by a negative regulator nra (Podbielski, 1999). Examination by electron microscopy demonstrated that the nra mutant exhibited higher adherence and internalization rates than did the corresponding wild type strain.

Overall, it can be concluded that streptococcal adherence to epithelial cells at the site of infection is a dynamic process determined by both host tissue and bacterial factors.

Invasion of GBS involves intimate attachment of streptococcal chains, engulfment of the adherent bacteria, entry of the bacteria and formation of membrane-bound vacuoles in which most of the intracellular streptococci reside. Streptococcal invasion of the host epithelial cells is mediated by specialized proteinaceous surface
components, which also may or may not play a role in adhesion. Among others, M proteins were suggested as streptococcal invasins (Table 1.3.4.1). Both M6 protein and protein F1, which act as adhesins of GAS, are required for an efficient invasion of host epithelial cells. It was demonstrated that entry of GAS into cultured cell requires the occupancy of protein F1 by fibronectin (Ozeri, 1998) and that expression of protein F1 may result in invasiveness of a normally non-invasive strain of GAS (Jadoun, 1997).

Invasiveness of Group B Streptococcus (GBS) is regulated by the growth rate of the culture (Malin, 2001) and may depend on the expression of a certain gene(s) that is expressed in a particular phase of bacterial growth.

Although higher adherence to host epithelium potentially could lead to a higher rate of intracellular invasion, invasiveness does not necessarily directly depend on the adhesiveness of streptococci (Adderson, 2003).

There is no consensus as to how the internalization of a Streptococcus cell or chain occurs. For *S. agalactiae* (GBS) it was demonstrated that the host cell microvilli wrap around the bacterial cell or groups of cells, resulting in eventual entry of the bacterium into the cell (Tyrrell, 2002). In contrast, during internalization of *S. pyogenes* (GAS), small cavities in the host cell membrane are formed close to adherent streptococci and the following invagination of a host cell membrane results in internalization of the bacterium (Rohde, 2003).

The ability of a Streptococcus to invade a host cell depends not only on the expression of proper invasins by the bacteria but also on the “competence” of a host cell and is probably defined by the cell lineage, its metabolic activity and a repertoire of the surface receptors. Invasion of GBS requires activation of the eukaryotic actin
microfilament system involving, at least partially, protein kinase signal transduction pathways. Invasion is inhibited in a dose-dependent manner by decreasing extracellular Ca$^{2+}$ levels as well as by substances known to interfere with eukaryotic calcium regulatory systems (Valentin-Weigand, 1997). This suggests that GBS invade epithelial cells by triggering calcium-dependent phagocytosis-like internalization mechanisms.

In experimental infection of cultured epithelial cells with GAS and GBS it was found that cytochalasin D almost completely inhibited internalization of bacteria, whereas colchicine had no effect, indicating that host microfilaments play a major role in bacterial internalization (Greco, 1995).

Depending on the streptococcal strain and on the strategy the strain uses for its dissemination, the fate of the host cell and of the intracellular bacteria may vary.

After 2 hours of infection of cultured A549 human respiratory epithelial cells with GBS strain COH-1, the invading bacteria were completely enclosed by the microvilli and were found deep in the cytoplasm (Rubens, 1992). Internalization of GBS by HEp-2 cells was observed within 20 minutes and live intracellular bacteria were detectable up to 48 hours post-infection (Valentin-Weigand, 1997) while other strains of GBS were able to destroy HeLa cells within 6 hours of co-incubation (Tyrrell, 2002). As was demonstrated in the HEp-2 / S. pyogenes model of infection, after internalization, the S. pyogenes continued to propagate intracellularly while the host cells underwent apoptosis (Marouni, 2004). This supports a hypothesis in which internalized bacteria can induce their own externalization into the medium by a process that requires both an intact host-cell cytoskeleton and de novo synthesis of bacterial proteins. Intracellular and, possibly, extracellular free bacteria induce apoptosis through
their cytotoxic activity, and a target host cell releases essential nutrients required for bacterial growth.

It was suggested that once internalized, GBS does not replicate, since similar numbers of bacteria were isolated from infected cultured epithelial cells after 2 and 8 hours of incubation of infected cells with antibiotics (Rubens, 1992). However, the reason for the similarity of numbers of CFU isolated from infected cells may be that streptococci tend to form chains upon replication and an increase in the number of cells may occur, but the number of CFU may remain constant. Electron micrographs presented by authors of that study show that the lines of division formed by internalized bacteria are clearly visible. The same study demonstrated that by up to 8 hours after internalization, GBS remained within the vacuole and did not escape into the cytoplasm.

The consequences of streptococcal invasion probably depends on the strain of the invading bacteria and expression of the bacterial surface components responsible for invasion, as well as on the lineage and competence of the host cell, which together determine the pathway of internalization of the bacteria and the sequence of molecular events following the internalization.

All of the above suggests that streptococcal adherence to and invasion of host epithelial cells is determined by the presence of one or more bacterial adhesins, by host cell receptors for the bacterial adhesins and, in some cases, by the presence of an intermediary host secreted component that may serve as a bridging molecule in the interaction between bacterial adhesins and host epithelial cell receptors responsible for adhesion.
1.4 Streptococcal Infection and Intercellular Signalling

1.4.1 Bacterial Signal Transduction Systems

Bacterial signalling pathways are activated by an array of surface components that sense changes in environmental and intracellular parameters and transmit these signals to various cellular mechanisms to cause adaptive changes in metabolism and physiology.

The existence of a eukaryotic-type serine/threonine kinase (Stk1) and its cognate phosphatase (Stp1) in GBS was demonstrated, suggesting the existence of signal transduction pathway(s) associated with protein phosphorylation. Mutants defective for Stk1 or both Stp1 and Stk1 expression exhibit affected growth, cell segregation, and virulence, suggesting a role for these enzymes in the regulation of various cellular processes (Rajagopal, 2003). This implies that a two-component transmembrane sensory kinase together with its response regulator may activate expression of the genes related to streptococcal virulence in response to changed environment following the entry of the Streptococcus into the site of infection.

1.4.2 Streptococci and Signal Transduction in Host Cells

Various signal transduction pathways are activated in different types of host cells upon contact with infectious agents (Fig. 1.4.2.1). Generally, activation of these pathways results in generation of the innate immune responses and elimination of the infecting bacteria [reviewed in (Moll, 2003; Li, 2003)]. Interference with induction of the pathways, leading to elimination of bacteria and activation of pathways allowing survival and perpetuation of the pathogen, would be beneficial for pathogenic
Figure 1.4.2.1. Intracellular signalling pathways affected by streptococcal factors.

A, Streptococcal species and factors playing a role in the host cell signal transduction; B, Host cell membrane with suggested surface receptors; “?” indicates unknown or hypothetical host cell receptor. C, Cellular processes or pathways influenced; D, Consequences of streptococci-associated signalling. 1, (Cleveland, 1996); 2, (Albanyan, 2000); 3, (von Hunolstein, 1997); 4, (Ali, 2003); 5, (Marodi, 2000); 6, (Cywes, 2001); 7, (Singleton, 2004); 8, (Turley, 2002); 9, (Zysk, 2001); 10, (Brown, 2001); 11, (Kobayashi, 2003); 12, (Lozupone, 2004); 13, (Fettucciari, 2003).
**Streptococci.** The major intracellular signalling pathways that were demonstrated to be affected by streptococci are summarized in Fig. 1.4.2.1. Streptococcal M- and M-like proteins were suggested to interfere with host intracellular signal transduction. The signal transduction pathways influenced by M-like streptococcal proteins are summarized in Fig. 1.4.2.2. M-like streptococcal proteins were implicated in signal transduction, affecting actin rearrangement of the host cell and subsequent intracellular invasion by *Streptococcus* (Purushothaman, 2003). M5 protein of GAS, which interferes with the killing of the bacteria by host neutrophils, was demonstrated to inhibit an activation of Cdc42, a component of a phagocytosis-related signalling pathway activated by complement receptor 3 (CR3) (Weineisen, 2004). Protein tyrosine phosphorylation is essential in CR3-dependent signal transduction in host neutrophils. Streptococcal M protein (M5) was demonstrated to interfere with CR3 signalling in neutrophils. Additionally, phosphorylation of β-integrin CD11b/CD18 itself on the surface of neutrophils is required for neutrophil activation (Buyon, 1997).

Integrin-linked kinase is associated with the contractile machinery and can phosphorylate myosin at the myosin light chain kinase sites. It was proposed that regulation of myosin phosphatase activity includes phosphorylation of the myosin phosphatase target subunit (Muranyi, 2002).

As was demonstrated (Dombek, 1999), streptococcal invasion may be mediated by interactions with host cell microvilli, and streptococci undergoing endocytosis are associated with polymerized actin. It was suggested that M protein of GAS activates a lipid kinase signalling pathway that is required for GAS entry into epithelial cells (Purushothaman, 2003). Both M protein and fibronectin binding protein were suggested
Figure 1.4.2.2. Cellular signal transduction pathways affected by streptococcal M proteins.

A, GAS M proteins, demonstrated to interfere with cell signalling pathways; B, Cellular membrane with host cell receptors implicated in M protein-associated signal transduction; C, Affected cellular pathways influenced by M proteins; D, Consequences of M protein-associated signalling. 1, (Weineisen, 2004); 2, (Buyon, 1997); 3, (Purushothaman, 2003); 4, (Cue, 1998); 5, (Molinari, 2000);
to initiate two different signalling pathways through the binding of the same ligand (fibronectin) and interaction with the same host cell receptor (α5β1 integrin, (Cue, 1998)), initiating cytoskeletal changes required for internalization of bacteria. Purified M1 protein promoted actin polymerization by PI 3-K-dependent mechanisms and the authors suggested that the downstream effector molecule from PI 3-K is the Akt (Ser/Thr) kinase and that phosphorylation of Akt and activation can occur only at the plasma membrane by interaction with phosphoinositides generated by PI 3-K. It was suggested that upon interaction of the eukaryotic cell with the M protein/fibronectin complex, the small protein G Ras is activated and it recruits PI 3-K to the cell membrane (Mansell, 2001), where membrane lipids are then phosphorylated. Phosphorylated products of PI 3-K have been reported to activate Rac, which in turn coordinates actin polymerization (Cantrell, 2001) required for the uptake of streptococcal cells.

Surprisingly, a GAS mutant lacking fibronectin binding protein and with reduced ability to bind fibronectin was more virulent in the murine infection model (Nyberg, 2004a), suggesting that streptococcal virulence and severity of the disease do not necessarily parallel the ability of the bacteria to adhere to and invade host epithelial cells. Activation of Akt and protein kinase C (PKC), which are downstream kinases of the phosphatidylinositol 3-kinase (PI-3K) pathway, was demonstrated in the interaction of an unencapsulated strain of *Streptococcus suis* with J774 macrophage-like cells (Segura, 2004) and the regulation of such activation was implicated in modulating a phagocytic activity of the host cells. Considering that PI-3K activation has been related to both receptors for the Fc portion of IgG- and CR3 (complement receptor 3,
CD11b/CD18)-mediated phagocytosis pathways (Cox, 2000), it is not surprising that bacteria may target PI-3K to modulate the bacteria-host interactions. Implicated in streptococcal adherence, lipoteichoic acid (LTA) was suggested to interact with actin upon host cell contact with bacterial cells (Sela, 2000), although no direct evidence of this was offered.

These data indicate that streptococcal M-related proteins (Fig. 1.4.2.2) as well as other surface components involved in host-pathogen contact and interaction (Fig. 1.4.2.1) play a role in activation of several distinct signal transduction pathways in the host cell, which potentially may affect a course and outcome of streptococcal infection.
2 HYPOTHESIS, OBJECTIVE AND SPECIFIC AIMS OF THIS STUDY

2.1 Hypothesis

We hypothesized that the lactoferrin-binding protein (Lbp) is a virulence factor of the environmental bovine pathogen *Streptococcus uberis*. This hypothesis was based on the structural similarity and amino acid sequence homology of Lbp to M-related proteins of Group A streptococci which suggested a possible functional similarity between Lbp and M-like proteins. The latter are involved in evasion of host antibacterial defenses, adhesion to host epithelial cells and intracellular invasion by the bacteria. High-affinity binding of milk lactoferrin in a species-specific manner suggested that Lbp might be involved in acquisition of iron by *S. uberis*. We also hypothesized that binding of lactoferrin (which is a major protein component in the host milk) to Lbp might trigger signal transduction in *S. uberis* during bovine intramammary infection.

2.2 Rationale and Overall Objective

The pathogenesis of bovine environmental intramammary infections is not completely understood. The overall objective of this project was to investigate the role of the lactoferrin-binding protein in the development of bovine intramammary infection with *S. uberis*. 
2.3 Specific Aims

Specific aims of this study included: (1) isolation of a defined \textit{lbp} mutant of \textit{S. uberis}; (2) comparison of the \textit{lbp} mutant and wild type \textit{S. uberis} in their abilities to resist host antibacterial factors, namely ingestion and killing by polymorphonuclear neutrophils and attack by serum complement; (3) comparison of the \textit{lbp} mutant and wild type \textit{S. uberis} in their abilities to adhere to and to invade host epithelial cells; (4) study of the capabilities of the two strains to acquire iron from bovine lactoferrin; (5) comparison of the pathogenicity of the two strains \textit{in vivo} and of their abilities to colonize the host mammary gland in experimental infection; and (6) study of the signal transduction events and regulation of gene expression activated by the two strains.
3 MATERIALS AND METHODS

3.1 Bacterial Strains and Culture Conditions

Bacterial strains and plasmids used in this study are listed in Table 3.1.1. For all experiments involving plasmid construction and amplification, *Escherichia coli* DH5α (Table 3.1.1) was used. *E. coli* was cultured with aeration in liquid Luria Bertrani (GIBCO BRL., Grand Island, NY) medium at 37°C. A clinical isolate of *Streptococcus uberis* designated strain Su-1 was subcultured on sheep blood agar and stored in a frozen stock in BHI broth containing 50% glycerol at −70°C. For routine laboratory experiments, all strains of *S. uberis* were cultured in Brain-Heart Infusion (BHI) broth (GIBCO BRL.). When necessary, bacterial growth media were solidified by adding Bacto-agar (final concentration 1%). When appropriate for the selection of antibiotic-resistant strains of either *E. coli* or *S. uberis*, antibiotics were used at the following concentrations: ampicillin, 100 µg/ml; spectinomycin, 100 µg/ml; erythromycin, 50 µg/ml. Selection of the temperature-sensitive antibiotic resistant strains was carried out at 28°C.
<table>
<thead>
<tr>
<th>Organism/plasmid</th>
<th>Strain</th>
<th>Description</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>DH5α</td>
<td>F- (φ80d lacZ ΔM15) Δ(lacZΔYA-argF) U169 recA1 endA1 hsdR17 (rk- mk+) supE44 thi1 gyrA relA1</td>
<td>(Hanahan, 1983), Gibco BRL</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>LBP5</td>
<td>Same as DH5α, carrying the plasmid pLBP5 with recombinant lbp</td>
<td>(Jiang, 1996b)</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>Su-1</td>
<td>Bovine mastitis isolate; Lbp+, Mga+, Cfu+</td>
<td>ATCC 9927 (Jiang, 1996a; Jiang, 1996b)</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>Su113</td>
<td>Same as Su-1, carrying the plasmid pMF113a (ErmR+, SpecR+, Ts, Lbp+, lbp)</td>
<td>(Moshynskyy, 2003), this study</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>Su3721</td>
<td>Same as Su113, with the pMF113a integrated into the chromosome; ErmR+, SpecR+, Ts, Lbp+ Δlbp</td>
<td>This study</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>SuM13</td>
<td>lbp mutant of Su-1 (SpecR+, lbp)</td>
<td>(Moshynskyy, 2003), this study</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>93-276</td>
<td>Bovine pinkeye isolate (LbpA+, LbpB+)</td>
<td>VIDO culture collection</td>
</tr>
<tr>
<td>pLBP5</td>
<td>DH5α</td>
<td>Lbp+, Mga+, AmpR+</td>
<td>(Jiang, 1996b)</td>
</tr>
<tr>
<td>pG+Host9</td>
<td>DH5α</td>
<td>ErmR+, Ts</td>
<td>(Maguin, 1995)</td>
</tr>
<tr>
<td>pEU904</td>
<td>DH5α</td>
<td>ErmR+, SpecR+, Ts,</td>
<td>Kindly provided by June Scott, Department of Microbiology and Immunology, Emory University Health Sciences Center, Atlanta, Georgia 30322, USA. See also (Podbielski, 1996a)</td>
</tr>
<tr>
<td>pGh9ΔK</td>
<td>DH5α</td>
<td>ErmR+, Ts, deleted KpnI site</td>
<td>(Moshynskyy, 2003), this study</td>
</tr>
<tr>
<td>pMF112a</td>
<td>DH5α</td>
<td>pGh9ΔK with Δlbp insertion</td>
<td>(Moshynskyy, 2003), this study</td>
</tr>
<tr>
<td>pMF113a</td>
<td>DH5α, Su113</td>
<td>pMF112a with aad9 insertion; ErmR+, SpecR+, Ts, Δlbp</td>
<td>(Moshynskyy, 2003), this study</td>
</tr>
</tbody>
</table>
The relationship between the optical density of an *S. uberis* culture and the number of bacteria was determined as follows. *S. uberis* was cultured in BHI broth overnight without aeration. The overnight culture was diluted 1:100 and grown without shaking at 37°C. Red light (600 nm) optical density of the culture (OD$_{600}$) was measured every hour and aliquots of the culture were serially diluted and plated on BHI agar for colony forming units (CFU) titration. A standard curve associating *S. uberis* culture optical density and number of CFU per ml was constructed to estimate the bacterial CFU titer of cultures used in the various experiments (Fig. 4.1.4.2) and to target the culture’s optical density to harvest bacteria at a desirable titer. Actual CFUs were measured for each individual experiment either by plating serial dilutions on agar plates or by counting bacterial chains using a haemocytometer. If the actual bacterial titer did not correspond to that predicted for the standard curve (Fig. 4.1.4.2), the results of the experiment were not analyzed further.

### 3.2 Recombinant DNA Techniques

Bacterial chromosomal DNA was extracted as described (Sambrook, 1989), using the phenol extraction method. Bacterial plasmid DNA was isolated using an alkaline mini scale plasmid extraction protocol (Birnboim, 1979). For extraction of large amounts of bacterial plasmid, the alkaline protocol was scaled up and the plasmid DNA was purified by CsCl gradient centrifugation (Sambrook, 1989). When necessary, DNA was purified using a plasmid isolation kit (QIAGEN Inc., Mississauga, Ontario) according to the manufacturer’s recommendations. Restriction endonucleases (Amersham Biosciences, Inc., Baie d’Urfé, Québec) were used according to the manufacturers’ recommendations. DNA fragments were separated by agarose gel
electrophoresis or, for the separation of fragments smaller than 200 base pairs, by non-denaturing polyacrylamide gel electrophoresis as described (Sambrook, 1989). To visualize DNA fragments, the gels were stained in 0.5mg/ml Ethidium Bromide (Sigma) and illuminated with 254 nm ultraviolet (UV) light. AlphaEase software (Alpha Innotech, San Leandro, CA) was used to analyze the gels. Restriction endonuclease cleavage maps were generated using Clone Manager Professional Suite (Scientific and Educational Software, Cary, NC) version 7.0 and adjusted manually.

PCR oligonucleotide primers were designed using Primer3 web-based algorithm at [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Suggested primers were analyzed using Clone Manager Professional Suite for optimization of annealing temperature, GC content, exclusion of self-complementarity and primer dimers. The specificity of the designed oligonucleotides was tested by BLAST searches for short nearly exact matches at [http://www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/). Custom PCR primers (Table 3.2.1) were synthesized by Sigma. PCR reactions were carried out using PCR 2X MasterMix (MBI Fermentas) in 50 µl volume using the Programmable Thermal Controller model PTC-100 (MJ Research, Watertown, MA).

T4 ligase (Amersham Biosciences) was used for ligation of both cohesive and non-cohesive end DNA fragments in the OPA buffer (Amersham Biosciences) containing 10 mM ATP at room temperature over 18 hours.

When required, overhanging nucleotides were removed from DNA fragments using the PCR Polishing Kit (Stratagene, Cedar Creek, TX) according to the manufacturer’s instructions.
Table 3.2.1. PCR oligonucleotide primers used in his study.

<table>
<thead>
<tr>
<th>Primer’s designated name</th>
<th>5’ to 3’ nucleotide sequence</th>
<th>Reference sequence / accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>lbp</em>#1</td>
<td>AAAGTCGACCCCTTAATATGG-CCAAGAATCGGT</td>
<td>pLBP5, (Jiang, 1996b)</td>
</tr>
<tr>
<td><em>lbp</em>#2</td>
<td>GAGGTCGACGGTGATCGA-AAGC</td>
<td>pLBP5, (Jiang, 1996b)</td>
</tr>
<tr>
<td><em>lbp</em>#3</td>
<td>TTTTTGGTACCTAAGCT-TCCCTGC</td>
<td>gbAY376838.1 (Moshynskyy, 2003)</td>
</tr>
<tr>
<td><em>lbp</em>#4</td>
<td>AAAAAAGGTACCCCGGGCAAAAGCGCAAAAAAA</td>
<td>gbAY376838.1 (Moshynskyy, 2003)</td>
</tr>
<tr>
<td><em>aad9</em>#1</td>
<td>TCGATAGCTTGCATGCC-GCAG</td>
<td>gbU50979.1 (Podbielski, 1996a)</td>
</tr>
<tr>
<td><em>aad9</em>#2</td>
<td>GAGGTCGACGGTGATCGA-AAGC</td>
<td>gbU50979.1 (Podbielski, 1996a)</td>
</tr>
<tr>
<td>ANX1F</td>
<td>GATCAAAGCGGGCTATCTGC</td>
<td>gbAW462573</td>
</tr>
<tr>
<td>ANX1R</td>
<td>GCTCTTCCGGCATCAAACTGG</td>
<td>gbAW462573</td>
</tr>
<tr>
<td>VIL1F</td>
<td>GAGAACAAGCGGACCACCAAA</td>
<td>gbBF045212</td>
</tr>
<tr>
<td>VIL1R</td>
<td>CCTTCACATGGCCCTCGAACT</td>
<td>gbBF045212</td>
</tr>
<tr>
<td>STM1F</td>
<td>AGACGCAAGTCCCCATGAAAGC</td>
<td>gbBF042135</td>
</tr>
<tr>
<td>STM1R</td>
<td>TCAGCTTCTCTCTGCGCCAT</td>
<td>gbBF042135</td>
</tr>
<tr>
<td>Elf1F</td>
<td>AGCCTCTGAACCACCAAGAGGG</td>
<td>gbBF045376</td>
</tr>
<tr>
<td>Elf1R</td>
<td>AGGCCAGCTGGGTGTGGTA</td>
<td>gbBF045376</td>
</tr>
<tr>
<td>IL13RF</td>
<td>CCCTTACTCCCCAAATGGT</td>
<td>gbBF040232</td>
</tr>
<tr>
<td>IL13RR</td>
<td>GGGTGAGGATGGAGCGCCTAA</td>
<td>gbBF040232</td>
</tr>
<tr>
<td>GAPbtF</td>
<td>TGACCCCTTCATTGACCTTC</td>
<td>AJ000039</td>
</tr>
<tr>
<td>GAPbtR</td>
<td>ATGGCCCTTCCATTGATGAC</td>
<td>AJ000039</td>
</tr>
</tbody>
</table>

Recognition sites for the restriction endonuclease *KpnI* are indicated in bold.
For Southern blot hybridization, DNA fragments were separated by agarose gel electrophoresis, transferred via capillary transfer to a nylon membrane (Bio-Rad Laboratories, Mississauga, Ontario) and hybridized as described (Southern, 1975). DNA probes for Southern blot hybridization were prepared by carrying out a PCR reaction into which an α[32P]dCTP was added. 32P-labeled DNA probes were purified by gel filtration through a Sephadex G50 (Amersham Biosciences) mini-column. Probed membranes were exposed to Kodak Scientific Imaging film (Eastman Kodak Company, Rochester, NY) and processed as recommended by the manufacturer.

Apoptosis in cultured epithelial cells was induced with either 2 µM of staurosporine or with live bacteria as described (Tsai, 1999). For assaying DNA laddering during MAC-T cell apoptosis, total cellular DNA was extracted as described (Tang, 1998) 24 hours after induction of apoptosis and separated by gel electrophoresis in 1.5 % agarose as described above.

3.3 Bacterial Transformation

All bacterial transformation experiments involved electroporation of competent bacterial cells. To prepare electrocompetent bacterial cells, a bacterial culture grown overnight was diluted 1:100 in fresh broth and grown to early logarithmic stage (OD$_{600}$ ≈ 0.2). Bacterial cells were collected by centrifugation, washed 3 times in ice-cold sterile deionized water, resuspended in cold water at approximately 10$^9$ CFU/ml, then stored in 100 µl aliquots at −70°C until use. Bacterial cells were transformed by electroporation in a 1 mm gap cuvette (Bio-Rad) at 12.5 kV/cm, 200 Ω in a GenePulser (Bio-Rad) using 100 µl of bacterial cells prepared as described above. Fifty ng of
ligated DNA (for transformation of *E. coli*), 500 ng of plasmid DNA (for transformation of *S. uberis*), or 10 pg of total DNA isolated from *S. uberis* carrying the plasmid pMF113a for re-transformation of *E. coli* were used for each electroporation.

### 3.4 Allele Replacement

Allele-replacement of the *lbp* gene was performed as described previously (Jones, 1991). Briefly, strain Su-1 transformed with pMF113a (ErmR⁺, SpecR⁺, Ts, Δ*lbp*; Fig. 4.1.2.1), resistant to both erythromycin and spectinomycin at 28°C (permissive for replication temperature) and susceptible to both spectinomycin and erythromycin at 42°C (non permissive for pMF113a replication), was cultured at 28°C to stationary phase, diluted 1:1000 in fresh BHI broth, spread on a BHI agar in the presence of both antibiotics and incubated overnight at 42°C. Incubation at the non permissive temperature ensured that the pMF113a plasmid did not replicate independently, while the antibiotic in the medium allowed selection for the presence of plasmid-encoded resistance genes, presumably integrated into the *S. uberis* chromosome. Several clones resistant to both antibiotics were selected and likely contained pMF113a integrated into the chromosomal DNA, resulting in two copies of the *lbp* gene: one originally present in the Su-1 strain and the other one the altered *in vitro*. To stimulate the second recombination event resulting in excision of the wild type *lbp* gene along with the pMF113 backbone, the recombinant strain was grown in fresh BHI without antibiotics at 28°C for 18 hours. The culture was diluted 1:100,000, plated on antibiotic-free BHI agar and incubated at 42°C for 18 hours. Colonies were replica-plated onto spectinomycin-containing and erythromycin-containing BHI agar. The
colonies resistant to spectinomycin and susceptible to erythromycin were considered replacement mutants and were characterized as described below.

3.5 Protein Techniques, Western Blot and ELISA

SDS polyacrylamide gel electrophoresis of total bacterial proteins was carried out as described (Laemmli, 1970) in a mini polyacrylamide gel apparatus model Mini-PROTEAN II Cell (BioRad). Protein bands were visualized by staining the gel with Coomassie Brilliant Blue R250 (BioRad). For immunoblotting, proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (BioRad) by electrophoretic transfer using an Electroeluter model 422 (BioRad) in cold transfer buffer at a constant current of 300 mA. The membranes were blocked with 0.3% casein at 4°C for 18 hours. Primary polyclonal anti-Lbp rabbit antibodies were described previously (Jiang, 1996b). Alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Kirkegaard and Perry Laboratories) was used as a secondary antibody. Dilutions of 1:1000 of primary antibodies and 1:5000 of the secondary antibody were used consecutively (30 minutes each, each followed by 3 washings with TBS, all at room temperature) to probe protein blots. Immunoreactive protein bands were visualized using nitroblue tetrazolium salt (NBT) / 5 bromo-4-chloro-3-indolyl phosphate (BCIP) alkaline phosphatase chromogenic reaction as described (De Jong, 1985). Bovine lactoferrin (Sigma) was labeled using the DIG-oxygenin Protein Labeling Kit (Roche Diagnostics, Laval, Quebec) as recommended by the manufacturer. Bound lactoferrin was detected using AP-conjugated Fab fragment of an anti-DIG monoclonal antibody (Roche) and the lactoferrin-binding protein bands were visualized as described above. DIG-labeled bovine lactoferrin was used for both
Western blot and ELISA (enzyme-linked immunosorbent assay)-based measurement of lactoferrin-binding. Developed nitrocellulose membranes were scanned with white fluorescent light and imaged using AlphaEase software as described above.

For ELISA, exponentially growing bacterial cultures were diluted to $10^8$ CFU/ml in 50 mM sodium carbonate, pH 9.6, buffer and heat-inactivated (15 minutes at 65°C). Whole bacterial cells were adsorbed to round-bottom High Binding 96-well polystyrene microtiter plates (Thermo Labsystems, Franklin, MA) in sodium carbonate buffer (50 µl, $10^8$ CFU/ml) overnight. All incubations were performed at room temperature. Excess bacteria were removed by washing with tris-buffered saline (TBS, 0.8% NaCl (w/v), pH7.0) containing 0.5% (v/v) of Tween 20. The plates were blocked with 100 µl/well of TBS-0.5% Tween 20 containing 0.3% (w/v) bovine serum albumin (BSA; Sigma, St. Louis, MO). The plates were washed once with TBS - 0.5% Tween 20. Either 50 µl of anti-Lbp rabbit serum or 50 µl of DIG-labeled bovine lactoferrin was added to the wells, then the plates were incubated for at least 3 hours and extensively washed with TBS-0.5% Tween 20. Both the rabbit anti-Lbp serum and the DIG-labeled lactoferrin (1 mg/ml) were serially diluted in fourfold increments from 1:20 to 1:81,520 in TBS-0.5% Tween 20-0.3 % BSA. The plates were washed as above and probed with either 50 µl of the 1:1000 diluted goat anti-rabbit IgG conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD.) or 50 µl of 1:1000 diluted Fab fragment of monoclonal anti-DIG antibodies conjugated to alkaline phosphatase (Roche), both diluted in TBS-0.5% Tween 20-0.5% BSA. The plates were incubated for 2 – 4 hours and washed 3 times as above. Para-nitrophenyl phosphate substrate (PNPP, 1 mg/ml; Sigma) in 50 µl of 1 M Tris-3 mM MgCl (pH 9.8) was
added. The optical densities were determined by a dual wavelength measurement, subtracting the OD$_{550}$ from the OD$_{405}$. ELISA titers were calculated as the reciprocal of the last positive dilution plus 2 standard deviations.

Protein phosphorylation was detected by incubation of the bacteria or mammalian cells in the presence of 10 µCi/ml of $\gamma^{32}$P[ATP (Amersham) for 1 hour with subsequent separation of the proteins by polyacrylamide SDS gel electrophoresis. The gels were stained with Coomassie Brilliant Blue R250 to visualize the protein bands and air-dried at 37°C, after which the gels were exposed to Kodak Scientific Imaging film to identify phosphorylated bands.

3.6 Bactericidal Assay and Phagocytosis

The bactericidal effect of whole bovine peripheral blood was assayed as described (Kotarsky, 2000) in a direct bactericidal assay. An overnight culture of bacteria in BHI broth was diluted 1:50 in fresh BHI and grown at 37°C without agitation to an OD$_{600}$ of ~ 0.2. The bacterial cultures were diluted 1:10$^4$ in BHI and 500 µl of the suspension, containing ~5000 CFU, was added to 4.5 ml of fresh bovine peripheral blood supplemented with 20 U/ml sodium heparin and 4 µg/ml of bovine lactoferrin (Sigma) in 5 ml polypropylene tubes. The mixture was incubated with rotation at 37°C for 3 hours. Bacterial numbers were determined every hour by plating serial 1:10 dilutions on BHI agar. The results are presented as a multiplication factor indicating a percentage of surviving bacteria in the mixture after each hour of incubation. The multiplication factor was calculated as described (Collin, 2002), by
dividing the number of CFU after each hour of incubation by the number of CFU at the previous hour.

To determine the opsonophagocytic killing of the bacteria in whole bovine peripheral blood, the mixtures above were supplemented with 0.5% of bovine anti-\textit{S. uberis} serum. The incubation of the mixtures and the calculation of multiplication factor were performed as above.

\textbf{Resistance to killing by PMN.} Neutrophils were isolated from fresh EDTA-supplemented bovine peripheral blood as described (Tithof, 1997), washed once in DMEM, counted using a haemocytometer (Amersham) and diluted to $10^7$ viable cells/ml in fresh DMEM (Sigma) in the presence of 0.5\% (v/v) bovine anti-\textit{S. uberis} serum and 4 µg/ml bovine lactoferrin. Exponentially growing bacteria (see above) were washed in fresh DMEM and added to the neutrophil suspension at a 1:1 ratio. The suspensions were incubated and multiplication indexes were determined as described above. After 3 hours of incubation, the suspensions were treated with 100 µg/ml of ampicillin and 50 µg/ml of gentamicin (both from Sigma)) for 3 hours at 37 °C with constant rotation in order to kill extracellular bacteria as described (Zlotkin, 2003). After each hour the bovine neutrophils were extensively washed and lysed with 0.1\% (w/v) saponin (Sigma) 1\% (w/v) trypsin (Sigma). Serial 1:10 dilutions of the lysates were plated on BHI agar and multiplication indices were calculated as above.

\textbf{Resistance to serum complement.} Bovine serum was used for assaying the bactericidal effect of serum complement on \textit{S. uberis} Su-1 and SuM13. Fresh bovine serum was centrifuged at 3000 g and, when required, heated at 56°C for 2 hours in order to completely inactivate complement enzymes. Exponentially growing bacteria were
mixed with bovine serum, incubated at 37°C with constant rotation, and bacterial multiplication factors were calculated as described above. Data are means ± standard deviations of at least three independent experiments.

**Phagocytosis assay.** The association of the bacteria with host peripheral blood polymorphonuclear cells was assessed by flow cytometry as described (Song, 2001). Exponentially growing bacteria were fluorescently stained using the PKH-2 Fluorescent Cell Linker kit (Sigma) as recommended by the manufacturer. Stained bacteria were washed 4 times in antibiotic-free DMEM and mixed with bovine PMNs in the presence of 4 µg/ml of bovine lactoferrin (Sigma) and 0.5% (v/v) of bovine anti-*S. uberis* serum at the CFU/PMN ratios of 1:10, 1:100 or 1:1000. Mixtures were incubated at 37°C in the dark in a slowly rotating 5 ml tube. After 1 hour of incubation, the mixtures were fixed with 2% formaldehyde (Sigma). A flow cytometry assay was performed using a FACScan flow cytometer (Becton Dickinson, Mississauga, Ontario). Ten thousand PMNs were counted for each sample. Granulocyte cell populations were selected by gating according to their granularity and cell sizes as described (Jain, 1991). The results are shown as representative dot plots of the particle size versus logarithm of the particle fluorescence as suggested previously (Song, 2001) for the gated cell population as determined in at least 3 independent experiments.

3.7 **Culturing of Bovine Mammary Epithelial Cells**

MAC-T bovine mammary epithelial cells (Huynh, 1991) ATCC CRL-10274 were cultured as described (Calvinho, 1998) in 75 cm² tissue culture flasks (Corning Inc., Corning, NY) to near confluence. Cells were treated with 0.5% (w/v) Trypsin
(GIBCO BRL.) in Versene Buffer (0.2 g/l sodium-EDTA, 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na$_2$HPO$_4$, 0.2 g/l KH$_2$PO$_4$, 0.2 g/l glucose), resuspended in fresh DMEM medium containing 5% (v/v) of fetal bovine serum (FBS) (GIBCO BRL.) and diluted to 5x10$^5$ cells/ml with DMEM. For adhesion and invasion experiments, 10$^6$ viable cells were placed in each well of 6 well tissue culture plates and incubated at 37°C in 5% CO$_2$ (v/v) atmosphere for 18 hours.

3.8 Light Microscopy

Counting of trypsinized bovine cultured epithelial cells was carried out in a haemocytometer (Amersham) according to the manufacturer’s guidelines. Viable cells were quantified using a trypan blue exclusion test as described (Sanchez, 1986). The trypsinized cells co-incubated with bacteria were fixed with 1% (v/v) formaldehyde 25% (v/v) glycerol solution and photographed using a fluorescent microscope Zeiss model Axiovert 200M (Carl Zeiss Vision GmbH., Hallbergmoos, Germany). Monolayers of cultured epithelial cells were examined and photographed as described above.

Bacterial cells were fluorescently labelled using a PHK-2 kit (Sigma) as recommended by the manufacturer. Prior to examination of infected cells by electron microscopy, both MAC-T cells and bacteria were stained with 2 µg/ml of Bis-Benzidine (Sigma) and analyzed by fluorescent microscopy.

3.9 Bacterial Adhesion and Invasion Assays

MAC-T cells were transferred to 6-well tissue culture plates at approximately 10$^6$ cells per well and cultivated overnight in DMEM tissue culture medium,
supplemented with 5% of fetal bovine serum. After replacement of the medium with 2.5 ml of fresh medium, the cells were infected with *S. uberis* at a multiplicity of infection (MOI) of 100:1 (approximately $10^8$ CFU), and incubated for 3 hours at 37°C. Since *S. uberis* grows in tissue culture medium thereby influencing the number of bacteria that can adhere to and invade the host cells, the number of bacteria after growth for 3 hours in tissue culture medium was set as the input inoculum as described (Gutekunst, 2003). To determine the number of cell-adherent bacteria, the infected host cells were washed three times with Versene buffer, lysed with 1% (w/v) trypsin 0.025% (w/v) saponin for 1 hour on ice and appropriate dilutions were plated onto agar plates. The CFUs were counted after incubation of the plates for 18 hours at 37°C. The amount of bacteria adhering non-specifically to the wells was determined in a similar way, except that the 6-well plates did not contain MAC-T cells. Due to the lysis of the eukaryotic cells, the calculation of cell-adherent bacteria also included bacteria that had invaded MAC-T cells. Therefore, the number of invasive bacteria (see below) was subtracted from the number of cell-adherent bacteria obtained to calculate the actual number of adherent bacteria. To determine the number of adherent bacteria per eukaryotic cell, the number of cell-adherent bacteria was divided by the number of MAC-T cells per assay. To determine the adhesion index, the number of viable adherent bacteria was divided by the total number of bacterial CFU in the well at the time of assay. All adhesion and invasion assays were performed in duplicate and the experiments were repeated at least 4 times.

The invasive bacteria were enumerated as described (Gutekunst, 2004). For invasion assays, the epithelial cells, grown in 6-well plates as described above, were
infected with $10^8$ CFU of streptococci, incubated for 2 hours at $37^\circ$C, and washed three times with Versene buffer. Subsequently, the infected cells were incubated for 2 h in tissue culture medium supplemented with ampicillin (100 µg/ml) and gentamicin (50 µg/ml) to kill extracellular bacteria. After three washes with Versene buffer, the epithelial cells were detached by the addition of trypsin-EDTA and lysed in 1 ml of 1% trypsin 0.025 % saponin for 1 hour on ice. The amount of invasive bacteria was determined by plating serial dilutions of the lysate onto BHI agar plates. The invasion index was calculated as follows: (number of invasive/total number of adherent bacteria immediately before addition of antibiotics) x 100%. To determine the number of intracellular bacteria per eukaryotic cell, the number of intracellular bacteria was divided by the number of MAC-T cells per assay and multiplied by 1000. The results are represented as the mean ± standard deviation.

In order to demonstrate the Lbp-dependent adherence of particles to bovine mammary tissue, latex beads (diameter 3 µm, Sigma) were coated with recombinant Lbp as described (Kang, 1998). Mammary tissue (bovine udder pieces, ~ 5cm$^3$) was aseptically taken and transported on ice from a slaughter facility to the laboratory within 2 hours in DMEM containing 50 µg/ml of gentamicin. It was aseptically minced and incubated with 2000 U of collagenase type III (Sigma) at $37^\circ$C in fresh DMEM containing 4 µg/ml of bovine lactoferrin for 2 hours. Approximately 0.1 ml of this suspension was mixed with $10^5$ CFU of bacteria or with $10^4$ latex microspheres coated with recombinant Lbp. The mixtures were microscopically examined every 10 minutes for a total period of 1 hour.
3.10 Transmission Electron Microscopy

To study the effect of Su-1 and SuM13 on subcellular components of cultured bovine mammary epithelial cells, infected MAC-T cells were examined by electron microscopy. Monolayers of MAC-T cells were trypsinized as described above and resuspended in fresh DMEM containing 5% FBS and 4 µg/ml of bovine lactoferrin without antibiotics. Exponentially growing bacteria, harvested at an OD \(_{600}\) of approximately 0.2, were washed in fresh DMEM once and added to the MAC-T cell suspension in 5 ml polypropylene tubes. The tubes were rotated overnight at 37 ° C. After 24 hours, MAC-T cells were centrifuged at 700 revolutions per minute (RPM) in a tabletop centrifuge (~45 g), washed with fresh DMEM and fixed in 0.2 M sodium cacodylate buffer containing 3% glutaraldehyde overnight at room temperature. Fixed samples were washed 3 times in 0.2 M sodium cacodylate buffer and post-fixed at room temperature for 1 hour in 1.25% sodium bicarbonate buffer pH 7.2 containing 1% OsO\(_4\). The samples were dehydrated by four consecutive washings with solutions containing progressively increasing ethanol concentrations as follows: ten minutes in 50% (v/v), 5 minutes in 60% (v/v), 5 minutes in 70% (v/v), 1 hour in 70% (v/v), 5 minutes in 80% (v/v), 5 minutes in 96% (v/v), 5 minutes in 100% ethanol solutions. During the dehydration, the samples were enbloc stained with 2 % uranyl acetate solution in 70% ethanol for 1 hour at room temperature. After dehydration, the samples were rinsed in propylene oxide and infiltrated into Epon/araldite in three steps with progressively increasing concentrations of Epon/araldite in propylene oxide. Samples were polymerized in moldo forms with fresh Epon/araldite at 60 ° C. The shaped samples were cut into 0.5 µm semi thick sections with a Jumdi diamond knife (Canemco Inc.,
Montreal, Quebec) using a Reichert-Jung ultramicrotome model E (Reichert-Jung, Vienna, Austria), and the sections were placed on 90 nm thick copper grids, mesh 200, 3mm in diameter. Sections were analyzed using a Phillips transmission electron microscope model 410LS at an electric field of 60-80 kV for electron beam acceleration. Images were taken on Kodak 4489 film.

3.11 Study of Iron Acquisition

The concentration of total iron in the BHI medium was measured by colorimetric titration as described (http://www.sonomoa.edu/users/b/brooks/115b/iron.html) using 1:1000 diluted medium with 0.25% (w/v) 1,10-phenanthroline in the presence of 1% hydroquinone and 2.5% (w/v) of sodium citrate in water at a wavelength of 508 nm.

To study the requirements of *S. uberis* for iron needed to support the bacterial growth, we depleted BHI broth of Fe(II) or Fe (III) by adding iron-chelating agents. Iron (III) restriction in BHI broth was achieved by supplementing the medium with 2,2-dipyridyl (GIBCO BRL) to a final concentration of 5 mM or with deferoxamine mesylate (Sigma) to a final concentration of 5 mM.

Divalent iron ions were chelated by either nitrilotriacetic acid (NTA, Sigma) or ethylenediamine di(o-hydroxyphenylacetic) acid (EDDA, Sigma), added to the BHI medium to a final concentration of 5 mM, after which MgCl₂, ZnCl₂, MnCl₂ and CaCl₂ were added to a concentration of 1 mM each as described (Eichenbaum, 1996b).

Bacterial cultures in the early exponential phase were split, with one part supplemented with an iron chelator and the other serving as a control (Fig. 3.11.1). Five hours after iron chelation, the culture supplemented with a chelator was again split and the iron source was added to one part of the culture (Fig. 3.11.1). Iron depleted medium
was supplemented with holo-lactoferrin to a final concentration of 4 µg/ml or with FeCl₃ to a final concentration of 10 mM. Optical densities of bacterial cultures were measured at a wave length of 600 nm (OD₆₀₀) using a spectrophotometer Ultraspec model 3000 (Pharmacia Biotech, Cambridge, England).

To measure acquisition of iron by strains Su-1 and SuM13, iron depleted BHI was supplemented with ⁵⁹Fe³⁺-saturated lactoferrin. Radioactive iron was obtained from Amersham Pharmacia Biotech as 100 mM FeCl₃ in 0.1 M HCl at an activity of 6 to 25 mCi/mg of Fe. For saturation of 8 mg of bovine apo-lactoferrin, 0.5 µl of 100 mM FeCl₃ was used. This 0.5 µl of FeCl₃ (3x10¹⁶ atoms of Fe³⁺) corresponded to 600,000 counts per minute (CPM) as measured by β-counting at the background radioactivity of 50 CPM. Unbound iron was separated from lactoferrin by gel filtration using G-25 Sephadex prepacked columns. Total β-radioactivity of the protein fraction was determined as 200,000 CPM, which corresponded to approximately 10¹⁶ atoms of Fe³⁺. This amount of iron was chelated by 8 mg of lactoferrin, as determined by the protein colorimetric titration. This amount corresponds to 15 - 30 % of saturation capacity of 3x10¹⁶ molecules (8 mg) of lactoferrin in the protein fraction. Four mg of such lactoferrin (1.5x10¹⁶ molecules of lactoferrin; 100 000 CPM; 5x10¹⁵ atoms of Fe³⁺) were added to the exponentially growing bacterial cultures pretreated with 10 mM of deferoxamine mesylate for 1 hour and containing 10⁹ CFU. After 1 hour of incubation, the total amount of intracellular iron proportional to cell radioactivity for each bacterial culture was determined.
Figure 3.11.1 Study of *S. uberis* requirements for iron and effect of iron depletion on growth of cultured bacteria.
Apo-lactoferrin and holo-lactoferrin were prepared as described (Mazurier, 1980). Measurement of β radioactivity of the protein fraction and the protein concentration in holo-lactoferrin samples indicated that the holo-lactoferrin was 15% saturated with iron (Lf:Fe ratio approximately 3). Bovine lactoferrin (Sigma) saturated with $^{59}\text{Fe}^{3+}$ was added to the bacterial suspension to a final concentration of 4 mg/ml and the suspension was incubated for 1 hour at 37°C. Bacteria were centrifuged, then washed 4 times with PBS to remove unbound iron and lactoferrin. Surface proteins, including iron-loaded lactoferrin, were removed by trypsin digestion (5% (w/v) trypsin, 15 min. at 30°C) of bacterial cells. Trypsinized cells were centrifuged and the radioactivity of the supernatant was measured using a β- liquid scintillation counter Beckman Coulter model LS1701 (Beckman RIIC Ltd, Glenrothes, Scotland). This corresponded to the amount of the surface-bound iron-saturated lactoferrin. The radioactivity of the cell pellets was measured as above and corresponded to the amount of intracellular iron in the bacteria. Moraxella bovis, which expresses two iron-repressible lactoferrin-binding proteins (Yu, 2002), requires iron for growth and is capable of iron acquisition from bovine lactoferrin, was used as a positive control. The data presented is the result of 6 independent experiments.

3.12 Experimental Infection

To study the role of the lactoferrin-binding protein of S. uberis in colonization of the bovine mammary gland and in order to determine the significance of expression of the Lbp in pathogenesis of S. uberis bovine mastitis, 6 lactating cows were infected with either Su-1 or SuM13.
Healthy Holstein cows, with no previous history of infection with *S. uberis*, in their 4th quarter of lactation were screened for the presence of bacteria in milk by plating of serial dilutions of the milk on BHI agar plates. Titers of anti-*S. uberis* IgG in milk were determined by ELISA. The presence of high background levels of anti-*S. uberis* antibodies in both milk and serum was possibly due to a previous likely exposure(s) to other streptococcal and/or staphylococcal species since there is considerable cross-reactivity between antigens of these pathogens. A total of 6 animals with bacteriologically negative milk and with the lowest anti-*S. uberis* IgG titers were selected and were divided into two groups of 3 animals each. The left hind and left front quarters of each animal were inoculated with sterile 0.8 % NaCl and served as internal negative controls. The right hind and right front quarters of the group 1 animals each were inoculated with 4.5 x 10⁶ CFU of *S. uberis* Su-1 harvested in the exponential phase of growth (OD₆₀₀ = 0.24), while the right hind and right front quarters of group 2 animals each were inoculated with 4.8 x 10⁶ CFU of *S. uberis* SuM13 also harvested in the exponential phase of growth (OD₆₀₀ = 0.25). Milk samples from each individual quarter were collected immediately before the challenge and then twice a day during milking for 10 days. Colonization of a mammary gland was assayed by plating serial dilutions of milk on BHI agar plates and on BHI agar plates supplemented with 100 µg/ml of spectinomycin, both in triplicate. For determination of somatic cell counts (SCC), milk was analyzed using a Beckman Coulter counter according to the International Dairy Federation guidelines (IDF, 1995). Briefly, milk samples were fixed in 0.3 % formaldehyde and the fat particles were dispersed in 2 % (v/v) TritonX100, 10% ethanol emulsifier solution at 80°C for 2 hours. Emulsified milk (0.3 ml) was
analyzed in a Beckman Coulter counter according to the manufacturer’s instructions.

Development of clinical mastitis and rectal temperature were monitored daily.

Colonies grown out of milk samples were randomly picked from BHI agar plates and examined microscopically for the presence of streptococcal chains. Additionally, several colonies representing milk samples from all challenged quarters were analyzed using API strep20 strips (Biomerieux Inc., Quebec) as recommended by the manufacturer for identification of the bacterial strains.

3.13 cDNA Microarray Hybridization

To study the transcriptional response in cultured bovine mammary epithelial cells infected with *S. uberis* Su-1 as opposed to that of the cells infected with SuM13, microarray hybridization was carried out. Bovine mammary epithelial MAC-T cells were maintained in DMEM culture medium containing 10% fetal bovine serum in 75-cm² tissue culture flasks as described above. At approximately 60% confluence, cells were trypsinized, counted in a haemocytometer and resuspended in fresh DMEM at a concentration of 10⁶ cells/ml in antibiotic-free medium. Cells were allowed a 2 hour period of recovery after trypsinization. Cells were then co-incubated with 4 µg/ml of bovine lactoferrin and with either *S. uberis* Su-1 or the isogenic *lbp* mutant SuM13 for 3 hours at 37°C in 5% CO₂ at a bacteria-to-cell ratio of 100:1. Total RNA was isolated from infected MAC-T cells using the Trizol reagent (Sigma) and subsequent extraction with chloroform as described (Baelde, 2001). RNA was treated with RNase-free DNase (Qiagen) and purified using disposable RNEasy mini columns (Qiagen) according to the manufacturer’s instructions. The integrity and purity of the RNA was analyzed by agarose gel electrophoresis and using an Agilent BioAnalyser, model 2100
(Agilent Technologies Canada Inc., Mississauga, Ontario) with RNA 6000 Nano kits (Agilent). To control for the presence of minor quantities of DNA contamination in the RNA samples, after synthesis of cDNA, PCR was carried out using a pair of oligonucleotides specific for bovine GAPDH (Table 3.2.1) and either initial RNA or cDNA samples as a template for the PCR. The products of the PCR amplification were analyzed by agarose gel electrophoresis.

Complementary DNA (cDNA) was synthesized by a reverse transcription reaction using a LabelStar Array Kit (Qiagen) as recommended by the manufacturer for direct cDNA labeling. RNA isolated from Su-1-infected MAC-T cells was used to synthesize biotin-labeled cDNA, while RNA isolated from SuM13-infected MAC-T cells was used to synthesize fluorescein-labeled cDNA. Labeling was carried out by incorporation of either Bio-11-dUTP or Fluorescein-12-dUTP (both from Enzo Life Sciences, Inc., Farmingdale, NY) into cDNA during the reverse transcription reaction. The cDNA was purified using a MinElute Reaction Cleanup Kit (Qiagen) and hybridized to bovine microarray glass slides containing individual spots for 7884 bovine open reading frames (ORF) (Pyxis Genomics, Inc., Chicago, IL) and probed with anti-biotin- and anti-fluorescein-coated resonance light scattering (RLS) particles using a Two-Color Nucleic Acid Microarray Toolkit (Genicon Sciences Corporation, San Diego, CA) as described (Aich, In Press). Next, the glass slides were scanned using a GSD-501 RLS Detection and Imaging instrument using ArrayVision software (Imaging Research Inc, St. Catharines, Ontario), and 16 bit tiff images were generated. Individual spots were identified and their intensities were determined using ArrayVision. Text files in the form of spreadsheets were generated from analyzed data.
consisting of spot IDs, signal intensities and background. Relative spot intensities were normalized for each individual spot using GeneSpring Expression Analysis software (Agilent) and the relative amount of mRNA was calculated as described (Aich, 2005). The data presented is the result of three independent experiments. The series of obtained normalized spot intensities of silver RLS were compared to the series of normalized spot intensities of gold RLS by t-test. Open reading frames with consistently altered mRNA levels in all three experiments at a 95 % confidence interval were selected for further analysis.

3.14 Quantitative RT PCR

To verify the microarray data, quantitative RT PCR was performed. Quantitative PCR was carried out using cDNA (synthesized as described above) as a template. A reaction was carried out in the volume of 15 µl in a 96 well plate using an iCycler qPCR instrument (Bio-Rad). The reaction was performed using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen Canada Inc., Burlington, Ontario) according to the manufacturer’s guidelines using a 3-step amplification cycle (95°C 15 s, 55°C 30 s, 72°C 30 s). The amplification of the PCR product was detected by measuring the amount of SYBR Green I dye incorporated in the PCR product and plotted as fluorescence versus cycle number. Accumulation of fluorescence was considered proportional to accumulation of a PCR product. The detection threshold was set at the average background fluorescence plus two standard deviations. A reaction mixture containing the oligonucleotide pair specific for bovine GAPDH was used as a positive control. The difference in expression of the gene was calculated as $2^{\Delta\Delta Ct}$. The parameter $C_t$ was defined as the cycle number at which the first detectable increase above the
threshold in fluorescence was observed. $\Delta C_t$ was calculated as a difference between the cycle number for the positive control and the tested gene at a fluorescence detection threshold of ($\Delta C_t = C_t[\text{target gene}] - C_t[\text{GAPDH}]$). $\Delta \Delta C_t$ was calculated as the difference between the $\Delta C_t$s of the control and the test samples. Quantitative RT PCR was carried out in triplicate using mRNA from each of the 3 microarray experiments. The data represent fold change in mRNA content ± standard deviation.

3.15 Statistical Analyses

In order to determine the role of Lbp in the studies comparing Su-1 and SuM13, two sets of data were compared. In each experiment, one set of data was generated in an assay involving *S. ubseris* Su-1; the other set was generated in an assay involving the isogenic *lbp* mutant SuM13. Positive or negative controls were included as indicated for each experiment. Differences between each of the two sets of data were calculated by the *t* test, with significance postulated as a *P* of <0.05.
4 EXPERIMENTAL RESULTS

4.1 Construction of a Defined \textit{lbp} Mutant of \textit{S. uberis}

4.1.1 Introduction

Random insertional inactivation of the streptococcal genes has been successfully carried out previously in \textit{S. uberis} (Ward, 2001; Smith, 2002) and in the other streptococcal species (Nida, 1983; McDaniel, 1987; Jadoun, 2000). However, in an insertion mutant there is always a possibility of a reverse mutation occurring by excision of the mobile insertion sequence from the target gene, leading to the restoration of the gene and to the reversing to the phenotype of a parent strain. Isolation of the desirable mutants requires screening of a large number of individual bacterial strains. Additionally, more than one gene may be inactivated during random insertional mutagenesis and this approach is not always suitable for the bacterial strains with low transformation efficiency. An insertion-duplication site-directed mutagenesis, when the gene is disrupted by an internal coding region of the gene (Berry, 1996) subcloned into a non-replicating or conditionally replicating plasmid construct, allows specific targeting of a designated gene. However, this does not solve the problem of instability of the generated mutants, since insertion mutants remain prone to reversion-mutation by homologous recombination and excision of an insert. Stability of the \textit{lbp} mutant was considered an important issue for the present study, since one of the objectives was to
determine whether Lbp is required for colonization of the host and for virulence of *S. uberis* in vivo.

Non-selectivity of the mutation, possible alterations in several genes in the same cell, and the necessity to screen large number of mutant strains, as well as instability of the generated mutants, also represent drawbacks of chemical and radiation-induced mutagenesis. For these reasons we carried out an allele replacement mutagenesis, which allows generating of stable irreversible mutants. Homologous recombination between wild type and mutant alleles specifically targets the designated gene, while screening for the antibiotic resistance and temperature sensitivity (as is described in the Materials and Methods) ensures the efficiency and specificity of this approach.

To study the role of the lactoferrin-binding protein (Lbp) in *S. uberis* pathogenesis, we used a genetic approach in order to construct an isogenic mutant of the wild type strain that would be unable to express this protein. A comparison of the biological properties of the isogenic *lbp* mutant versus the parent strain should allow an analysis of the role of this surface component both in vivo during infection of the host and in vitro using specific biological assays.

### 4.1.2 Construction of the Temperature-sensitive Plasmid

A conditionally replicating plasmid construct carrying an altered allele of the *lbp* gene of *S. uberis* was used for the allele replacement mutagenesis. The construct was based on the thermosensitive pG+Host9 (Maguin, 1995) derivative of the pWV01 broad host range replicon (Kok, 1984). The use of a conditionally replicating plasmid bearing an antibiotic resistance genetic marker allows selecting for the insertion (primary) recombinants in the bacterial strain transformed with such a plasmid. Changing the
culture condition to those permissive for plasmid replication allows stimulating of the excision (secondary) recombination (Moshynskyy, 2003) from the chromosome of a primary recombinant. For this reason, we constructed a temperature-sensitive plasmid pMF113a (Table 3.1.1) bearing the altered lbp gene in which its central portion was replaced by a spectinomycin-resistance cassette aad9 (Fig. 4.1.2.1). This plasmid was used to carry out an allele replacement in S. uberis Su-1. A deletion of the central portion of the lbp gene was constructed by PCR-amplification of the upstream portion and 5’-coding region and the 3’-coding sequence plus downstream region, in two separate reactions using the primers lbp#01 and lbp#03, and lbp#02 and lbp#04 (Table 3.2.1), respectively (Fig. 4.1.2.1). A stop codon was included in the amplified 5’ region. PCR fragments were digested with KpnI (bold type in primer sequences above), ligated and reamplified with lbp#01 and lbp#02. Extended bases were removed from the PCR product using the PCR Polishing Kit (Stratagene, Cedar Creek, TX) and cloned into SmaI digested pGh9ΔK, a derivative of pG+host 9 lacking the KpnI restriction endonuclease site. The resulting construct was designated pMF112a. The aad9 spectinomycin resistance gene (LeBlanc, 1991; Podbielski, 1996a) was amplified from the pEU904 plasmid with the primers aad9#01 and aad9#02 (Table 3.2.1). Extended bases were removed from the PCR-amplified aad9 product and from KpnI-digested pMF112a. These were ligated and electroporated into E. coli DH5α, and transformants were selected at 28°C in the presence of spectinomycin and erythromycin. The resulting plasmid was designated pMF113a (Fig. 4.1.2.1.). The insertion of the aad9 cassette and its orientation were verified by sequencing of the pMF113a plasmid with
Figure 4.1.2.1. Construction of the temperature sensitive plasmid pMF113a used for allele replacement of the *lbp* gene of *S. uberis*.

A, two regions adjacent to the central coding region of the *lbp* gene were amplified using *lbp*#01/*lbp*#03 and *lbp*#04/*lbp*#02 PCR primers pairs (Table 3.2.1), respectively; B, PCR products were digested with restriction endonuclease *Kpn*I (K) and ligated together; C, the product of ligation was amplified using primers *lbp*#01 and *lbp*#02; extending nucleotides were removed from the PCR product; D, temperature-sensitive plasmid pGh9ΔK was digested with *Sma*I restriction endonuclease (S); E, PCR product generated in the step C was cloned into a linearized pGh9ΔK, which resulted in generation of the pMF112a plasmid; F, a spectinomycin-resistance cassette *aad9* was PCR-amplified from pEU904 plasmid using *aad9#1* and *aad9#2* primers and extending nucleotides were removed from the PCR product; G, *aad9* cassette was cloned into *Kpn*I site of pMF112a, containing temperature-sensitive replication factor (Ts) and the gene coding for resistance to erythromycin (*Erm*), resulting construct pMF113a was used for transformation of *S. uberis* Su-1 followed by allele replacement of its *lbp* gene.
or lbp#02 oligonucleotide primers using an ABI 373 stretch DNA sequencer (Applied Biosystems, Foster City, CA), the service kindly provided by Ingeborg A. (Inge) Roewer, DNA Technologies, Plant Biotechnology Institute (PBI), National Research Council (NRC), Canada.

4.1.3 Two-step Homologous Recombination

*S. uberis* Su-1 was transformed with the plasmid pMF113a as described in Materials and Methods. The presence of the independently replicating plasmid pMF113a in *S. uberis* cultured at 28°C (temperature permissive for the plasmid replication) was verified by Southern blot hybridization using an *aad9*-specific probe (Fig. 4.1.3.2). Probe-specific bands corresponding to different forms of the pMF113a plasmid were found in both *E. coli* DH5α and *S. uberis* that were transformed with pMF113a, while the corresponding bands were not present in the Su-1 strain of *S. uberis* (Fig. 4.1.3.2). Additionally, the total DNA extracted from erythromycin- and spectinomycin-resistant transformants of *S. uberis* Su-1 was electroporated into *E. coli* DH5α as described in Materials and Methods. Plasmids isolated from randomly picked *E. coli* transformants were verified as pMF113 by agarose gel electrophoresis of uncut plasmids and by restriction endonuclease digestion analysis (data not shown). Allele-replacement of the *lbp* gene was performed as described in Materials and Methods and illustrated in Fig. 4.1.3.3. Integration of the plasmid into the *S. uberis* chromosome was selected for by isolation of erythromycin- and spectinomycin-resistant colonies at 42°C, a non-permissive temperature for replication of the plasmid. Subculturing of the integrated recombinants at 28°C allowed subsequent excision of the plasmid.
Figure 4.1.3.2. Southern blot hybridization of plasmid DNA samples isolated from *S. uberis*.
Lane 1, total DNA from *S. uberis* Su-1; Lane 2, DNA from Su-1 transformed with pMF113a; Lane 3, *E. coli* DH5α used for cloning and amplification of pMF113a.
M: linear DNA molecular weight standards. Different forms of the pMF113a plasmid are present in lanes 2 and 3. Arrow indicates the linear (A) and the circular covalently closed (B) forms of the plasmid. The *aad9*-specific probe was used for hybridization.
Figure 4.1.3.3 Replacement of the wild type lbp allele with the in vitro altered allele by two step homologous recombination.

I, at permissive temperatures, when the plasmid replication factor encoded by the Ts is active, and at applied spectinomycin and erythromycin selection, the pMF113a plasmid in S. uberis cells is capable of replicating independently of the chromosome since it carries sequences coding for erythromycin (ErmR) and spectinomycin (aad9) resistance; II, at non-permissive temperatures the plasmid is not capable of replicating independently and at continuing antibiotics selection only colonies with the plasmid integrated into the chromosome would grow. Integration occurs due to the homologous recombination between identical regions (A or B) in the plasmid and in the bacterial chromosome. III, Su3721 (Table 3.1.1), the primary recombinant strain with the pMF113a incorporated into the chromosome. Su3721 is capable of growing at temperatures non-permissive for the plasmid replication and in presence of both antibiotics. IV, culturing of the primary recombinant at permissive temperatures stimulates the secondary homologous recombination event of an excision of the plasmid elements from the S. uberis chromosome. The secondary homologous recombination takes place at identical regions A or B, resulting in either reversion to the wild type or in the mutant with the replaced lbp allele (V).
by homologous recombination. Phenotypic screening for the spectinomycin-resistant erythromycin-sensitive phenotype allowed selection for the secondary recombinant strains with the wild type lbp replaced by the deleted lbp allele. These mutants were characterized for the presence of the altered lbp allele by Southern blot hybridization, for expression of the Lbp by immunoblotting and for the ability to bind bovine lactoferrin by ELISA as described below.

4.1.4 Characterization of the lbp Mutant of S. uberis

Both Su-1 and SuM13, when cultured either in TH or in BHI broth, demonstrated the same rates of growth (Fig. 4.1.4.1) and numbers of colony forming units (CFU) per millilitre of culture (Fig. 4.1.4.2). Microscopic examination demonstrated their similar morphology and the length of chains (5-30 cells per chain in exponentially growing culture and 15-150 cells per chain in overnight culture). The only differences detected between the two strains included alterations in the bacterial genome within the lbp gene, inability of SuM13 to express Lbp, significantly reduced binding of bovine lactoferrin by SuM13, and resistance of SuM13 to high concentrations of spectinomycin.

One of the allele replacement mutants was designated SuM13 and the deletion of the internal lbp coding sequence was confirmed by Southern blotting. Chromosomal DNA samples isolated from S. uberis strain Su-1, from the recombinant strain with pMF113a integrated into the chromosome and from the strain with replaced lbp allele
Figure 4.1.4.1. Kinetic of growth of Su-1 and SuM13 in BHI broth.

The overnight cultures were diluted 1:100 in fresh BHI and incubated at 37°C without aeration. The OD$_{600}$ of the cultures was measured every hour for a total of 13 hours. The data is representative growth curves of 3 independent experiments.

■: *S. uberis* Su-1, ◆: *lbp* mutant SuM13
Figure 4.1.4.2. Relation between OD$_{600}$ and CFU/ml for *S. uberis* Su-1 and SuM13 in BHI broth at 37°C.

BHI broth was inoculated with approximately $10^7$ CFU/ml of an overnight bacterial culture and incubated without aeration at 37°C. Culture OD$_{600}$ was measured every hour and the corresponding bacterial titers were determined by plating of serial dilutions of the culture on BHI agar.

■, ◆: *S. uberis* Su-1; △, ○: *lbp* mutant SuM13: Each type of symbol represents an individual experiment.
were digested with HindIII restriction endonuclease. The fragments of the digested DNA were separated by agarose gel electrophoresis as described in Materials and Methods, transferred to a hybridization membrane and hybridized with an lbp-specific $^{32}$P labelled probe. Analysis of the autoradiograph demonstrated that integration of pMF113a into the S. uberis chromosome occurred at the region upstream of the lbp gene. Digestion of the S. uberis chromosomal DNA with HindIII endonuclease generated two fragments (B, 3.7 kbp and E, 0.965 kbp, Fig. 4.1.4.3, lane 1) homologous to the lbp probe. HindIII digestion of the independently replicating pMF113a plasmid resulted in two fragments of 4.038 and 1.123 kbp (data not shown) containing regions homologous to the lbp coding sequence. No such combination of the HindIII fragments was detected in the digests of S. uberis DNA with pMF113a integrated into the chromosome (Fig. 4.1.4.3, lane 2), indicating that there was no independently replicating pMF113a in the integration (primary) recombinant. The size of the HindIII fragments suggested that the integration event took place at the region upstream to the lbp, in which case fragment B remained unchanged in the primary recombinant and corresponds to fragment B in the wild type strain. The excision (secondary) recombination event occurred at the region downstream of the lbp, since fragment D after the secondary recombination event (Fig. 4.1.4.3, lane 3) remained corresponding to fragment D of the primary recombinant (Fig. 4.1.4.3, lane 2). The size of the HindIII fragments of the secondary recombinant strain (Fig. 4.1.4.3, lane 3) suggested that the wild type lbp gene in S. uberis SuM13 was replaced by the altered in vitro allele containing a spectinomycin resistance cassette.
Figure 4.1.4.3  Southern blot hybridization of chromosomal DNA from S. uberis.

Su-1 (1), S. uberis with pMF113a integrated into the chromosome (2) and SuM13 mutant (3) digested with HindIII (left) and restriction map of the corresponding chromosomal loci (right).

H: position of the HindIII recognition site; Size of each HindIII DNA fragment (arrows, A through F) is indicated in brackets in kilobase pairs (kbp); M: DNA molecular weight standards with size of the fragments indicated on the left.

Region homologous to the coding sequence of the lbp and used as a probe; aad9 gene;
The Lbp⁻ phenotype of SuM13 was confirmed by immunoblotting (Fig. 4.1.4.4). Total cellular streptococcal proteins were separated by SDS polyacrylamide gel electrophoresis as described in Materials and Methods, transferred to the nitrocellulose membrane, probed with rabbit anti-Lbp polyclonal serum (primary antibodies) and then with the goat-anti-rabbit Alkaline Phosphatase conjugated IgG (secondary antibodies) as described in Materials and Methods, (Fig 4.1.4.4, panel B). An identical polyacrylamide gel was stained with Coomassie Brilliant blue R-250 to ensure that the samples contained equal amounts of streptococcal proteins and that no degradation of protein occurred (Fig. 4.1.4.4, panel A). Analysis of immunoblots suggested that SuM13 did not express Lbp, since no protein band corresponding to Lbp and reactive to anti-Lbp serum (Fig. 4.1.4.4, panel B, lane 3) was found. Alternatively, bovine lactoferrin (Sigma) was labelled with digoxigenin as described in Materials and Methods and was used in place of the primary rabbit anti-Lbp serum as described above. Bound lactoferrin was detected using AP-conjugated anti-DIG monoclonal antibodies (Roche). The absence of the lactoferrin-binding band(s) among SuM13 proteins (Fig. 4.1.4.4, panel C, lane 3) suggests that bovine lactoferrin is not bound by SuM13 protein(s) specifically, which is consistent with Lbp being not expressed.

Binding of the DIG-labelled bovine lactoferrin by Su-1 and SuM13 was assayed by ELISA, as described in Materials and Methods. It was demonstrated that immobilized whole cells of SuM13 had significantly reduced ability to bind bovine lactoferrin compared to the parental Su-1 strain and to E. coli pLBP5, which expresses recombinant Lbp (Fig. 4.1.4.5). This is consistent with the absence of the protein band
Figure 4.1.4.4 Immunoblotting demonstrating that the Lbp is not expressed in the lbp mutant.

Lbp expression by *S. uberis* Su-1 (lane 1), by Su-1 containing pMF113a integrated into the chromosome (lane 2) and by *lbp* mutant SuM13 (lane 3).

A: Polyacrylamide gel stained with Coomassie blue R-250; B: Blot of the gel identical to that shown on the panel A, probed with anti-Lbp polyclonal serum; C: Blot of the gel identical to that shown on the panel A, probed with DIG-labelled bovine lactoferrin. The arrow indicates the position of Lbp protein band. Protein molecular standard sizes are indicated to the left of each gel.
Figure 4.1.4.5 Reduced binding of DIG-labelled bovine lactoferrin by the \textit{lbp} mutant.

The binding of bLf was determined by ELISA using titration of the DIG-labeled bovine lactoferrin against 96-well plates coated with heat-inactivated whole bacterial cells. Lactoferrin-binding by \textit{E. coli} LBP5 expressing a recombinant secreted Lbp was used as a positive control (bar 1) and was considered 100\%. Bar 2, Su-1; Bar 3, SuM13. The data is the mean of 3 experiments; Error bars represent standard deviations.
corresponding to the Lbp on the Western blot, probed with DIG-labeled bovine lactoferrin and anti-DIG labelled antibodies (Fig. 4.1.4.4, C).

4.1.5 Discussion

The prevalence of mastitis caused by environmental pathogens is growing in well-managed herds. For this reason, virulence of environmental pathogens is studied in greater detail. One classical approach to the study of virulence determinant function is the isolation of mutant strains with defined alterations in the gene coding for the determinant. The possibility of generating mutants of environmental streptococci allows one to study the role of each individual putative virulence factor in these bacteria. Several mutant strains of *S. uberis* were isolated, including those generated by insertional inactivation of the *mtuA* lipoprotein receptor (Smith, 2002), *hasA* hyaluronic acid synthesis (Field, 2003), *oppF* oligopermease (Smith, 2002) and other genes. Allele replacement has also been carried out to inactivate the *mig* gene coding for the immunoglobulin receptor of *S. dysgalactiae* (Song, 2001). We reported isolation of the isogenic *lbp* allele replacement mutant of *S. uberis* Su-1 (Moshynskyy, 2003). Allele replacement using conditionally replicating constructs allowed us to overcome the drawbacks of other mutagenesis approaches since it relies on a naturally occurring homologous DNA recombination in bacterial cells (Fig. 4.1.3.3). Reliable phenotypic selection procedure and easy verification of the gene replacement event by conventional methods (Southern and Western blotting) makes allele replacement a valuable tool in the study of individual bacterial genes and their roles in bacterial physiology. The generation of the isogenic *lbp* mutant of *S. uberis* Su-1 suggests that neither the *lbp* gene itself nor its protein product are required for normal *in vitro* growth of *S. uberis*
A dramatic reduction of the lactoferrin binding ability of the \textit{lbp} mutant as compared to the parent Su-1 strain (Fig. 4.1.4.5), suggests that Lbp is the only surface protein of \textit{S. uberis} Su-1 responsible for binding of bovine lactoferrin. All these make the isogenic \textit{lbp} mutant SuM13 a potentially valuable tool for studying the role of Lbp in \textit{S. uberis} physiology and in pathogenesis of bovine mastitis by comparative analyses of the \textit{lbp} mutant and parent Su-1 strain. Irreversibility of the \textit{lbp} mutant suggests that the comparison of the two strains can be carried out both \textit{in vivo} and \textit{in vitro}.

4.2 Study of the Role of Lbp of \textit{S. uberis} in Evasion of the Host Immune Response

4.2.1 Introduction

The lactoferrin-binding protein of \textit{S. uberis} is homologous to M protein of GAS in terms of both amino acid sequence and putative structure. M proteins were implicated in evasion of host immune defenses, allowing the bacteria to survive and multiply in host blood (Courtney, 1997; Poirier, 1989). Surface-associated M proteins have been shown to be members of a family of structurally related M-like proteins that includes several subtypes known as Mrp, Emm, and Enn. M-like proteins, as well as M proteins, have been demonstrated to contribute to streptococcal resistance to phagocytosis (Podbielski, 1996b; Thern, 1998; Kihlberg, 1999). However, not all M-like proteins have antiphagocytic activity, as has been demonstrated for the Arp4 protein (Husmann, 1995) of \textit{S. pyogenes}. Analysis of the predicted secondary structure and genetic
organization of the Lbp of *S. uberis* (Jiang, 1996b) demonstrated a similarity between Lbp and M-like streptococcal proteins (Fig. 1.2.3.1).

The structural similarity of M-like proteins and Lbp of *S. uberis* suggests a possible functional similarity. Therefore, we hypothesized that Lbp might be involved in resistance of the bacteria to antimicrobial defenses of the bovine mammary gland during intramammary infection. Indeed, during such infection, *S. uberis* is exposed to bovine milk neutrophils, milk complement and antibodies present in the milk of the lactating animal. Expression of surface-associated antiphagocytic factors would provide an advantage for the infecting bacteria and promote the survival of *S. uberis* in the presence of bovine neutrophils, lactoferrin and complement, while an *lbp* mutant should be more susceptible to the host antimicrobial defenses. In order to determine the role of Lbp of *S. uberis* in overcoming the bactericidal effect of host neutrophils and complement, we compared the ability of *S. uberis* Su-1 and *lbp* mutant SuM13 to survive and multiply in host serum and blood.

### 4.2.2 Role of Lbp in Survival of *S. uberis* in Whole Bovine Peripheral Blood

Peripheral bovine blood contains both major components of the host innate antibacterial machinery – polymorphonuclear neutrophils and serum complement, which represent factors to which pathogenic bacteria may be exposed. Although *S. uberis* normally does not spread systemically and rarely can be found in the host blood, it can be exposed to complement and neutrophils present in the milk during infection. We used peripheral bovine blood as the source for complement and neutrophils to model bactericidal conditions for *S. uberis* Su-1 and its *lbp* mutant. The role of
Table 4.2.2.1. Multiplication indices of *S. uberis* Su-1 and *lbp* mutant SuM13 during first 3 hours of incubation in fresh nonimmune bovine peripheral blood.

<table>
<thead>
<tr>
<th>Time of incubation, hours</th>
<th>Multiplication index(^1), %,</th>
<th>p value (t Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. uberis</em> Su-1</td>
<td>lbp mutant SuM13</td>
</tr>
<tr>
<td>1</td>
<td>107.4 ± 31.6</td>
<td>97 ± 14.9</td>
</tr>
<tr>
<td>2</td>
<td>100.4 ± 17.3</td>
<td>109 ± 22.5</td>
</tr>
<tr>
<td>3</td>
<td>81.23 ± 15.1</td>
<td>85.4 ± 20.1</td>
</tr>
</tbody>
</table>

\(^1\)Multiplication index was determined as the number of bacterial CFU isolated from the mixture of the bacteria with immune blood divided by the number of bacterial CFU isolated an hour earlier and multiplied by 100%. The data is presented as average multiplication index ± standard deviation, the result of three experiments, each performed in triplicate.

Bacteria were harvested at the exponential phase of growth, diluted in PBS, mixed with fresh heparinized bovine blood and incubated at 37\(^{\circ}\)C. Bacterial numbers in the mix were determined every hour by plating serial 1:10 dilutions on BHI agar. The results are presented as multiplication indices indicating a percentage of survival bacteria in the mixture after each hour of incubation.
Table 4.2.2.2. Multiplication indices of *S. uberis* Su-1 and *lbp* mutant SuM13 during first 3 hours of incubation in fresh bovine peripheral blood supplemented with anti-Su-1 antibodies.

<table>
<thead>
<tr>
<th>Time of incubation, hours</th>
<th>Multiplication index¹, %,</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. uberis</em> Su-1</td>
<td><em>lbp</em> mutant SuM13</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>84.5 ± 9.5</td>
<td>113.8 ± 31.7</td>
<td>0.146655</td>
</tr>
<tr>
<td>2</td>
<td>103 ± 5.4</td>
<td>96.7 ± 17.4</td>
<td>0.291</td>
</tr>
<tr>
<td>3</td>
<td>130.1 ± 17.3</td>
<td>120.8 ± 15.2</td>
<td>0.334251</td>
</tr>
</tbody>
</table>

¹Multiplication index was determined as the number of bacterial CFU isolated from the mixture of the bacteria with immune blood divided by the number of bacterial CFU isolated an hour earlier and multiplied by 100%. The data is presented as average multiplication index ± standard deviation, the result of three independent experiments, each performed in triplicate.

Bacteria were harvested at the exponential phase of growth, diluted in PBS, mixed with fresh heparinized bovine blood supplemented with anti-Su-1 antibodies and incubated at 37°C. Bacterial numbers in the mix were determined every hour by plating serial 1:10 dilutions on BHI agar. The results are presented as multiplication indices indicating a percentage of survival bacteria in the mixture after each hour of incubation.
lactoferrin-binding protein in the resistance of *S. uberis* Su-1 to host antibacterial defense was analyzed in the bactericidal tests as described in Materials and Methods. The results of the bactericidal test indicated that the SuM13 mutant strain survived in whole bovine blood as well as did Su-1 (Table 4.2.2.1).

Both strains appeared to be relatively resistant to the bactericidal properties of bovine peripheral blood since the numbers of bacteria in the bactericidal mix did not decrease significantly during the first 3 hours of incubation, indicating that there was no killing by blood bactericidal components. The presence of anti-*S. uberis* antibodies in the mixture of Su-1 or SuM13 with bovine blood did not have a bactericidal effect on the survival of either Su-1 or SuM13 (Table 4.2.2.2), suggesting that expression of the Lbp on the surface of the bacteria does not affect the resistance of *S. uberis* to the antibacterial properties of blood from an immune host.

### 4.2.3 Lbp and Resistance to Killing of the *S. uberis* by Serum Complement

Resistance of bacteria to serum bactericidal activity is determined at the surface of the bacterial cell. Antibodies and complement cooperate to kill serum-sensitive bacteria and very small concentrations of antibodies confer a high degree of killing activity of complement (Taylor, 1983). Although it is possible that the cell wall and hyaluronic acid capsule protect streptococcal cells from lysis by the membrane attack complex that is formed on the cell surface by activated serum complement, there is no direct data concerning the bactericidal effect of fresh serum on *S. uberis*. To study the susceptibility of *S. uberis* to lysis by host serum complement and the possible role of Lbp in resistance to complement-mediated lysis, we compared multiplication indices of *S. uberis* Su-1 and SuM13 in fresh non-immune bovine serum and in heated serum in
Table 4.2.3.1. Multiplication indices of *S. uberis* Su-1 and *lbp* mutant SuM13 during first 3 hours of incubation in heated bovine serum.

<table>
<thead>
<tr>
<th>Time of incubation, hours</th>
<th>Multiplication index, %, <em>S. uberis</em> Su-1</th>
<th>lbp mutant SuM13</th>
<th>p value (t Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100.8 ± 10.4</td>
<td>109.8 ± 16.6</td>
<td>0.191303</td>
</tr>
<tr>
<td></td>
<td>107.2 ± 7.8</td>
<td>102.7 ± 10.0</td>
<td>0.225227</td>
</tr>
<tr>
<td></td>
<td>107.1 ± 5.4</td>
<td>111.4 ± 4.6</td>
<td>0.105658</td>
</tr>
</tbody>
</table>

Multiplication index was determined as the number of bacterial CFU isolated from the mixture of the bacteria with heated bovine serum divided by the number of bacterial CFU isolated an hour earlier and multiplied by 100%. The data is presented as average value ± standard deviation, the results of three independent experiments, each performed in triplicate.

Bacteria were harvested at the exponential phase of growth, diluted in PBS, mixed with heat-inactivated bovine serum and incubated at 37°C. Bacterial numbers in the mix were determined every hour by plating serial 1:10 dilutions on BHI agar. The results are presented as multiplication indices indicating a percentage of survival bacteria in the mixture after each hour of incubation.
Table 4.2.3.2. Multiplication indices of *S. uberis* Su-1 and *lbp* mutant SuM13 during first 3 hours of incubation in fresh bovine serum.

<table>
<thead>
<tr>
<th>Time of incubation, hours</th>
<th>(^1\text{Multiplication index, %},)</th>
<th>(\text{p value (t Test)})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. uberis</em> Su-1</td>
<td><em>lbp</em> mutant SuM13</td>
</tr>
<tr>
<td>1</td>
<td>90.3 ±4.7</td>
<td>88.5 ± 1.3</td>
</tr>
<tr>
<td>2</td>
<td>105.6 ±12.6</td>
<td>96.6 ± 8.7</td>
</tr>
<tr>
<td>3</td>
<td>99.8 ± 9.5</td>
<td>97.5 ± 8.1</td>
</tr>
</tbody>
</table>

\(^1\text{Multiplication index was determined as the number of bacterial CFU isolated from the mixture of the bacteria with heated bovine serum divided by the number of bacterial CFU isolated an hour earlier and multiplied by 100\%. The data is presented as average value ± standard deviation, the results of three independent experiments, each performed in triplicate.}

Bacteria were harvested at the exponential phase of growth, diluted in PBS, mixed with fresh bovine serum and incubated at 37°C. Bacterial numbers in the mix were determined every hour by plating serial 1:10 dilutions on BHI agar. The results are presented as multiplication indices indicating a percentage of survival of the bacteria in the mixture after each hour of incubation.
which complement enzymes were inactivated. Both strains were capable of surviving in heat-inactivated and in fresh bovine serum (Table 4.2.3.1).

The initial inhibition of growth of the bacterial cultures transferred into blood or bovine serum may be explained simply by adaptation of the cells to a new environment, including new sources of nutrients requiring expression of a new set of genes. The effects of these putative factors on multiplication of Su-1 and SuM13 were similar, suggesting that susceptibility of *S. uberis* to antimicrobial properties of host blood is not affected by the presence of Lbp on the surface of the bacteria.

The major blood bactericidal factors are represented by serum complement and phagocytic polymorphonuclear leukocytes. Streptococci are protected by a thick peptidoglycan cell wall and hyaluronic acid capsule and are therefore not readily susceptible to lysis by serum complement with or without anti-streptococcal antibodies present. This is consistent with observed similarities in survival of both *S. uberis* and *lbp* mutant in heated host serum (Table 4.2.3.1), as well as in fresh naïve serum (Table 4.2.3.2).

### 4.2.4 Role of Lbp in Resistance of *S. uberis* to Killing by Phagocytes

In order to determine the possible role of Lbp in resistance of *S. uberis* to killing by host polymorphonuclear leukocytes (PMN), the two *S. uberis* strains were incubated with isolated bovine peripheral blood PMN and the bactericidal effect on both strains was assessed in direct bactericidal assays. The results indicated that in the presence of anti-streptococcal antibodies, *lbp* mutant was as resistant to killing by PMNs as *S. uberis* Su-1 (Table 4.2.4.1).
Table 4.2.4.1. Multiplication indices of *S. uberis* Su-1 and *lbp* mutant SuM13 during first 3 hours of incubation with bovine peripheral blood PMNs in the presence of anti-Su-1 polyclonal serum.

<table>
<thead>
<tr>
<th>Time of incubation, hours</th>
<th>(^1)Multiplication index, %, S. uberis Su-1</th>
<th>(^1)Multiplication index, %, lbp mutant SuM13</th>
<th>p value (t Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>105.2 ± 13.6</strong></td>
<td><strong>106 ± 15.7</strong></td>
<td>0.454291</td>
</tr>
<tr>
<td>1</td>
<td>103.9 ± 11.7</td>
<td>125.7 ± 17.9</td>
<td>0.07642</td>
</tr>
<tr>
<td>3</td>
<td>110.1 ± 16.5</td>
<td>109 ± 27.2</td>
<td>0.467138</td>
</tr>
</tbody>
</table>

\(^1\)Multiplication index was determined as the number of bacterial CFU isolated from the corresponding mixture of PMNs with bacteria divided by the number of bacterial CFU isolated an hour earlier and multiplied by 100%. The data is presented as average value ± standard deviation, the results of three independent experiments.

Bacteria were harvested at the exponential phase of growth, diluted in PBS, mixed with bovine peripheral blood PMNs and incubated at 37°C. Bacterial numbers in the mix were determined every hour by plating serial 1:10 dilutions on BHI agar. The results are presented as multiplication indices indicating a percentage of survival bacteria in the mixture after each hour of incubation.
Table 4.2.4.2. Multiplication indices of *S. uberis* Su-1 and *lbp* mutant SuM13 inside bovine peripheral blood PMNs.

<table>
<thead>
<tr>
<th>Time of incubation, hours</th>
<th>(^1)Multiplication index, %, &amp; p value (t Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. uberis</em> Su-1 &amp; <em>lbp</em> mutant SuM13</td>
</tr>
<tr>
<td>2</td>
<td>97.6 ± 10.1 &amp; 94.3 ± 8.5 &amp; 0.305419</td>
</tr>
<tr>
<td>3</td>
<td>82.8 ± 9.9 &amp; 89 ± 8.8 &amp; 0.177037</td>
</tr>
</tbody>
</table>

\(^1\)Multiplication index was determined as the number of bacterial CFU isolated from the corresponding mixture of PMNs with bacteria divided by the number of bacterial CFU isolated an hour earlier and multiplied by 100%. The data is presented as average value ± standard deviation, the results of three independent experiments.

Bacteria were harvested at the exponential phase of growth, diluted in PBS, mixed with bovine peripheral blood PMNs and incubated at 37\(^\circ\)C. Extracellular bacteria were killed by added antibiotics. PMNs were washed, lysed and numbers of viable ingested bacteria were determined by plating serial 1:10 dilutions of the lysate on BHI agar. The results are presented as multiplication indices indicating a percentage of survival of bacteria inside PMNs after each hour of incubation.
To assess the ability of *S. uberis* to survive within phagocytic cells and to determine whether Lbp is required for survival, Su-1 and SuM13 were ingested by PMNs, after which the extracellular bacteria were killed with antibiotics as described in Materials and Methods. PMNs were thoroughly washed and lysed, and viable bacteria released from lysed PMNs were scored.

The results indicate that both Su-1 and SuM13 strains have similar levels of resistance to intracellular killing by host peripheral blood neutrophils in the presence of immune serum.

### 4.2.5 Resistance to Phagocytosis by Peripheral Blood Polymorphonuclear Leukocytes

The abilities of Su-1 and SuM13 to resist phagocytosis by host blood polymorphonuclear leukocytes were analyzed by flow cytometry as described in Materials and Methods. The scatter plots of the bovine blood neutrophils co-incubated with fluorescently-labeled bacteria demonstrated that as the neutrophil/CFU ratio decreased tenfold, the percent of gated neutrophils on the plot also decreased one order of magnitude (Fig. 4.2.5.1).

The results imply that at CFU/PMN ratios ranging from 1:10 to 1:1000, essentially 100% of the bacteria (both Su-1 and SuM13) were being ingested by bovine blood neutrophils. This indicates that Su-1 did not differ from SuM13 in the ability to resist ingestion by host neutrophils.
Figure 4.2.5.1 Scatter plot of the fluorescence of bovine peripheral blood neutrophils after 1 hour of co-incubation with fluorescently-labelled S. uberis.

A, neutrophils prior to incubation with bacteria; B, neutrophils incubated with fluorescently-labelled Su-1; C, neutrophils incubated with fluorescently labelled SuM13
*R, targeted CFU/neutrophil ratio;
**Gated, %, the portion of neutrophils fluorescent due to phagocytosis of or association with fluorescently labelled bacterial cells (upper right corner of each plot).
4.2.6 Discussion

It has been suggested that both true M proteins and M-like proteins of streptococci might be important in overcoming host innate immune mechanisms (Staali, 2003). The ability of GAS to survive and to multiply in whole host blood was correlated to the ability of the bacteria to resist phagocytosis by host neutrophils (Schnitzler, 1995a). The role of the streptococcal M- and M-like proteins in evasion of phagocytosis has been demonstrated (Dominigue, 1965; Thern, 1998) and several molecular models were proposed to describe the details of the process and the possible mechanisms involved. One mechanism involves binding of host regulators of complement activators. Acquisition of such regulatory proteins as serum factor H, factor H-like protein or C4-binding protein can inhibit complement-mediated killing of the bacteria or can limit deposition of opsonins on the surface of the bacterial cell, decreasing the pathogen’s susceptibility to phagocytosis. However, expression of the M-like streptococcal protein Lbp on the surface of *S. uberis* Su-1 apparently does not affect resistance of the bacteria to phagocytosis by host neutrophils (Fig. 4.2.5.1). Since fresh bovine serum and heat inactivated serum exhibit similar effects on the survival of both Su-1 and SuM13 (Tables 4.2.3.1 and 4.2.3.2), it is unlikely that Lbp is important in the resistance of *S. uberis* against serum complement attack. Both FH- and FHL-1 binding activity were reported for the Fba protein of *S. pyogenes*, which does not require the presence of M protein to contribute to the survival of the organism incubated with human blood or to inhibit C3 deposition on bacterial cells (Pandiripally, 2002). Similarly, Lbp is not required by *S. uberis* for resistance to the host serum complement. Because Su-1 and SuM13 exhibited similar sensitivity to bactericidal effects of the whole bovine blood in
the presence of an active serum complement and intact neutrophils (Table 4.2.2.1), it is unlikely that Lbp of *S. uberis* plays a role in inhibiting the alternative pathway of activation of serum complement. This is consistent with similar resistance to the antibacterial properties of fresh naïve bovine serum (Table 4.2.3.2) exhibited by Su-1 and SuM13.

It was also demonstrated that streptococcal M and M-like proteins can bind the Fc regions of immunoglobulins, thereby inhibiting activation of the classical complement pathway (Berge, 1997). Both Su-1 and SuM13 were similarly resistant to bactericidal effects of bovine blood in the presence of anti-*S. uberis* antibodies (Table 4.2.2.2) and ingested by peripheral blood neutrophils in the presence of anti-*S. uberis* antibodies with similar efficiency (Fig. 4.2.5.1), suggesting that expression of Lbp did not affect the resistance of *S. uberis* to opsonophagocytosis and to the bactericidal effect of the serum complement activated by the classical pathway.

The ability of virulent streptococci to survive and multiply in whole host blood might be explained not only by an antiphagocytic effect of bacterial surface components. As was suggested (Staali, 2003), the evasion of host defenses by streptococci may occur intracellularly and the survival inside neutrophils may contribute to the pathogenesis of streptococci. Both *S. uberis* Su-1 and its *lbp* mutant SuM13 are ingested by host blood neutrophils with the same efficiency (Fig. 4.2.5.1) and have the same ability to survive within neutrophils (Table 4.2.4.2) or in the presence of neutrophils (Table 4.2.4.1), suggesting that Lbp does not provide resistance to killing by neutrophils intracellularly after ingestion of the bacteria.
It is possible that the immune serum resistance observed with *S. uberis* (Table 4.2.2.2) was not the result of blocking the activation of complement, but rather of impaired insertion of the membrane attack complex into the bacterial membrane. This impairment may be caused by the hyaluronic acid capsule (Almeida, 1993) and thick peptidoglycan layer on the surface of *S. uberis*.

Although the concentration of all complement components in normal bovine milk is not well described, it is known that the C3 opsonic fragment is relatively abundant and can be deposited on the surface of bacteria as was demonstrated for the bovine pathogen *Streptococcus agalactiae* (Rainard, 1995). During mastitis, deposition of both C3 and C4 on bacteria is more effective, reflecting higher amounts of complement and its contribution to the classical pathway of activation (Rainard, 1995). In the presence of antibodies, both alternative and classical pathways of complement activation can occur in bovine milk (Rainard, 1992), suggesting that an ability of bacteria to inhibit or avoid activation of complement may be beneficial during intramammary infection. The data presented here indicate that Lbp is not essential for conferring resistance of *S. uberis* to serum complement activation (Table 4.2.3.2) or its bactericidal effect in the presence of anti-*S. uberis* antibodies (Table 4.2.2.2).

The results of flow cytometry suggest that essentially 100% of both *S. uberis* and *lbp* mutants were ingested by bovine blood neutrophils in our *in vitro* phagocytosis model during 1 hour of incubation. At the same time, it is known that in normal bovine milk, PMNs are less effective phagocytes compared to the blood PMNs used in our study, partially because they contain less glycogen (Newbould, 1973) and hence have
Figure 4.2.6.1 Host bactericidal pathways, in which the role of Lbp of S. uberis was addressed in this study.

A, antibody-dependent phagocytosis of the bacteria by peripheral blood neutrophils (Fig. 4.2.5.1); B, killing of the bacteria by host serum complement (Table 4.2.3.1 and Table 4.2.3.2); C followed by D, opsonophagocytic killing of the bacteria by host blood neutrophils (Table 4.2.4.1); E, killing of ingested bacteria by bovine blood neutrophils (Table 4.2.4.2); B, C, D and E combined, killing of the bacteria by host fresh non-immune blood (Table 4.2.2.1). A, B, C, D and E combined, killing of the bacteria by fresh immune bovine blood (Table 4.2.2.2).
lower energy reserves. Additionally, for efficient phagocytosis, PMNs require large quantities of plasma membrane to form pseudopodia for engulfing bacteria, but many milk PMNs phagocytose casein micelles and/or fat globules (Paape, 2003), reducing the amount of plasma membrane available for phagocytosis of bacteria and formation of phagosomes. In this respect, the model presented is more relevant to mastitic milk neutrophil phagocytosis and the results suggest that Lbp is not essential for resistance of *S. uberis* to phagocytosis by bovine neutrophils.

Apparently, Lbp of *S. uberis* does not play a significant role in any of the processes related to host’s antibacterial defense suggested above and summarized in Fig. 4.2.6.1. The data presented here implies that, unlike M-like proteins of many streptococcal strains, Lbp of *S. uberis* is not essential for inhibition of complement activation and opsonization of the bacteria in the presence of immune serum whether due to the binding of the Fc regions of immunoglobulins, the regulators of complement activation or other serum component(s).

### 4.3 Role of Lbp in Adhesion of *S. uberis* to and Internalization by Host Epithelial Cells

#### 4.3.1 Introduction

Bacterial adhesion to host tissue is considered a critical initial step in colonization and establishment of infection by pathogenic microorganisms. Because M proteins as well as M-like proteins have been implicated in adhesion of human streptococci to host epithelial cells (Caparon, 1991; Courtney, 1992; Wang, 1994; Frick, 2003), we hypothesized that Lbp of *S. uberis* may take part in colonization of bovine
mammary epithelium by promoting adhesion of the organism, since Lbp combines the characteristic structural features of M-like protein with the binding of bovine lactoferrin (Jiang, 1996b). Binding of milk protein(s) (Almeida, 2003), notably lactoferrin (Fang, 2000), was demonstrated to promote adhesion of bovine streptococci, including some strains of *S. uberis*, to cultured mammary gland cells. We addressed the question of whether lactoferrin-binding protein of *S. uberis* Su-1 promotes bacterial adhesion to and invasion of host epithelial cells.

Cell invasion might be an important virulence trait of *S. uberis* that enables the pathogen to enter deeper tissues, to avoid immune surveillance and effector mechanisms at the site of colonization and to gain access to intracellularly located nutrients. Both M and M-like proteins of GAS are associated with the ability of streptococci to invade cultured host epithelial cells (Jadoun, 1997). Therefore, we compared the adherence and invasiveness of the *lbp* mutant SuM13 to those of the wild type strain Su-1.

4.3.2 Adhesion of *S. uberis* Su-1 and SuM13 to and Invasion of the Host Epithelial Cells

To determine the potential role of Lbp in adhesion of *S. uberis* to host epithelial cells, we studied the adherence of *S. uberis* strains Su-1 and SuM13 to cultured MAC-T bovine mammary epithelial cells and to fragments of bovine mammary tissue. Adherence of the bacteria to mammary gland tissue obtained from farm animals *post mortem* should accurately reflect the processes occurring *in vivo*, since besides epithelial cells, all the other cell types and extracellular matrix proteins of the gland are present. However, the accurate quantification of bacterial adherence to such tissue samples poses certain challenges: streptococcal chains can be easily entrapped in
Figure 4.3.2.1. Adhesion of *Streptococcus uberis* Su-1 and *lbp* mutant SuM13 to bovine mammary cells.

A, Adhesion of *S. uberis* Su-1 to trypsinized MAC-T cells; B, Adhesion of *S. uberis* SuM13 to trypsinized MAC-T cells. MAC-T cells were grown in monolayer, trypsinized and resuspended in fresh DMEM containing 4 µg/ml of bovine lactoferrin without antibiotics. Exponentially growing bacteria were added to the MAC-T cell suspension at a cell/bacteria ratio of 1/100 and rotated at 37 °C. The suspension was examined every 10 minutes for the total period of 1 hour. C, Adhesion of *S. uberis* Su-1 to bovine mammary gland explants tissue; D, Adhesion of *S. uberis* SuM13 to bovine mammary gland explants tissue. Mammary tissue was taken and transported from a slaughter facility to the laboratory. It was aseptically minced and incubated with 2000 U of collagenase type III at 37°C in fresh DMEM containing 4 µg/ml of bovine lactoferrin. Approximately 0.1 ml of this suspension was mixed with 10^6 CFU of either strain of *S. uberis* harvested in exponential phase of growth. The suspension was examined every 10 minutes for the total period of 1 hour. Arrows indicate streptococcal chains adherent to bovine mammary cells. Magnification bar: 10 µm.
collagen fibrils, and the viability and integrity of the epithelium can be compromised depending on the method of tissue sampling and on the time elapsed after the animal’s death. Also, deeper tissues with different bacterial adhesion properties can be exposed. For these reasons, we limited the study of *S. uberis* adhesion *ex vivo* to direct microscopic examination of tissue samples incubated with either *S. uberis* (Fig. 4.3.2.1, C; D) or with latex beads coated with partially purified recombinant Lbp (Fig. 4.3.2.2, B).

The results of microscopic examination of trypsinized MAC-T cells co-incubated with either Su-1 or SuM13 suggest that both strains are able to adhere to trypsinized MAC-T cells (Fig. 4.3.2.1, A, B). Both strains also adhered to cells extracted from a bovine mammary gland (Fig. 4.3.2.1, C, D) in the presence of 4 µg/ml of bovine lactoferrin. Adhesion of both strains was also observed microscopically when no lactoferrin was added with identical results (data not shown).

If Lbp promoted adhesion of *S. uberis* to host epithelial cells, adhesion of particles coated with recombinant Lbp to host cells would be easily observed. Latex microspheres, 3 µm in diameter and coated with partially purified recombinant Lbp as described (Kang, 1998), did not adhere to cultured trypsinized MAC-T cells or to the cells extracted from a bovine mammary gland (Fig. 4.3.2.2) in the presence of bovine lactoferrin.

In order to determine if attachment of *S. uberis* to cultured mammary epithelial cells could be inhibited by anti-Lbp antibodies, the bacteria were labelled fluorescently
Figure 4.3.2.2 Adhesion of latex beads coated with recombinant Lbp to bovine mammary cells.

A, Adhesion of latex beads to trypsinized MAC-T cells; MAC-T cells were grown in monolayer, trypsinized and resuspended in fresh DMEM containing 4 µg/ml of bovine lactoferrin. This suspension was mixed with latex microspheres coated with recombinant Lbp at the cell/microsphere ratio of 1/10 and examined microscopically every 10 minutes for the total period of 1 hour. B, Adhesion of latex beads to bovine mammary tissue explants. Mammary tissue was aseptically minced and incubated with collagenase type III in fresh DMEM containing bovine lactoferrin for 2 hours. Approximately 0.1 ml of this suspension was mixed with $10^4$ latex microspheres coated with recombinant Lbp. The mixture was microscopically examined every 10 minutes for a total period of 1 hour. Arrows indicate latex beads. Magnification bar: 10 µm.
Figure 4.3.2.3  Adhesion of fluorescently-labelled bacteria to trypsinized cultured bovine mammary epithelial cells in the presence of polyclonal anti-Lbp rabbit serum.

A. Adhesion of *S. uberis* Su-1 to MAC-T cells; B, adhesion of SuM13 to MAC-T cells. Trypsinized MAC-T cells were allowed to recover for 90 minutes in fresh DMEM without antibiotics, mixed with fluorescently labelled bacteria pretreated with anti-Lbp rabbit serum. The cell/bacteria ratio used was approximately 1/100. The mixture was incubated for 1 hour at 37°C in the presence of 5% rabbit anti-Lbp serum. Magnification bar, 10 μm.
as described in Materials and Methods, incubated with trypsinized MAC-T cells in the presence of anti-Lbp serum and examined microscopically. The results of this fluorescent microscopy indicated that both Su-1 and SuM13 strains were capable of adhering to mammary epithelial cells (Fig. 4.3.2.3), suggesting that since the presence of anti-Lbp antibodies did not inhibit adhesion of \textit{S. uberis} to MAC-T cells, Lbp probably does not play a role in promoting of adhesion of \textit{S. uberis} to host epithelial cells.

The multiplicity of infection used in adherence assays can influence streptococcal invasion (Calvinho, 1998) and thus may potentially affect the experimental results. We determined that the MOI which allowed saturation of the monolayer surface during 3 hours of infection was approximately 50 CFU per cell (Fig. 4.3.2.4). Later in infection, higher numbers of bacterial CFU per cell could be isolated from infected MAC-T cell monolayers (data not shown), but at that time (6-8 hours) damage to MAC-T cells becomes apparent and non-specific bacterial adherence may have taken place.

Although the results of microscopic examination of infected bovine mammary cells suggest that both Su-1 and SuM13 were capable of adherence to host epithelial cells, binding of the host lactoferrin and expression of a lactoferrin-binding protein other than Lbp by \textit{S. uberis} can potentially affect the efficiency of bacterial adherence and the number of adherent bacteria (Fang, 2000). In order to determine whether the Lbp of \textit{S. uberis} Su-1 promoted adhesion of the bacteria to host cells, adhesion of Su-1 and SuM13 to cultured monolayers of MAC-T cells was assayed. Quantitation of the adherent bacteria of the two strains to the monolayers of cultured MAC-T cells
Figure 4.3.2.4 Adherence of S. uberis to MAC-T cell monolayers at different multiplicities of infection.

Monolayers containing 5 \times 10^5 – 8 \times 10^5 MAC-T cells per well were infected at different multiplicities of infection in 6-well plates in antibiotic-free DMEM with S. uberis harvested during the exponential phase of growth. After 3 hours of co-culturing with bacteria, monolayers were washed and lysed with a trypsin/saponin solution. The number of bacteria in the lysate was determined by plating of 1/10 serial dilutions on BHI agar.
Table 4.3.2.1. Adherence of *S. uberis* Su-1 and *lbp* mutant SuM13 to cultured bovine mammary epithelial cells.

<table>
<thead>
<tr>
<th>Strain of <em>S. uberis</em></th>
<th>Adherent CFU/host cell</th>
<th>Adherence index(^1) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Su-1</td>
<td>24.9±5.18</td>
<td>6.6±2.8</td>
</tr>
<tr>
<td>SuM13</td>
<td>29.7±10.6</td>
<td>8.7±4.8</td>
</tr>
</tbody>
</table>

\(^1\)Adherence index was calculated as the number of bacteria adhering to the host cell monolayer divided by the number of bacteria in the culture medium at the time of assay and multiplied by 100.

Monolayers containing 5 x 10^5 – 8 x 10^5 MAC-T cells per well were infected with *S. uberis* at an MOI approximately 100 in 6-well plates in antibiotic-free DMEM. After 3 hours of co-culturing with the bacteria, monolayers were washed and lysed with a trypsin/saponin solution. The number of bacteria in the lysate was determined by plating of 1/10 serial dilutions on BHI agar in duplicate. The result is the average of four independent experiments ± standard deviation.
demonstrated that there was no statistically significant difference between two strains in either the number of adherent CFU per host cell or adherence index (Table 4.3.2.1). These results indicate that the Lbp of S. uberis Su-1 does not play a role in adhesion of the bacteria to host epithelial cells.

*S. uberis* is internalized by epithelial cells (Matthews, 1994) and we hypothesized that Lbp may play a role in this process. In order to study the possible role of Lbp in internalization of *S. uberis* by host mammary epithelial cells, we compared the abilities of Su-1 and SuM13 to invade cultured bovine epithelial cells and studied the consequences of co-incubation of these two streptococcal strains with cultured mammary epithelial cells.

Both *S. uberis* Su-1 and the *lbp* mutant SuM13 were found intracellularly after incubation with bovine epithelial cells for a total period of 4 hours. The number of bacteria recovered from infected MAC-T cell monolayers did not significantly differ between the two strains (Table 4.3.2.2).

Transmission electron microscopy performed on trypsinized MAC-T cells infected with either *S. uberis* Su-1 or SuM13 confirmed that both strains could be found inside host cells. Bacterial cells of both strains were found within membrane-bound vacuoles and some bacteria were dividing since the line of division between two cells was visible in some of the cocci (Fig. 4.3.2.5). This indicated that both Su-1 and SuM13 strains are capable of invasion of host epithelial cells and of intracellular growth. Prolonged (24 hours) co-culturing of *S. uberis* with host epithelial cells resulted in extensive cell injury, expressed as cytoplasm vacuolization, loss of microvilli, loss of
Table 4.3.2.2 The numbers of viable intracellular bacteria recovered from monolayers of MAC-T cells co-incubated with the respective strain of *S. uberis*.

<table>
<thead>
<tr>
<th><em>S. uberis</em> strain</th>
<th>Intracellular CFU/cell, x10^{-3}</th>
<th>Invasion index^{1} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Su-1</td>
<td>35±14</td>
<td>0.7±0.3</td>
</tr>
<tr>
<td>SuM13</td>
<td>39±24</td>
<td>0.9±0.7</td>
</tr>
</tbody>
</table>

^{1} Invasion index was calculated as the number of bacteria recovered from infected host cell monolayer treated with ampicillin and gentamicin divided by the sum of the numbers of adherent and intracellular bacteria at the time of addition of ampicillin and gentamicin to the medium multiplied by 100%.

Monolayers containing 5 x 10⁵ – 8 x 10⁵ MAC-T cells per well were infected at MOI approximately 100 in 6-well plates in antibiotic-free DMEM with *S. uberis* harvested during the exponential phase of growth. After 2 hours of co-culturing with the bacteria, monolayers were treated with antibiotics for 2 hours to kill extracellular bacteria. MAC-T cells were washed with fresh DMEM and lysed with a trypsin/saponin solution. The number of bacteria in the lysate was determined by plating of 1/10 serial dilutions on BHI agar in duplicate. The data is the average of four independent experiments ± standard deviation.
Figure 4.3.2.5  Transmission electron microscopy of MAC-T cells infected with *S. uberis*.
A, MAC-T cells infected with *S. uberis* Su-1; B, MAC-T cells infected with SuM13; C, uninfected MAC-T cells. Arrows indicate internalized bacteria within vacuoles. M, microvilli. CM, cellular membrane. Magnification bar, 1 µm.
MAC-T cells were infected for 24 hours with bacteria harvested in the exponential phase of growth. Transmission electron microscopy was done on samples stained with osmium oxide/uranil acetate.
cellular membrane integrity and destructurizing of the cytoplasm and nucleus (Fig. 4.3.2.5). We do not have an indication whether these morphological changes are the result of the activity of *S. uberis* cells (external or internal).

### 4.3.3 Discussion

The abilities of *S. uberis* Su-1 and SuM13 to adhere to and invade host epithelial cells were studied to determine whether the Lbp plays a role in these processes. Two different *in vitro* approaches were used to compare the adherence of *S. uberis* Su-1 and SuM13 to the host epithelium: co-incubation of the bacteria with cultured bovine mammary epithelial cells and with mammary gland tissue explants from uninfected dairy cows. The use of trypsinized cultured cells has certain drawbacks. Trypsinization of mammalian cells can damage required adhesion receptors or accessory protein(s) on the surface of the cells due to proteolytic activity of trypsin. In addition, trypsin can disturb the surface membrane integrity of cells, affecting their viability, transmembrane electric potential, ion channel functioning and distribution of phosphate moieties. To minimize the potential effect of these on adherence of *S. uberis* and its *lbp* mutant, we harvested cultured MAC-T cells in their exponential stage of growth (60-80% confluence) and allowed cells to recover for 60-90 minutes after trypsinization in fresh DMEM. Su-1 and SuM13 were attached to trypsinized MAC-T cells (Fig. 4.3.2.1) within 10 minutes, suggesting that the factors described above did not affect any proteinaceous components of the host cells responsible for adherence of *S. uberis*. The results of microscopic examination of suspensions of MAC-T cells demonstrated that the Lbp is not required for adhesion of *S. uberis* Su-1, since SuM13, which does not express Lbp, still was able to adhere as efficiently as the parent strain.
The results of the adhesion assay indicate that both *S. uberis* Su-1 and SuM13 could adhere to bovine epithelial cells at a multiplicity of approximately 25 - 30 CFU/cell (Table 4.3.2.1). The presence of the lactoferrin-binding protein, and, consequently, the ability to bind bovine lactoferrin on the bacterial cell surface do not affect the number of bacteria attached to host epithelial cells. This seems contradictory to the results obtained for other *S. uberis* strains (Fang, 2000). Other studies suggest that *S. uberis* is capable of binding to laminin and collagen (Almeida, 1996) and may use these extracellular matrix proteins (Almeida, 1999b) for adherence to cultured bovine mammary epithelial cells, indicating that adherence of *S. uberis* to host epithelium is a multifactorial and possibly strain-dependent process (Calvinho, 1996). Additionally, streptococcal adherence may depend on the characteristics of the host tissue or infection model. The results of an *ex vivo* study of *S. uberis* adherence (Thomas, 1992) suggested that it does not adhere to healthy explanted epithelial tissues. This is in agreement with similar findings (Ditcham, 1996) which demonstrated that *S. uberis* adhered more readily to non-microvillated host cells than to cells with abundant microvilli. Because the presence and abundance of microvilli may serve as an indicator of epithelial cell viability, its membrane integrity and metabolic activity, these findings support the hypothesis (Thomas, 1992) that *S. uberis* adheres primarily to injured host tissue or cells and not to healthy mammary epithelium. This is consistent with our findings that the number of adherent cells of *S. uberis* and its *lbp* mutant per host cell increases with time of incubation (data not shown), possibly because of the increase in the number of injured cultured cells in monolayers co-incubated with the bacteria. Injury to epithelial cells may be due to the secretion of proteolytic or cytotoxic products by streptococcal
cells. For example, *S. uberis* Su-1 has been demonstrated to produce cytotoxin CAMP factor (Jiang, 1996a).

Because anti-Lbp polyclonal serum (Fig. 4.3.2.3) could not prevent attachment of Su-1 and SuM13 to mammary epithelial tissue and because Su-1 adheres to MAC-T cells as efficiently as does SuM13 (Table 4.3.2.1), we conclude that Lbp of *S. uberis* does not play a role in adherence of the organism to host epithelial cells.

Our results also suggest that Lbp is not essential for effective uptake of *S. uberis* by non-phagocytic cells, since the number of Su-1 CFU recovered per ampicillin- and gentamicin-treated MAC-T cells did not differ from that of the isogenic *lbp* mutant (Table 4.3.2.2). Our results also indicate that lactoferrin binding capacity did not affect the invasiveness of *S. uberis* because although SuM13 binds significantly less bovine lactoferrin, cultured bovine mammary cells can internalize the SuM13 strain with the same efficiency. Although preincubation of *S. uberis* with bovine milk was demonstrated to promote bacterial invasion of mammary epithelial cells (Almeida, 2003), our results suggest that bovine lactoferrin was not the component responsible for this enhanced uptake. Overall, *S. uberis* Su-1 may be characterized as a strain with a very low potential for intracellular invasion since only ~1% of adherent bacteria were found intracellularly 3 hours post infection (Table 4.3.2.2) at an MOI of 100 CFU/host cell.

In contrast, the mastitis-causing pathogen *S. dysgalactiae* has a much higher ability to invade mammary epithelial cells (Calvinho, 1998) at the same MOI as was used in our study, although this ability also greatly varies between strains (Almeida, 1995). Like *S. dysgalactiae, S. uberis* and its *lbp* mutant SuM13 damage host epithelial
cells, as apparent from morphological changes of MAC-T cells co-cultured with either Su-1 or SuM13 (Fig. 4.3.2.5). Examination of MAC-T cells co-cultured with *S. uberis* for 24 hours by electron microscopy demonstrated that intracellular *S. uberis* remains viable and possibly propagates since lines of cell division were observed in some intracellular as well as in remaining extracellular bacteria. As shown on the transmission electron micrographs (Fig. 4.3.2.5), after 24 hours of co-incubation with either strain, MAC-T cells show a pronounced necrotic phenotype: liquidified enlarged nucleus, vacuolated cytoplasm, absent or scarce microvilli and destructurized cytosol with a reduced number of membrane-containing organelles. This cell-damaging property may also be a strain-specific trait, since it was previously observed that 24 hours of co-culturing of MAC-T cells with a different strain of a mastitis-causing *S. uberis* isolate did not reveal apparent epithelial cell injury (Matthews, 1994). This may be due to an accumulation of toxic components such as the CAMP factor or other proteolytic enzymes.

Our findings suggest that Lbp is not essential for adherence of *S. uberis* to host epithelial cells. Lbp of *S. uberis* also is not required for internalization of the bacterium by host epithelial cells.

### 4.4 The role of Lbp in Iron Acquisition

#### 4.4.1 Introduction

Iron is required in most organisms for growth and for crucial metabolic pathways. The redox potential of Fe^{2+}/Fe^{3+} favours its use in a number of protein complexes, especially those involved in electron transfer. A number of proteins require
iron for their activity in the form of haem or iron-sulfur clusters in order to transfer electrons. For this reason, iron is abundant in mammals. However, because of its potential toxicity, iron is normally complexed with chelating molecules, which makes it unavailable to infecting bacteria. Bacteria have evolved several mechanisms for acquisition of iron in the host, including production and uptake of ferric iron chelator siderophores, decreasing pH to make ferric iron more soluble, and reducing ferric iron to its more soluble ferrous state, as well as acquisition of ferric iron from host haem, transferrin, lactoferrin or possibly intracellular iron-containing proteins and iron storage components (ferritins). For iron acquisition from host lactoferrin or transferrin, some organisms express lactoferrin- and transferrin-binding proteins. Thus, a plausible role for Lbp of *S. uberis* would be acquisition of iron from lactoferrin. We investigated the possibility that Lbp is a receptor involved in iron acquisition from bovine milk lactoferrin during streptococcal mastitis.

In order to test this hypothesis, we analyzed the dynamics of bacterial growth using four different iron chelators: deferoxamine mesylate, 2,2-dipyridyl, sodium nitroprussi triacetate (NTA) and sodium salt of ethylenediamine-di(o-hydroxyphenylacetic) acid (EDDA). Additionally, we cultured *S. uberis* strains Su-1 and SuM13 in the presence of bovine lactoferrin saturated with $^{59}$Fe$^{3+}$ and assayed the amount of radioactivity acquired by the two strains either on the cell surface or intracellularly.

### 4.4.2 Iron Acquisition by *S. uberis* Su-1 and SuM13

As a first step in determining whether Lbp plays a role in iron acquisition by *S. uberis*, we compared the growth of *S. uberis* Su-1 and SuM13 in a rich complex media...
Figure 4.4.2.1. Growth of *S. uberis* Su-1 and SuM13 in presence of 2,2-dipyridyl. (A), A representative plot of a growth of *S. uberis* Su-1 in BHI broth supplemented with 5 mM of 2,2-dipyridyl; (B), A representative plot of growth of *S. uberis* SuM13 in BHI broth supplemented with 5 mM of 2,2-dipyridyl. The arrow (I) indicates the point of addition of the iron chelator; The arrow (II) indicates addition of 10 mM of FeCl₃ for reconstitution of ferric iron.

- **•**: Control culture in iron-replete medium;  **■**: Culture growth after addition of 2,2-dipyridyl;  **▲**: Growth after reconstitution of ferric iron.
Figure 4.4.2.2. Growth of *S. uberis* Su-1 and SuM13 in presence of deferoxamine mesylate.

(A), A representative plot of growth of *S. uberis* Su-1 in BHI broth supplemented with 5 mM of deferoxamine mesylate; (B), A representative plot of growth of *S. uberis* SuM13 in BHI broth supplemented with 5 mM of deferoxamine mesylate. The arrow (I) indicates the point of addition of the chelator; the arrow (II) indicates the point of addition of 10 mM of FeCl₃ for reconstitution of ferric iron.

- : Control culture in iron-replete medium; ■ : Culture growth after addition of deferoxamine mesylate; ▲ : Growth after reconstitution of ferric iron.
Figure 4.4.2.3. Dynamics of the growth of *S. uberis* Su-1 in the medium depleted of divalent ions.

A, Representative growth curves of the cultures in the medium supplemented with 5 mM EDDA; B, Representative growth curves of the cultures in the medium supplemented with 5 mM NTA. The point of addition of the chelator is indicated with the arrow (I); the point of addition of FeCl₃ for the iron reconstitution is indicated with the arrow (II).

[•] : Control culture; [●] : Culture after supplementation with a divalent ion chelator; [□] : Iron-depleted culture after supplementation it with 10 mM FeCl₃.
in which free iron had been bound by iron chelators. The BHI broth was determined to have a total iron concentration of 80±10 µM and the iron chelators were used at concentrations of 5 mM. Depletion of BHI broth of iron with chelators which specifically remove iron ions from their soluble form did not inhibit the growth of *S. uberis*. Supplementation of the culture medium with 5 mM of 2,2-dipyridyl (Fig. 4.4.2.1) or with 5 mM of deferoxamine mesylate (Fig. 4.4.2.2) at an early exponential phase of growth of *S. uberis* did not inhibit the growth of the culture. Addition of EDDA or NTA to the culture medium at a concentration of 5 mM effectively inhibited growth of *S. uberis* (Fig. 4.4.2.3), but supplementation of the medium with 10 mM of FeCl₃ did not reconstitute bacterial growth, similar to results obtained with *S. mutans* (Martin, 1984).

In order to determine if *S. uberis* could acquire iron directly from bovine lactoferrin, we measured the uptake of ⁵⁹Fe from the ⁵⁹Fe-saturated lactoferrin by Su-1 and SuM13. For assay control we used *Moraxella bovis*, which was demonstrated to express a lactoferrin receptor that is used for iron acquisition (Yu, 2002). Measurement of the amount of intracellular ⁵⁹Fe³⁺ acquired from bovine lactoferrin demonstrated a low level of iron acquisition from bovine lactoferrin by both wild type *S. uberis* and its *lbp* mutant.

In 1 hour, less than 200 CPM (approximately 10¹³ atoms of Fe³⁺) were found intracellularly in 10⁹ CFU of *S. uberis*, (Fig. 4.4.2.4). *M. bovis* was used for a positive control as an organism capable of iron acquisition from bovine lactoferrin. After 1 hour of incubation with ⁵⁹Fe³⁺ lactoferrin, its average total intracellular radioactivity was
Figure 4.4.2.4 Acquisition of $^{59}$Fe from bovine lactoferrin by *S. uberis* Su-1, *lbp* mutant SuM13 and Gram negative bovine pathogen *Moraxella bovis*.

Bacteria were incubated with $^{59}$Fe-saturated bLf for 1 hour and the surface-associated proteins were removed by trypsin treatment. Intracellular $^{59}$Fe was determined as proportional to the total cell radioactivity. The data is the mean of 6 experiments; Error bars represent standard deviation.
determined as approximately 500 CPM per $10^9$ CFU. The bovine pathogen *M. bovis* accumulated approximately 4-5 times more radioactivity than *S. uberis* Su-1 in 1 hour. This corresponds to approximately $5 \times 10^4 - 10^5$ Fe atoms per colony forming unit, which is 5 - 20 times less than the amount typically required by Gram-negative bacteria (McIntosh, 1977). These results indicate that *S. uberis* Su-1 accumulated a significantly higher amount of $^{59}$Fe from iron-saturated lactoferrin than did the *lbp* mutant. However, since growth of both strains was not inhibited in iron-depleted media, and since the total amount of iron accumulated by both bacterial strains was exceedingly low, we suggest that Lbp of *S. uberis* is not required for iron acquisition by the organism.

### 4.4.3 Discussion

It was demonstrated previously that expression of Lbp of *S. uberis* is not regulated by the concentration of iron in the growth medium (Jiang, 1996b). However, the dynamics of *S. uberis* growth in iron-depleted media was not addressed, nor was the Su-1 requirement for iron studied. Our finding that *S. uberis* needs very little iron to support its metabolism is not surprising and is consistent with similar results obtained for porcine pathogen *S. suis* (Niven, 1999) and for the human oral pathogen *S. mutans* (Martin, 1984). However, acquisition of significant amounts of iron has been demonstrated for other streptococcal species such as human pathogens *S. pyogenes* (GAS) (Janulczyk, 2003) and *S. pneumoniae* (Brown, 2001), suggesting that requirements of streptococci for iron may vary between species. Addition of up to 5 mM of dipyridyl (Fig. 4.4.2.1) or 5 mM of deferoxamine mesylate (Fig. 4.4.2.2) was not able to inhibit growth of either *S. uberis* or its *lbp* mutant, suggesting that iron can be
specifically chelated in the medium without an apparent effect on growth of the bacteria. Growth of both Su-1 and SuM13 strains was effectively inhibited by 5 mM of NTA or EDDA, but supplementing the medium with 10 mM FeCl₃ did not reverse this inhibitory effect (Fig. 4.4.2.3). It is likely that NTA and EDDA chelated some divalent ions other than iron essential for growth of *S. uberis*. These may be essential for growth of *S. uberis* and cannot be reconstituted by addition of ferric chloride (Fig. 4.4.2.3). This is in agreement with observations that often iron can be replaced by other metals to support streptococcal metabolism. One example might be manganese, as was shown in the case of *S. mutans* (Martin, 1984).

Although according to one series of data, *S. mutans* requires only Mg for aerobic growth and either Mn or Fe in anaerobic conditions (Paik and Kitten, 2003), other experimental studies suggest that this organism needs iron for optimal growth. Culturing of *S. mutans* in the presence of ⁵⁵Fe results in a significant uptake of radioactivity - up to 7 cpm/CFU (Spatafora, 2001) - and the amount of iron acquired by *S. mutans* reaches 1 - 3 x 10⁶ of iron atoms per cell (Paik and Kitten, 2003). This amount of intracellular iron is similar to that required by *E. coli* cells for optimal growth (Hantke, 1997). The amount of radioactivity acquired intracellularly by *S. uberis* Su-1 corresponds to approximately 10⁴ atoms of ⁵⁹Fe³⁺ per CFU. Considering that cultured *S. uberis* forms chains and thus that 1 CFU potentially represents several individual bacterial cells, this suggests that the requirement of *S. uberis* for iron is approximately 3 orders of magnitude lower than that of *E. coli*. A possible *in vivo* source of metabolic iron and putative components of iron acquisition and metabolism were identified in other streptococcal strains (Lei, 2002; Ricci, 2002). The dynamics of ⁵⁵Fe accumulation
by *S. pyogenes* cells suggested that only $10^3$ - $10^4$ atoms of iron per bacterial cell are accumulated during the exponential phase of growth of the culture in an iron-depleted culture medium supplemented with ferric chloride (Janulczyk, 2003). This amount of intracellular iron corresponds to that determined above for *S. uberis*.

*S. pneumoniae* was suggested to require either Mn or Zn, but not iron, for reconstitution of the growth in a medium supplemented with a divalent ion chelator (Dintilhac, 1997). The requirement for a yet unspecified divalent ion(s) may be the case with *S. uberis* Su-1, since its growth in a metal-depleted (EDDA- of NTA-containing) media could not be reconstituted with ferric chloride. Because both NTA- and EDDA-supplemented media were reconstituted with Ca, Mg, Mn and Zn salts as described in Materials and Methods, we suggest that concentrations of these ions are not growth-limiting factors in the used metal-depleted media.

The very low amount of accumulated radioactive iron by both *S. uberis* strains supports the hypothesis that Lbp is not involved in iron acquisition by the organism. The indication that *S. uberis* Su-1 accumulated significantly more radioactivity from $^{59}$Fe-saturated bovine lactoferrin may be explained by higher local concentration of the lactoferrin bound to the surface of the bacterial cells. Acidification of the growth medium can decrease the affinity of lactoferrin to ferric ion (Brochu, 1998), causing a dissociation of Fe-Lf complex and a passive diffusion of iron released from the lactoferrin molecules into streptococcal cells along the gradient of Fe concentration.

Similar affinities of binding of both apo- and holo-lactoferrin by Lbp of *S. uberis* (Jiang, 1996b); (Moshynskyy, 2003) indirectly support the data above, suggesting that Lbp is not essential for iron acquisition by *S. uberis*. As was
demonstrated for other microorganisms that use host lactoferrin and/or transferrin for iron acquisition, the respective receptors are able to distinguish, at least partially, between apo- and holo- forms of their ligands (Powell, 1998; Retzer, 1998) and bind iron-loaded ligands with higher affinity than the apo- forms.

*M. bovis* has been demonstrated to bind bovine lactoferrin specifically using a two-component lactoferrin receptor typical for Gram-negative bacteria. Although in our study *M. bovis* acquired a significantly higher amount of radioactive iron than any of the *Streptococcus* strains (Fig. 4.4.2.4) representing a positive control of iron acquisition from bovine lactoferrin, the total amount of iron acquired by *M. bovis* was determined as 5 - 20 times less than that required by Gram-negative bacteria in exponentially growing culture. This may be explained by two main reasons. First, in our assay we used iron-starved bacteria pre-incubated with deferoxamine mesylate; therefore, the culture of *M. bovis* was no longer in the optimal conditions of growth. Secondly, in iron-depleted conditions Gram-negative pathogens may use multiple mechanisms of iron acquisition, in which case uptake of iron from bovine lactoferrin would account for only a portion of the total amount of iron internalized by *M. bovis*.

Considering an observed uninhibited growth of *S. uberis* in an iron depleted medium, as well as an exceedingly low amount of radioactive iron acquired from bovine lactoferrin, we conclude that lactoferrin-binding protein is not essential for iron acquisition by *S. uberis* from host lactoferrin.
4.5 Role of the Lbp in \textit{S. uberis} Infection \textit{in vivo}

4.5.1 Introduction

Inflammation of the udder, commonly known as mastitis, arises as a result of intra-mammary gland infection by various microorganisms. Infection of the mammary gland can be caused by either contagious pathogens such as \textit{Staphylococcus aureus} and \textit{Streptococcus agalactiae} or by organisms such as \textit{S. uberis}, \textit{S. dysgalactiae}, \textit{E. coli} and others found in the cow's environment. Clinical signs of mastitis include abnormal milk appearance and composition, swollen quarters, pain, high fever, depressed appetite and elevated body temperature. In subclinical infections, no visible changes occur in the appearance of the milk or the udder but milk production decreases, bacteria are present in secretions, and milk composition is altered.

The origin and mechanisms of development of the inflammatory reaction during streptococcal mastitis differ from those caused by \textit{E. coli} and other Gram-negative infections. For the latter, endotoxin (lipopolysaccharide) triggers an inflammatory reaction and neutrophils attracted to the site of infection effectively control and quickly resolve the infection (Hill, 1979). The role of neutrophils in resolving streptococcal infection is unclear at present. A massive influx of neutrophils during \textit{S. uberis} mastitis (Thomas, 1994) is usually associated with a variable degree of inflammatory reaction (Vaarst, 1997). Immunization of experimental animals with heat-killed \textit{S. uberis} was demonstrated to elicit at least partial protection from challenge with the identical strain (Hill, 1994), suggesting it is possible to develop protective immunity against environmental streptococci, although the molecular basis for such resistance remains obscure. An observation that quarters that had recovered from \textit{S. uberis} in the past are at
higher risk of being infected with a different strain of *S. uberis* (Zadoks, 2001) suggests that besides the possible development of protective immunity, other host-related factors may be responsible for the outcome of an infection.

Lactoferrin receptors, as well as receptors for the related serum metalloprotein transferrin, were suggested as important components of colonization and infection by members of the *Neisseriaceae* and *Pasteurellaceae* families of Gram-negative bacteria (Gray-Owen, 1996).

To study the role of Lbp in virulence during an infection of the mammary gland by *S. uberis* and the role of this protein in the development of clinical signs of mastitis, we carried out an experimental infection of lactating animals with *S. uberis* strain Su-1 and with *lbp* mutant SuM13.

If the lactoferrin-binding protein of *S. uberis* is necessary for the colonization of bovine mammary gland (important for adhesion at the site of infection or for allowing the organism to invade host epithelial tissue more effectively), or if it plays a role in nutrient(s) acquisition or in evasion of the host innate immune response, then it would be expected that *lbp* mutant SuM13 would be less virulent and would be cleared more rapidly.

### 4.5.2 Characterization of Experimental Animals

A total of six Holstein dairy cows in the third quarter of the fifth lactation were used for challenge with live *S. uberis* Su-1 and SuM13. All cows were free from intramammary infection as was determined by microbiological examination of their milk. Anti-*S. uberis* serum IgG titers were measured prior to infection and did not change significantly by day 9 of infection (Table 4.5.2.1). Animals were divided in two
Table 4.5.2.1 Titors of anti-*S. uberis* antibodies in experimental animals.

<table>
<thead>
<tr>
<th>Animal No</th>
<th>Strain used for challenge</th>
<th>Serum IgG titer</th>
<th>Milk IgG titer</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Prior to infection</td>
<td>Post infection</td>
</tr>
<tr>
<td>1</td>
<td>Su-1</td>
<td>7181</td>
<td>4733</td>
</tr>
<tr>
<td>2</td>
<td>Su-1</td>
<td>25960</td>
<td>7989</td>
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<tr>
<td>3</td>
<td>Su-1</td>
<td>7772</td>
<td>6755</td>
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<tr>
<td>4</td>
<td>SuM13</td>
<td>6459</td>
<td>6685</td>
</tr>
<tr>
<td>5</td>
<td>SuM13</td>
<td>24650</td>
<td>5570</td>
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<tr>
<td>6</td>
<td>SuM13</td>
<td>28726</td>
<td>24919</td>
</tr>
</tbody>
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Antibodies were titrated in 96-well round bottom plates coated with whole *S. uberis* Su-1 cells using 1:10 serial dilutions of the serum or milk of the respective animal. This was followed by a titration using 1:2 serial dilutions to determine antibody titers, which were calculated as reciprocal of the last positive plus 2 standard deviations.
groups, one of which was challenged with *S. uberis* Su-1 and the other with SuM13. Live bacteria harvested at the early exponential phase of growth at an OD$_{600}$~0.2 were resuspended in sterile PBS to approximately $1.5 \times 10^6$ CFU/ml. Three ml of the bacterial suspension were inoculated into the right front and right hind quarters of the animals immediately after the morning milking (Day 0). The left quarters were used as internal controls. Somatic cell count (SCC) in milk prior to infection (Day 0) averaged $1.8 \times 10^7$ per ml. Quarters that were inoculated with bacteria and from which either strain of *S. uberis* was isolated after day 3 post infection, were defined as “challenged and infected”. Those quarters that were inoculated with bacteria but from which neither strain of *S. uberis* was isolated after day 3 post infection, were designated “challenged but not infected”. No significant changes in SCC of challenged but not infected quarters were observed (data not shown).

### 4.5.3 Colonization of Bovine Mammary Gland

Out of 6 quarters, each challenged with $4.5 \times 10^6$ CFU of *S. uberis* Su-1, 3 were considered colonized by the bacteria, since it was detected in milk samples during the entire experiment. Of the 6 quarters challenged with $4.8 \times 10^6$ CFU of the *lhp* mutant SuM13, bacteria were recovered from 3 quarters for the duration of the experiment. Bacteria were detected in all challenged quarters 12 hours after challenge, but none were isolated for the next 24 hours. From day 3 of the trial until the end, low numbers of *S. uberis* were isolated from milk samples (Fig. 4.5.3.1). Organisms were occasionally isolated from non-challenged quarters, but these were identified as non-streptococcal species. Bacterial colonies isolated from the challenged quarters were randomly selected and microscopically examined. Those from infected quarters were
Figure 4.5.3.1. Numbers of bacteria isolated from challenged infected bovine mammary glands.

A, quarters infected with *S. uberis* Su-1; B, quarters infected with *lbp* mutant. The limit of detection was 10 CFU/ml. Bars represent average bacterial titers in milk from challenged and infected quarters.
identified as *Streptococcus* species based on the results of microscopic examination. Three randomly picked colonies isolated on day 7 from each quarter were analyzed using an Api20Strep strip test (see Materials and Methods) and all organisms from challenged infected quarters were identified as *S. uberis*. None of the randomly picked colonies from the “challenged but not infected” or from the control quarters were identified as *S. uberis*. All of the streptococcal colonies isolated from SuM13-infected quarters were resistant to 100 µg/ml of spectinomycin, which is consistent with the SuM13 phenotype. None of the bacteria isolated from Su-1-infected quarters were resistant to spectinomycin, suggesting that there was no cross-infection between challenged quarters during the experiment.

No clinical manifestation of mastitis was observed in any of the experimental or control quarters. The milk samples did not present any visual abnormalities such as protein aggregates or clots in the milk. No redness or swelling of mammary glands was observed and rectal temperature remained normal in all animals. All of the above suggest that the infection of the challenged lactating mammary glands with both Su-1 and SuM13 resulted in subclinical infection, a common occurrence with *S. uberis* (Zadoks, 2003).

### 4.5.4 Somatic Cell Count

The number of somatic cells detected in the milk of infected animals was high throughout the trial (Fig. 4.5.3.2). It should be noted that the somatic cell count in the milk prior to the infection was already relatively high due to the state of lactation of the animals. However, that in itself cannot account for the high levels of SCC observed in experimental animals. Since the animals were not screened for mycoplasma, fungal or
Figure 4.5.3.2. Somatic cell count in milk from challenged infected quarters of bovine mammary glands.

A, quarters infected with *S. uberis* Su-1; B, quarters infected with *lbp* mutant. Bars represent average SCC in milk from challenged and infected quarters.
viral infections, this might account for the high cell counts. Their SCC ranged from 5x10^6/ml to 2x10^8/ml. There was no statistically significant difference in SCC between quarters through the duration of the experiment. The average somatic cell count from the quarters infected with Su-1 was slightly higher than that from the quarters infected with SuM13 (Fig. 4.5.3.2), but since the difference was not statistically significant, it was not considered indicative of a higher virulence of the Su-1 strain.

4.5.5 Discussion

M-like protein(s) have been suggested as one of the virulence factors of *S. uberis* (Oliver, 1998). Binding of bovine lactoferrin by *S. uberis* was suggested to promote adherence of the bacteria to the host epithelial cells, aiding in colonization of the host (Fang, 2000). Since Lbp of *S. uberis* is structurally related to M-like proteins and binds bovine lactoferrin, we hypothesized that during infection, Lbp might play a role in colonization of the host or in pathogenicity of infection. However, no dramatic difference in virulence between *S. uberis* Su-1 and lbp mutant SuM13 was observed during in vivo experimental infection. From the pattern of bacterial shedding, it may be suggested that a substantial amount of time (up to 72 hours) is required for the establishment of a productive infection by *S. uberis* Su-1 (Fig. 4.5.3.1, A). The presence of the bacteria in the first post-challenge milking samples indicates that a large portion of inoculated bacteria were washed out of the gland as expected and as reported by others (Rambeaud, 2003). In contrast to the infections with Su-1 and SuM13, a progressive inflammation of mammary tissues has been reported following experimental *S. uberis* infection as early as 8 hours, with bacteria detected in macrophages and neutrophils and within alveoli (Pedersen, 2003). It is possible that the
length of lag in shedding of *S. uberis* is a strain-dependent feature (Doane, 1987).

Milk from the mammary gland contains leukocytes, including macrophages, neutrophils, and lymphocytes, typically at <150,000 cells/ml or 200,000 - 300,000 cells/ml (Zadoks, 2001). According to published survey results, herds with low bulk SCC counts are at a higher risk of contracting mastitis caused by Gram-negative bacteria (Suriyasathaporn, 2000), while herds with higher somatic cell counts tend to be more prone to clinical mastitis caused by *Staphylococcus aureus* and streptococci (Barkema, 1999b). Experimental infection of the mammary gland with virulent strains of *S. uberis* results in the appearance of large numbers of neutrophils in the interstitial tissues and secretions (Smits, 1998). Infection usually results in an inflammatory response, which leads to an increase in the number of cells, primarily due to the influx of neutrophils from the peripheral circulation. Milk from clinically infected quarters usually contains more than 2,000,000 cells/ml, over 90% of these being neutrophils (Field, 2003). As suggested by the results of our experiment, somatic cell counts in the milk of animals late in lactation may increase without other apparent inflammatory responses independent of colonization of the mammary gland by *S. uberis*, although as was mentioned above, the SCC were extremely high, perhaps due to viral, mycoplasma or fungal infection. However, the data are consistent with higher rates of clinical *S. uberis* mastitis during early lactation (Hockett, 2000).

The duration of *S. uberis* Su-1 and SuM13 experimental intramammary infections (>9 days) was consistent with the duration of subclinical mastitis reported for environmental Gram-positive cocci (12.5 days) (Todhunter, 1995), indicating that a lack of Lbp expression on the bacterial surface does not result in elimination of *S. uberis* by
the host and does not abolish the ability of \textit{S. uberis} to survive within the mammary gland for an extended period of time.

The dynamics of bacterial concentration in milk from infected quarters differed from that of previously reported data. While both Su-1 and SuM13 were detectable in the milk at low concentrations through the whole period of experiment, infections with more virulent strains resulted in higher bacterial counts ($10^4$ to $10^7$ CFU/ml) in the milk during a period between 24 and 60 hours post infection, after which time the bacterial counts decreased significantly (Smits, 1998). The appearance of clinical signs of mastitis coincided with the presence of large numbers of \textit{S. uberis} in the milk (Rambeaud, 2003), suggesting that in the experimental Su-1 and SuM13 infections, low bacterial concentrations in milk may be responsible for development of mainly subclinical mastitis in infected animals. The relatively high number of somatic cells in the milk prior to inoculation of bacteria also might have contributed to the limited number of viable \textit{S. uberis} Su-1 and SuM13 in the mammary glands of experimental animals. As was demonstrated earlier (Finch, 1994), the presence of specific anti-streptococcal antibodies in the serum and in the milk of experimental animals prior to challenge with \textit{S. uberis} may prevent the development of clinical symptoms of mastitis and keep milk bacterial titers at a very low level (often under 500 CFU/ml). The exact mechanism underlying such apparent partial immunity is not clear since no increased opsonic activity of serum or milk was detected with increased levels of \textit{S. uberis}-specific IgGs (Hill, 1994; Finch, 1994). We did not find an association between the ability of \textit{S. uberis} to colonize bovine mammary glands and antibodies titers in either milk or serum (Table 4.5.2.1).
Titers of *S. uberis* in the lactating mammary gland may reach $10^7$ CFU/ml of milk (Rambeaud, 2003), provided the bacteria resist the bactericidal action of neutrophils. The results of the Su-1 and SuM13 experimental infection indicate that somatic cell counts were generally inversely proportional to bacterial titers in milk (Fig. 4.5.3.1 and Fig. 4.5.3.2), suggesting that the bacterial population in milk was at least to some extent controlled by host cells, possibly neutrophils. Despite significant fluctuations in all quarters studied, average SCC in quarters infected with Su-1 was higher than in quarters infected with SuM13. We did not consider this indicative of more severe mastitis development since the difference in SCC values between quarters infected with Su-1 and SuM13 was not statistically significant.

The absence of cross-infection between quarters agrees with a previous suggestion that intramammary infections caused by environmental organisms are predominantly transmitted between cows but not between quarters of the same animal (Baxter, 1992).

Both Su-1 and SuM13 were able to colonize experimental animals and to persist in the mammary glands for the whole period of experiment (10 days). We suggest that Lbp of *S. uberis* is not essential for colonization of the host, whether its function is related to adherence, intracellular invasion, resistance to host antibacterial immunity, nutrient acquisition, or other function(s).

### 4.6 Role of Lbp in Regulation of Gene Expression

#### 4.6.1 Introduction

We hypothesized that Lbp of *S. uberis* may be involved in signal transduction as
a part of a two-component sensor-effector bacterial system as a sensor surface component that binds lactoferrin, a ligand abundant at the site of infection. Prokaryotic signal-transduction systems as well as many eukaryotic pathways use phospho transfer schemes involving two conserved components: a protein kinase and a response regulator protein (Stock, 2000). For example, several key virulence determinants in GAS are controlled by the two-component sensor-regulator system CsrS/CsrR, depending on phosphorylation of its regulator component CsrR (Gryllos, 2003). For Group B streptococcus (GBS), it was reported that tyrosine phosphorylation by CpsD regulates capsular polysaccharide production (Cieslewicz, 2001) in response to environmental stimuli.

It was suggested that the presence of certain surface bacterial proteins or a combination of such proteins may influence transcription of various host genes potentially involved in the host-pathogen relationship during streptococcal infection. When exposed to bacterial products, host cells can initiate the activation of genes encoding proteins that modulate the innate immune response (Strieter, 2002). For example, the local and systemic inflammatory response is orchestrated by a complex cytokine network. Cytokines are mainly synthesized de novo (Taniguchi, 1988) following stimulation by the bacterial cellular and secreted products and are secreted by the cells present at the site of infection. Although the transcriptional response to streptococcal infection of phagocytic cells was studied earlier (Kobayashi, 2003), professional phagocytes are not the only type of host cells encountering the bacteria and potentially capable of mediating inflammation in the mammary gland. The other cell types that eventually encounter pathogens include T- and B-lymphocytes, natural killer
(NK) cells and secretory epithelial cells. The interaction of the host epithelial cells with *Streptococcus uberis* was studied previously (Matthews, 1994), although an altered expression of host genes was not demonstrated. To date, several streptococcal components have been implicated in regulation of a host cell gene expression. One such component is the pneumococcal choline-binding protein CbpA, capable of binding secretory IgA (Hammerschmidt, 1997), the third component of complement (C3) (Cheng, 2000) and human complement factor H (Dave, 2001). The secondary structure of CbpA (NCBI protein accession number NP_357715) predicted by COIL algorithm (http://www.ch.embnet.org/software/COILS_form.html) suggests that a large portion of this protein may form a coiled-coil structure similar to the Lbp of *S. uberis*. This protein also was implicated in adherence (Rosenow, 1997) and invasion (Zhang, 2000) of *S. pneumoniae*. The spectrum of the ligands the Cbp binds, a predicted secondary structure and the suggested role in adherence and invasion are consistent with CbpA of *S. pneumoniae* being related to M-like streptococcal proteins. The choline binding protein was demonstrated to increase the intracellular content of mRNA for chemokines and for the intercellular adhesion molecule 1 (ICAM-1, CD54) by human alveolar epithelial cells upon infection with *S. pneumoniae in vitro* (Murdoch, 2002), and chemokines and ICAM1 are considered important for the regulation of inflammation and leukocyte trafficking, respectively. This suggests that Cbp of *S. pneumoniae* may play a role in the host response to streptococcal infection, by regulation of host gene expression. Importantly, this protein has a homology to the Lbp of *S. uberis*. This allowed us to hypothesize that Lbp of *S. uberis* may play a role in the host cell transcriptional response, possibly affecting the course and outcome of mastitis infection.
One of the results of *Streptococcus*-host cell interaction was shown to be host cell apoptotic death (Braun, 2001; Fettucciari, 2000; Marouni, 2004). Transcriptional activation of a set of growth factors has been demonstrated in bovine mammary tissues of animals infected with *S. agalactiae* (Sheffield, 1997). These were implicated in cell cycle regulation and included apoptosis markers.

Human mononuclear cells stimulated with GBS were demonstrated to produce a soluble factor(s), affecting reactive oxygen production and expression of inducible nitric oxide (NO) synthase by cultured epithelial cells (Goodrum, 2002), which suggested a role of streptococci-induced signalling in free-radical tissue injury or antibacterial tissue defense. This implies that infection of host epithelial cells with live streptococci may result in upregulation or downregulation of the expression of host genes, affecting the consequences of the infection for both the bacterial and the host cells. Indeed, GBS induce synthesis and secretion of TNF-α, IL-1, IL-6, IL-8, IFN-γ and IL-12 by host mononuclear cells (Kwak, 2000). *Streptococcus bovis* increases production and secretion of adrenomedulin, which belongs to cationic antimicrobial peptides (Allaker, 1999). Antimicrobial peptides are synthesized at mucosal surfaces as effectors of innate host defenses in response to the presence of microorganisms, as was demonstrated *in vitro* for gastric adenocarcinoma cells (Allaker, 2003). Pneumolysin of *S. pneumoniae* up-regulates expression of interleukin 2 receptor β, interleukin 15 receptor α, down-regulates complement receptor 2 and affects expression of other inflammatory mediators and antibacterial components by human mononuclear cells (Rogers, 2003).

An example of signal transduction triggered by streptococcal M protein is the
activation of phosphatidylinositol 3-kinase (PI 3-K) leading to epithelial cell cytoskeleton rearrangement and promoting internalization of the bacterium by host epithelial cells (Purushothaman, 2003). Although the transcriptional response of epithelial cells to the presence of M protein has not been studied, PI 3-K is considered one of the key components of intracellular signalling pathways, regulating transcription of an array of eukaryotic genes (Wang, 1999), some of them crucial for cell differentiation and regulation of inflammation (Klein, 2002). Activation of PI 3-K is also important for the transcriptional regulation of the inducible nitric oxide synthase (Pahan, 1999), an enzyme responsible for production of NO (a reactive oxygen species playing a role both in killing of intracellular bacteria by phagocytic cells and in tissue damage during inflammation). In order to determine if the Lbp of \textit{S. uberis} plays a role in signal transduction as a part of a bacterial two-component signal transduction system, we analysed differential protein phosphorylation of Su-1 and SuM13 treated with bovine lactoferrin. To study the role of Lbp in bacteria-host interaction, we analyzed differential protein phosphorylation of MAC-T cells infected with Su-1 and with SuM13. For analysis of host cell transcriptional response affected by Lbp of \textit{S. uberis}, we carried out a cDNA microarray hybridization of the cells infected with Su-1 and with SuM13.

### 4.6.2 Role of Lbp in Differential Phosphorylation of Proteins and Host Cell Morphological Changes

Since the M-like protein genes may be central to streptococcal virulence and because regulatory elements must respond to environmental signals and control the expression of virulence genes in streptococci, we hypothesized that M protein may play
a role in the control of expression of virulence genes. We hypothesized that binding of bovine lactoferrin resulting from *S. uberis* entering into the mammary gland may serve as a signalling event for the streptococcal cell, which includes a protein phosphorylation event.

In order to detect a phosphorylated protein product(s) formed in *S. uberis* cells upon interaction of the bacterial surface Lbp with host lactoferrin, we incubated *S. uberis* Su-1 and its *lbp* mutant SuM13 with bovine lactoferrin in the presence of $\gamma^{32}$[P]ATP and analysed the protein phosphorylation profiles. Similarly, we analyzed the host cell protein phosphorylation profiles after exposing MAC-T cells to *S. uberis* Su-1 and SuM13 in the presence of $\gamma^{32}$[P]ATP. The amount of total protein loaded on the gel per lane was verified by staining of the protein gels with coomassie brilliant blue. Following autoradiography, only one band of total cellular streptococcal proteins (Fig. 4.6.2.1, A) was detected in the Su-1 strain. The electrophoretic mobility of this protein coincided with that of bovine lactoferrin present in the cultures (Fig. 4.6.2.1, B, lane Lf) and was much more faint in Su-1 protein samples with no lactoferrin added (Fig. 4.6.2.1, A, lane 2) and in SuM13 protein extracts (Fig. 4.6.2.1, lanes 3 and 4). Similar amounts of phosphorylated bovine lactoferrin were detected in the culture medium protein samples (Fig. 4.6.2.2, B) of both strains. Higher amounts of lactoferrin in Su-1 protein extracts (Fig. 4.6.2.1, A) can be explained by the capability of Su-1 cells to bind significantly larger amounts of bovine lactoferrin. Phosphorylation of the bovine lactoferrin was detected in the absence of bacterial cells (Fig. 4.6.2.1, B, lane Lf), albeit to a lesser degree.
When bacterial cells were treated with trypsin prior to extraction of cellular proteins, there was an additional phosphorylated protein, distinct from bovine lactoferrin, present in both Su-1 and SuM13 cells, with electrophoretic mobility similar to that of bovine lactoferrin (Fig. 4.6.2.1, B, the band is indicated by the arrow).

These results did not support the hypothesis that Lbp of *S. uberis* is involved in bacterial signal transduction as a part of a two-component bacterial regulatory system that employs an event of protein phosphorylation.

To check the hypothesis that Lbp of *S. uberis* is involved in the regulation of those host cell signal transduction pathways that include protein phosphorylation, we analysed protein phosphorylation profiles of the cultured MAC-T epithelial cells infected with *S. uberis* Su-1 and SuM13. No differential protein phosphorylation was observed in cells infected with the two strains (Fig. 4.6.2.3, lanes 3 and 5; lanes 4 and 6). However, protein bands were detected in the extracts from the cells infected with *S. uberis* (Fig. 4.6.2.3 lanes 3, 4, 5 and 6) but not in those treated with lactoferrin alone (Fig. 4.6.2.3 lane 2) or in untreated cells (Fig. 4.6.2.3 lane 1). Additionally, distinct bands of phosphorylated proteins were present in protein extracts from the cells infected with *S. dysgalactiae* (Fig. 4.6.2.3, lanes 7 and 8), indicating that differential protein phosphorylation can be detected using this approach. This indicates that infection with *S. uberis* Su-1 and SuM13 induced protein phosphorylation-associated events in host epithelial cells. There also was no indication of whether these protein bands are of bacterial or of host cell origin. However, comparing the autoradiographs of the protein profiles of streptococcal cells (Fig. 4.6.2.1, A) with those of the MAC-T cells proteins (Fig. 4.6.2.3, B) suggests that at least some bands of phosphorylated proteins are host
Figure 4.6.2.1  Autoradiographs of total phosphorylated cellular proteins of *S. uberis*.

A, Total cellular proteins of the bacteria; B, Cellular proteins of the bacteria treated with 1% trypsin; Lane 1, Su-1 in the presence of bLf; lane 2, Su-1 without bLf; lane 3, SuM13 in the presence of bLf; lane 4, SuM13 without bLf; lane Lf, bovine lactoferrin incubated with $\gamma^{32}$PATP without bacteria. The arrow indicates the position of the major bLf protein band in SDS PAGE.
Figure 4.6.2.2 Phosphorylated proteins detected in the growth media of *Streptococcus uberis*.

Proteins present in the growth media of bacteria treated with bovine lactoferrin in the presence of $\gamma^{32}$P[ATP] were separated by SDS polyacrylamide gel electrophoresis. The gel was stained with Coomassie brilliant blue R-250 (A) and exposed to X-ray film (B); Lanes 1, 2 and 3, protein extracts from the Su-1 culture media; Lanes 4, 5 and 6, protein extracts from the SuM13 culture media; Lanes 1 and 4, protein extracts from the culture media with no lactoferrin added. H, high molecular weight protein standards; L, low molecular weight protein standards, both from Bio-Rad; Molecular weight of the proteins indicated in kDa. The arrow indicates the position of the major protein band of bovine lactoferrin.
Figure 4.6.2.3 Protein phosphorylation in *Streptococcus*-infected bovine mammary epithelial cells (MAC-T).

Cultured bovine mammary epithelial cell were infected with *S. uberis* in the presence of bovine lactoferrin and γ${}^{32}$P[ATP. Cells were lysed and proteins were separated by SDS polyacrylamide gel electrophoresis. The gel was stained with Coomassie R-250 (A) and exposed to the X-ray film (B); Lanes: 1, MAC-T cells alone; 2, MAC-T cells with 4 μg/ml of bovine lactoferrin; 3, MAC-T cells infected with Su-1; 4, MAC-T cells infected with Su-1 in presence of 4 μg/ml of bovine lactoferrin; 5, MAC-T cells infected with SuM13; 6, MAC-T cells infected with SuM13 in presence of 4 μg/ml of bovine lactoferrin; 7 and 8, MAC-T cells infected with *S. dysgalactiae*. H, high molecular weight protein standards; L, low molecular weight protein standards; both from Bio-Rad.
cell proteins differentially phosphorylated during infection with *S. uberis*. Since no differences in protein phosphorylation profiles were detected between cells infected with Su-1 and SuM13, there was no evidence that Lbp of *S. uberis* was responsible for the protein phosphorylation-associated signalling in the host cells.

The consequences of the host cell interaction with streptococci have been demonstrated to have no effect (Matthews, 1994), to result in complete cell lysis by streptococcal proteolytic enzymes (Sierig, 2003), or to result in apoptotic death (Marouni, 2004). To address the question of whether Lbp of *S. uberis* promotes apoptosis of host epithelial cells, we studied changes that occurred in cultured bovine mammary epithelial cells exposed to either *S. uberis* Su-1 or SuM13. These included both morphological changes as well as chromosomal DNA fragmentation, the latter being a characteristic marker of apoptosis. Cells grown in monolayers and co-incubated with either strain exhibited a necrotic phenotype, including enlarged, rounded, non-detached cells with enlarged nuclei, often appearing to possess several nuclei per cell (Fig. 4.6.2.4, B and C). This morphology was independent of the presence of lactoferrin in the culture medium, suggesting that neither binding of lactoferrin nor expression of the Lbp by the bacteria was required for induction of the described morphological changes in epithelial cells. Infection of the monolayers with heat-inactivated or with formalin-fixed Su-1 or SuM13 did not produce obvious morphological changes in cultured epithelial cells (Fig. 4.6.2.4, D and E), regardless of the presence of lactoferrin.

Cultured MAC-T cells were infected with either Su-1 or SuM13 harvested in the exponential phase of growth. After co-incubation with bacteria for 24 hours, cells were examined by light microscopy. Trypsinized cells infected with either strain also
Figure 4.6.2.4  Morphological changes in cultured epithelial cells co-incubated with *S. uberis* in the presence of bovine lactoferrin.

A: uninfected MAC-T epithelial cells; B, MAC-T cells co-incubated with live Su-1; C, MAC-T cells co-incubated with live *lbp* mutant SuM13; D, MAC-T cells co-incubated with formaldehyde-fixed Su-1; E, MAC-T cells co-incubated with formaldehyde-fixed SuM13. Arrows indicate enlarged MAC-T cells, some of them with more than one nucleus; Magnification X 300.
Figure 4.6.2.5  Morphological changes in trypsinized MAC-T cells co-incubated with live *S. uberis*.

A, light microscopy of uninfected epithelial cells stained with Bis-Benzidine, magnification x 400; B light microscopy of the cells infected with Su-1 in presence of bovine lactoferrin, stained with Bis-Benzidine, magnification x 400; C, electron microscopy image of a cell from panel A, magnification x 4500; D, electron microscopy image of a cell from panel B, magnification x 4500. Arrows indicate: M, mitochondria; MV, microvilli; CM, cellular membrane; NM, nuclear membrane; N, nucleus; V, vacuolae.
exhibited a necrotic phenotype (Fig. 4.6.2.5, B), which included enlarged cells with swollen nuclei. The presence of lactoferrin had no effect on this phenotype. Electron microscopy of bovine epithelial cells co-cultured with live Su-1 or with live SuM13 demonstrated that in infected MAC-T cells, the cytoplasm became liquidified, the number of vacuoles increased, cell membrane integrity was lost, most subcellular structures disappeared and infected cells no longer possessed microvilli. Cells infected with live bacteria of both strains had enlarged nuclei, decreased electron density of both nuclei and cytoplasm and breaches in the nuclear membrane (Fig. 4.6.2.5). All these features are characteristics of the necrotic cell death phenotype, suggesting that neither Su-1 nor SuM13 induced apoptosis in MAC-T cells.

Although no morphological changes consistent with an apoptotic phenotype were detected in MAC-T cells infected with either strain of *S. uberis*, cell enlargement occurring as a result of the damage to the cellular membrane might mask ongoing apoptosis. Such damage may be caused by the CAMP factor or by non-specific proteolytic enzymes expressed by *S. uberis*. For this reason, MAC-T cells infected with Su-1 and SuM13 were analyzed for their DNA fragmentation, which is a distinctive feature of cells undergoing apoptosis. As a positive control for DNA fragmentation, MAC-T cells were treated with 20 µM of staurosporin, which induces apoptosis in all known cell types and is a non-specific inhibitor of protein phosphorylation. Characteristic DNA fragmentation was detected in MAC-T cells treated with staurosporin (Fig. 4.6.2.6, lanes 1 – 4). No DNA fragmentation, which occurs when a significant proportion of tested cells undergo apoptosis, was detected in the cells co-cultured with either live Su-1, live SuM13, heat-inactivated Su-1 or heat-inactivated
Figure 4.6.2.6  Analysis of DNA “laddering” in MAC-T cells infected with *S. uberis*.

Total DNA from the cells treated with staurosporin (Lanes 1-4), from cells co-incubated with live *S. uberis* Su-1 (lane 5), with heat-inactivated *S. uberis* (lane 6), with live *lbp* mutant (lane 7) and with heat inactivated *lbp* mutant SuM13 (lane 8), all in presence of lactoferrin;
M, 100 base pair ladder DNA marker.
SuM13, all in the presence of bovine lactoferrin (Fig. 4.6.2.6). This result is consistent with the data obtained by studying the morphology of infected MAC-T cells. It indicates that neither \textit{S. uberis} Su-1 nor \textit{lbp} mutant SuM13 induced apoptosis in cultured host epithelial cells. This suggests that Lbp of \textit{S. uberis} does not play a role in induction of apoptosis in host cells.

4.6.3 Transcriptional Response to \textit{S. uberis} Infection

The reaction of the host tissue to bacterial infection can determine the outcome of infection. We studied the transcriptional response of cultured bovine mammary epithelial cells following infection with Su-1 and SuM13 in order to determine the role of Lbp in regulation of host gene expression. To study the transcriptional response of host epithelial cells to the presence of Lbp of \textit{S. uberis} during infection \textit{in vitro}, a cDNA microarray hybridization of MAC-T cells infected with each strain was carried out. Microarray hybridization of Su-1-infected versus SuM13-infected cells was selected in order to limit the number of analyzed host genes to those specifically regulated by Lbp.

Twenty eight individual bovine genes were identified as repressed in the presence of \textit{S. uberis} relative to the \textit{lbp} mutant (Table 4.6.4.1). The expression of these genes was significantly altered (p<0.05) in all 3 hybridization experiments. None of the genes on the array were found to be consistently up-regulated. The results of 3 independent experiments demonstrated that genes that play roles in cell cycle regulation and/or cell motility and plasticity were consistently down-regulated in the presence of Lbp of \textit{S. uberis}. Human ortholog open reading frames were identified for all probe sequences (Table 4.6.4.1).
Table 4.6.3.1. Genes significantly down-regulated in cultured mammary epithelial cells infected with *S. uberis* Su-1, as determined by microarray hybridization, compared to the cells infected with *lbp* mutant SuM13.

Microarray slide IDs in bold were selected for further analysis.

<table>
<thead>
<tr>
<th>Microarray Slide ID</th>
<th>Name, synonyms</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AW462573</td>
<td>Lipocortin; phospholipase a2 inhibitor; annexin I.</td>
<td>Mimics the effect of steroids; mediates anti-inflammatory activity; inhibits cell replication, forces entering differentiation; inhibits phospholipids generation; affects cytoskeleton arrangement</td>
</tr>
<tr>
<td>AW462679</td>
<td>ATPase, Na+/K+ transporting, β 3 polypeptide</td>
<td>Takes part in IFN-γ, IL-2, IL-4 and IL-10 production, influences T and B lymphocyte activation</td>
</tr>
<tr>
<td>BF039948</td>
<td>IK cytokine, down-regulator of HLA II</td>
<td>IK factor almost completely abolishes HLA class II expression; associated with cancer.</td>
</tr>
<tr>
<td>BM362648</td>
<td>Ribosomal protein L5</td>
<td>Regulator of activity or subcellular localization of Casein KinaseII; shuttling protein (nucleocyttoplasmic transport), affecting thyroid hormone receptor dependent regulation of transcription;</td>
</tr>
<tr>
<td>BF041596</td>
<td>Hypoxanthine phosphoribosyltransferase 1(HPRT)</td>
<td>Transcription activator, initiates of DNA replication</td>
</tr>
<tr>
<td>BF042536</td>
<td>Sin3-associated polypeptide</td>
<td>Transcriptional repressor</td>
</tr>
</tbody>
</table>
| Accession | Description | Details
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BM365597</td>
<td>ATP synthase, H+ transporting, mitochondrial F1 complex, α subunit</td>
<td>Its expression is being regulated by transcriptional factors Sp1, AP-2 and GCF.</td>
</tr>
<tr>
<td>BF045212</td>
<td>Villin 2 (ezrin) (VIL2)</td>
<td>Invasive phenotype formation in malignantly transformed esophageal epithelial cells; elongation of microvillus-type parallel actin bundles; when associated with phosphatidylinositol 4,5-bisphosphate, regulates the actin cytoskeleton</td>
</tr>
<tr>
<td>BF042064</td>
<td>MKI67 (FHA domain) interacting nucleolar phosphoprotein (MKI67IP)</td>
<td>Cell cycle regulation</td>
</tr>
<tr>
<td>BF042135</td>
<td>Stathmin 1/oncoprotein 18 (STMN1)</td>
<td>Polyploidisation; regulation of the microtubule (MT) filament system; a substrate for extracellular signal-regulated kinase (ERK)</td>
</tr>
<tr>
<td>BF042903</td>
<td>Ribosomal protein L27 (RPL27)</td>
<td>Unknown function; In E. coli - assembly and activity of 50S ribosomal unit</td>
</tr>
<tr>
<td>AW462218</td>
<td>RAB11A, member of the RAS oncogene family (RAB11A)</td>
<td>Associated with both constitutive and regulated secretory pathways</td>
</tr>
<tr>
<td>BF045376</td>
<td>PDZ and LIM domain 1 (elfin); CLIM1, CLP36, ELFIN, CLP-36, hCLIM1</td>
<td>CLP-36 PDZ-LIM protein is an adapter, recruiting the Clik1 kinase to actin stress fibers in nonmuscle cells. Clik1 is a regulator of actin stress fibers. : CLP-36 contains a PDZ- and LIM domain interacts with actinin-1, actinin-2 and actinin-4, and localizes to actin stress fibers</td>
</tr>
<tr>
<td>Accession</td>
<td>Description</td>
<td>Function</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------------------------------</td>
<td>-------------------------------------------------------------------------</td>
</tr>
<tr>
<td>BF043378</td>
<td>High-mobility group box 2 (HMGB2)</td>
<td>This locus controls region regulation of the β-globin gene cluster; HMG2 facilitates the nucleosome assembly, transcriptional activation, and DNA repair functions of SET (nucleosome assembly protein) and/or APE (base excision repair enzyme); down-regulates the p53- and p73-dependent sequence-specific transactivation from the human Bax gene promoter</td>
</tr>
<tr>
<td>BF041607</td>
<td>T-cell activation protein</td>
<td>Mode of action unknown</td>
</tr>
<tr>
<td>BF044056</td>
<td>Transcriptional adaptor 3</td>
<td>Interacts with p-53, required for p-53 mediated apoptosis</td>
</tr>
<tr>
<td>AW465140</td>
<td>Moesin-like 1 (MSNL1) pseudogene, related to ezrin (see row 9)</td>
<td>Function unknown; suggested as a substrate for tyrosine phosphorylation in receptor-mediated cytoskeletal reorganization; cell shape control, linking the cytoskeleton to the membrane; its expression is stimulated by lipopolysaccharide</td>
</tr>
<tr>
<td>AW463190</td>
<td>Hypothetical protein H41</td>
<td>Overexpressed in human breast cancer</td>
</tr>
<tr>
<td>AW465027</td>
<td>Myosin regulatory light chain MRCL3</td>
<td>Regulates actin filament assembly and reorganization in nonmuscle cells;</td>
</tr>
<tr>
<td>AW464991</td>
<td>Decorin (DCN)</td>
<td>A key regulator of tumor growth by acting as an antagonist of the epidermal growth factor; supports adhesion and activation of human platelets</td>
</tr>
<tr>
<td>BF041950</td>
<td>Heat shock 90kDa protein 1</td>
<td>Cellular response to stress</td>
</tr>
<tr>
<td>Accession</td>
<td>Description</td>
<td>Function/Role</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>BF045606</td>
<td>PPAR binding protein</td>
<td>Transcriptional coactivator</td>
</tr>
<tr>
<td>BF046046</td>
<td>Mitochondrial translational release factor</td>
<td>Strong anti-apoptotic endogenous factor</td>
</tr>
<tr>
<td>AW463135</td>
<td>Solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator)</td>
<td>Expression of ANT2 is activated by cell growth stimulation</td>
</tr>
<tr>
<td>BF043205</td>
<td>Retinoblastoma binding protein 4 (RBBP4)</td>
<td>Chromatin remodelling; transcriptional repression; histone deacetylation; cell cycle regulation</td>
</tr>
<tr>
<td>BF040232</td>
<td>Interleukin 13 receptor α 1 (IL13RA1)</td>
<td>Binds tyrosine kinase TYK2, mediates the signalling processes that leads to the activation of JAK1, STAT3 and STAT6 induced by IL13 and IL4.</td>
</tr>
<tr>
<td>BF039701</td>
<td>Protein AHNAK</td>
<td>Function unknown. Resides in nucleus</td>
</tr>
<tr>
<td>BF041471</td>
<td>Heterogeneous nuclear ribonucleoprotein H3 (2H9) (HNRPH3)</td>
<td>mRNA splicing; shock-induced splicing arrest</td>
</tr>
</tbody>
</table>
The interaction of streptococci with either host epithelial or professional phagocytic cells is probably a strain-specific process (Segura, 1998) activating several different signalling pathways. Signal transduction in host cells during bacterial infection is often associated with internalization of bacterial particles. Internalization of a particle by a cell depends on its ability to rearrange its cytoskeleton, in which process actin is a central component (May and Machesky, 2001). This suggestion is corroborated by the results of the study of streptococcal M1 protein involvement in signal transduction resulting in host cell actin rearrangement in host epithelial cells (Purushothaman, 2003). It is also consistent with our results from the cDNA microarray hybridization experiment (Table 4.6.3.1), suggesting a role for Lbp of S. uberis in the regulation of annexein1, ezrin, stathmin and elfin expression. The products of these genes were demonstrated to play roles in regulation of cellular plasticity and morphogenesis through interaction with actin and microtubules, and thus affecting organization of the cytoskeleton.

From the list of those genes which are down-regulated in the presence of Lbp during S. uberis infection (Table 4.6.3.1), we selected genes whose functions were associated with cytoskeleton organization for verification of the results of microarray hybridization by RT PCR. We also quantitated the content of mRNA for IL-13 receptor to test the possibility that Lbp may play a role as an immunomodulatory component.

RNA samples quality control was performed using Agilent Bio-analyser as described in Materials and Methods. Additionally, polymerase chain reactions were carried out on each total RNA sample and on synthesized cDNA using oligonucleotide
Figure 4.6.3.1 RNA quality control assessed by PCR.

PCR of 4 different RNA samples was carried out using a pair of primers specific for the bovine gene coding for GAPDH before (1, 3, 5 and 7) and after (2, 4, 6 and 8) synthesis of cDNA. The arrow indicates the position of the GAPDH-specific PCR product; M, DNA molecular weight standards.
Table 4.6.3.2. Changes in expression levels of selected genes following incubation of MAC-T cells with *S. uberis* Su-1 relative to the *lbp* mutant SuM13.

<table>
<thead>
<tr>
<th>Probe ID and ortholog’s name</th>
<th>Fold change in relative amount of mRNA</th>
<th>Microarray hybridization</th>
<th>Quantitative RT PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AW462573</strong>, Lipocortin; phospholipase a2 inhibitor; annexin I.</td>
<td>1.68 ± 0.5</td>
<td>1.33±0.29</td>
<td></td>
</tr>
<tr>
<td><strong>BF045212</strong>, Villin 2 (ezrin) (VIL2)</td>
<td>1.45 ± 0.2</td>
<td>3.27±0.02</td>
<td></td>
</tr>
<tr>
<td><strong>BF042135</strong>, Stathmin 1/oncoprotein 18 (STMN1)</td>
<td>1.45 ± 0.25</td>
<td>1.6±0.7</td>
<td></td>
</tr>
<tr>
<td><strong>BF045376</strong>, PDZ and LIM domain 1 (elfin); CLIM1, CLP36, ELFIN, CLP-36, hCLIM1</td>
<td>1.63 ± 0.48</td>
<td>1.02±0.3</td>
<td></td>
</tr>
<tr>
<td><strong>BF040232</strong>, Interleukin 13 receptor α 1 (IL13RA1)</td>
<td>1.37 ± 0.16</td>
<td>3.01±1.4</td>
<td></td>
</tr>
</tbody>
</table>

The data is the result of 3 independent experiments each performed in duplicate and presented as an average fold change ± standard deviation.
Figure 4.6.3.2  A representative plot of fluorescence versus cycle number for the RT PCR of the IL-13 receptor specific cDNA, which was synthesized from the total RNA of mammary epithelial cells infected with *S. uberis* Su-1 or with *lbp* mutant SuM13. Fluorescence of the PCR product specific for the gene coding for bovine GAPDH as a positive control is shown for both cDNA samples; total RNA from Su-1 infected cells used as a PCR substrate without cDNA synthesis for a negative control. Threshold value determined as 807 units of fluorescence.

- : IL-13R in the cells infected with *S. uberis* Su-1; ✶: GAPDH in the cells infected with *S. uberis* Su-1; ■: GAPDH in the cells infected with *S. uberis* SuM13; ▲ : IL-13R in the cells infected with *S. uberis* SuM13; ●: No PCR template.
primers specific for bovine glyceraldehyde phosphate dehydrogenase (GAPDH) to confirm the absence of DNA contamination in RNA preparations (Fig. 4.6.3.1).

The results of RNA quality control suggested that the samples were free from host DNA contaminations since no PCR product could be amplified from the RNA samples prior to the synthesis of cDNA (Fig. 4.6.3.1).

SYBR Green-based quantitative RT PCR was carried out on the cDNA samples using oligonucleotide primers specific for annexin I, villin 2, stathmin 1, elfin, GAPDH and IL-13 receptor α (Table 3.2.1) as described in Materials and Methods. The amount of mRNA coding for annexin I, villin 2, stathmin 1, elfin and the IL-13 receptor α relative to the GAPDH mRNA in mammary epithelial cells infected with *S. uberis* Su-1 was compared to the amount of respective mRNA content in the cells infected with *lhp* mutant SuM13 (Table 4.6.3.2).

The plot of CYBR Green fluorescence versus RT PCR cycle number suggested that relative amounts of the tested transcripts in the mRNA samples (Fig. 4.6.3.2) was consistent with the results of cDNA hybridization, indicating that annexin I, villin 2, stathmin 1, elfin and the IL-13 receptor α were down-regulated in MAC-T cells infected with Su-1 relative to that of MAC-T cells infected with SuM13.

Although the results of quantitative RT PCR confirmed the results obtained by cDNA microarray hybridization, discrepancies in the fold change of transcripts were noted. The discrepancies between fold change in the genes expression determined by two different methods using same RNA samples (Table 4.6.3.2) may be explained by the semi-quantitative nature of cDNA hybridization approach, while RT PCR offers more accurate quantitation of an mRNA content.
4.6.4 A Possible Mechanism for the Regulation of Host Genes by Lbp of *S. uberis*

Lactoferrin is homologous and is related to serum transferrin, a major metalloprotein important for iron transport and cellular iron metabolism (Metz-Boutigue, 1984). Although it has been suggested that lactoferrin is not crucial in the maintenance of intracellular iron homeostasis in mammals (Ward, 2003), it potentially can serve as a source of iron for a cell. In order to search for possible post-transcriptional mechanisms of iron-associated regulation of the genes we identified as down-regulated by the Lbp of *S. uberis* Su-1 in the presence of bovine lactoferrin, we analyzed the predicted secondary structure of the mRNA for the genes used for verification of the microarray data (Table 4.6.4.2) and studied how the overload of Fe⁢³⁺ influenced the expression of these genes.

Putative stem-loop structures containing an IRE motif CAGUG were predicted for annexin 1 (sequence accession number X56649, position 107), ezrin (accession number X51521, position 2427) and stathmin 1/oncoprotein 18 (accession number NM_203401, position 1253) mRNAs (Fig. 4.6.4.1) using Software for Statistical Folding and Rational Design of Nucleic Acids algorithm as described in Materials and Methods. The free energy for each structure was predicted as -13.4 kJ/M, -30.2 kJ/M and -18.8 kJ/M, respectively, at the folding temperature of 37°C and the ionic strength corresponding to 1M NaCl, with no divalent ions.

Quantitative RT PCR was carried out on total RNA extracts from the MAC-T cells treated with bovine lactoferrin and with 10 mM of FeCl₃ in order to model iron overload. One of each primer pair specific for annexin 1, villin 2 and stathmin1 (Table 3.2.1) were used. The amplification profiles obtained (Fig. 4.6.4.2) suggest that both iron and bovine lactoferrin can reduce the content of mRNA for annexin 1, stathmin 1 and villin 2 in bovine mammary epithelial cells, although this reduction is more obvious and probably more effective when cells are treated with an excess of ferric chloride than
A; $\Delta G = -13.4$ kJ/M  
B; $\Delta G = -30.2$ kJ/M  
C; $\Delta G = -18.8$ kJ/M

Figure 4.6.4.1 Putative IRE-like structures with CAGUG motif in the stem-loop regions of mRNA.

A, mRNA for human annexin 1; B, mRNA for human villin 2; C, mRNA for human stathmin 1. $\Delta G$, estimated free energy of folding of the fragment of the mRNA molecule spanning approximately 60 nucleotides in both directions from CAGUG motif (boxed).
Figure 4.6.4.2  A representative plot of accumulation of the fluorescence in RT PCR reaction using oligonucleotide pair specific for bovine villin 2.

- Villin2 in the cells treated with 4 mg/ml of bovine lactoferrin;  \(\times\) : GAPDH in the cells treated with 10mM FeCl\(_{3}\);  \(\blacksquare\) : Villin2 in untreated cells;  \(\blacktriangle\) : Villin2 in the cells treated with FeCl\(_{3}\);  \(\circ\) : No template.
when they are treated with lactoferrin. Representative RT PCR amplification curves are shown in Fig. 4.6.4.2.

4.6.5 Discussion

Two-component signal transduction pathways of bacteria involve events of phosphorylation of a cytoplasmic response regulator following recognition of an environmental signal by a sensor surface molecule (Stock, 2000). Therefore the possibility that Lbp of *S. uberis* plays a role in the functioning of this type of hypothetic two-component signal transduction pathway was addressed by examining differential protein phosphorylation of bacterial proteins following incubation with bovine lactoferrin. Although no differential phosphorylation of streptococcal proteins was detected following binding of lactoferrin, the presence of such proteins cannot be ruled out based on these results. One reason is that the direct detection of incorporated radioactive phosphorus may not be sensitive enough to detect minor changes in phosphorylation. For example, the half-life of mRNAs of the proteins of two-component signal transduction systems in bacteria can be as short as 1.5 - 3 minutes, as was demonstrated for the histidine kinase genes (Aiso, 2003). Secondly, the phosphorylated intermediates are often short-lived or transiently phosphorylated proteins.

Our results indicated that bovine lactoferrin was phosphorylated in the presence of both Su-1 and SuM13 strains of live *S. uberis* (Fig. 4.6.2.2). The significance or mechanisms of such phosphorylation are not clear, although *S. uberis* Su-1 is capable of binding significantly larger amounts of phosphorylated lactoferrin (Fig. 4.6.2.1). Since equal amounts of lactoferrin with similar intensities of the protein bands on
autoradiograph were detected in culture media of both strains, it is unlikely that Lbp plays a role in lactoferrin phosphorylation.

Alternatively, environmental signalling can result in phosphorylation of an effector protein (enzyme, DNA-binding components), which may affect its activity. This may result in the altering of one or several cell metabolic pathways. None of such phosphorylated effector proteins was detected in either Su-1 or SuM13 strains of S. uberis upon stimulation with bovine lactoferrin, suggesting that the mere presence of bovine lactoferrin in an environment and its binding by the S. uberis cells is not sufficient for altering signal transduction pathways.

Infection of bovine mammary cells with Su-1 and SuM13 in the presence of γ\(^{32}\)[P]ATP revealed that several host proteins are modified (phosphorylated) upon infection (Fig. 4.6.2.3). The presence of unique differentially phosphorylated protein bands in the Streptococcus uberis-infected host suggests that these products might represent components of the host cell signalling pathways specific for infection with S. uberis.

Several major protein bands were identified as host cell proteins differentially phosphorylated during infection with S. uberis (Fig. 4.6.2.3, B), suggesting that infection with either Su-1 or SuM13 possibly altered phosphorylation-related pathways in MAC-T epithelial cells. However, no differences in protein phosphorylation profiles were detected between cells infected with S. uberis Su-1 and those with SuM13, indicating that Lbp of S. uberis does not play a role in a phosphorylation-dependent signal transduction in host cells.

Examination of the morphologic changes in cultured bovine epithelial cells co-
incubated with viable *S. uberis* indicated that the bacteria exhibited a cytotoxic effect on cultured host cells and no indication of ongoing apoptosis was found. This cytotoxic effect is expressed as a marked necrotic phenotype of cultured bovine epithelial cells co-incubated with live *S. uberis* and suggests severe cytotoxicity associated with the bacteria, possibly due to the activity of proteolytic enzymes of streptococcal origin and/or expression of CAMP cytotoxin. However, certain proteolytic secreted products, e.g., pneumolysin of *S. pneumoniae* (Zysk, 2001), can also cause host cell apoptosis, as characterized by cell shrinking, nuclear condensation, endonuclease-mediated DNA fragmentation and preservation of cell membrane integrity. In contrast, *S. uberis* caused cell enlargement (Fig. 4.6.2.4, B, C; Fig. 4.6.3.2, B), nucleus swelling and disruption of the cellular membrane (Fig. 4.6.2.5, D). Additionally, no DNA laddering was detected in cultured epithelial cells infected with either strain of *S. uberis* (Fig. 4.6.2.6). These features are consistent with necrosis rather than with apoptosis, indicating that *S. uberis* either does not promote apoptosis in host epithelial cell or that death by necrosis masks the apoptotic phenotype. The presence of bovine or human lactoferrin at physiological concentrations has been demonstrated to have an anti-apoptotic effect on some types of cells and also to stimulate cell proliferation and differentiation (Cornish, 2004). Thus, the absence of typical features of cell apoptosis in the presence of lactoferrin bound by Lbp of *S. uberis* is not surprising. Both Su-1 and SuM13 have similar cytopathic effects on cultured epithelial cells, suggesting that Lbp of *S. uberis* does not promote or inhibit apoptosis in host epithelial cells.

Analysis of the transcriptional response of infected MAC-T cells dependent on the expression of Lbp by *S. uberis* was assayed by cDNA microarray hybridization. The
analysis of the microarray spot intensities suggests that a total of 28 bovine genes were consistently downregulated in cells infected with *S. uberis* Su-1 as compared to cells infected with *lbp* mutant SuM13 (Table 4.6.4.1). These changes in expression were confirmed by quantitative RT PCR for the genes coding for annexin I, villin 2, stathmin 1, elfin and IL-13 receptor α (Table 4.6.4.2). The results of microarray hybridization may be affected by several variables, depending on the design of the experiment such as multiplicity of infection, phase of growth of the bacterial culture and time point of host RNA sampling, as well as by the design of the microarray slides. Since only approximately 8% of *S. uberis* CFU present in the bacteria/host cell mixture adhere to MAC-T cells (Table 4.3.2.1) and only approximately 1% of adherent bacteria are internalized by host cells at an MOI of 100 (Table 4.3.2.2), it is probably mainly extracellular bacteria and bacterial secreted products that influence host cell gene expression. Since we intended to study regulation of host gene expression in response to infection with live bacteria, we limited the time of incubation to 3 hours, a period that should allow for detection of changes in transcription but is not long enough for the bacteria to induce extensive damage to the cell by proteolytic enzymes.

A reduced amount of annexin I in membranes of the phagosome has been demonstrated to be associated with an impaired maturation of the phagolysosome during phagocytosis of *Mycobacterium avium* (Pittis, 2003), suggesting a possible bacteria-induced redistribution of the host intracellular annexin or a down-regulation of its synthesis. In this respect, the down-regulation of expression of annexin 1 by host cells in the presence of an M-like protein Lbp of *S. uberis* is consistent with the functions of M and M-like proteins, which were demonstrated as antiphagocytic or as
favouring survival of phagocytosed streptococci factors. Although we did not demonstrate either enhanced phagocytosis (Fig. 4.2.5.1) or reduced survival of the *lbp* mutant in the presence of bovine blood neutrophils (Table 4.2.4.1), the reduced content of mRNA for annexin I may indicate that phagocytosis and/or phagosome maturation and, consequently, intracellular survival of *S. uberis* may be associated with expression of Lbp. Considering the demonstrated role of annexins in intracellular redistribution of endosomes with transferrin receptor during transferrin uptake and recycling (Zobiack, 2003), down-regulation of annexin I in the presence of the adherent *S. uberis* with lactoferrin-loaded Lbp on its surface may indirectly indicate a possible process of an iron-dependent redistribution of endosomes that contain lactoferrin receptor, analogous to the altered transferrin receptor recycling in iron-overloaded cells (Malorni, 1998).

Annexin I also functions as an inhibitor of phospholipase A2 (Oh, 2000), which has been implicated in the adherence of GAS. Group A *Streptococcus* secretes a prophage-encoded phospholipase A2 implicated in host-pathogen interactions, increasing its synthesis when co-cultured with host pharyngeal epithelial cells (Nagiec, 2004). Host cellular phospholipase A is also rapidly activated by pneumolysin of *S. pneumoniae* in host artery endothelial cells (Rubins, 1994). Down-regulation of expression of the phospholipase inhibitor annexin I by *S. uberis* Su-1 may be a way to reduce the presence of one of the cellular components that interfere with *Streptococcus*-host cell interaction.

The synthesis of annexin I increases when cells are stimulated by IL-1-β (Miyachi, 2001) or by tumor necrosis factor (TNF) (Wu, 2000), inhibiting TNF-mediated cytotoxicity (Beyaert, 1990) and inflammation-related cell damage. Annexin I
is essential for hepatocyte growth factor-induced proliferation of epithelial cells (Skouteris, 1996) and can abolish phospholipase A2 induction of the Rac-dependent nuclear signalling pathway (Kim, 1997). Interference with host cytokine signalling by down-regulation of expression of annexin I potentially may be beneficial for *S. uberis* and may aid survival of the bacteria. Hence three major consequences of the reducing of the presence of annexin I in cells co-incubated with *S. uberis* may be outlined: (1) it may be essential for altering cell plasticity and motility, possibly affecting internalization of the bacterial cell; (2) a reduced quantity of phospholipase A2 inhibitor may increase the background activity of phospholipase and affect bacterial adherence to the host cell; and (3) decreasing of the intracellular content of annexin I may interfere with cytokine signalling pathways.

Ezrin is a protein that belongs to the ezrin-radixin-moesin (ERM) family of homologous membrane/cytoskeleton linker proteins involved in the organization of cytoskeleton, especially in the formation of microvilli (Franck, 1990), (Yonemura, 1999). Simultaneous inactivation of all three ERM in epithelial cells by antisense oligonucleotides caused disappearance of microvilli and altered cell-cell adhesion (Takeuchi, 1994), while overexpression of full-length ezrin enhanced cell adhesion (Martin, 1995) and formation of microvilli and pseudopodia (Lamb, 1997). Upon infection with *S. uberis*, the number of microvilli on the surface of MAC-T cells was greatly reduced (Fig. 4.6.2.5, B), which is consistent with down-regulation of expression of ezrin. However, a similar effect was observed in cells infected with SuM13, suggesting that Lbp may not be involved in regulation of microvilli formation. This apparent contradiction may be explained by the length of time (24 hours) of co-
incubation of MAC-T cells and bacteria prior to examination by electron microscopy. This suggests that the reason for the disappearance of microvilli on the surface of infected cells 24 hours after infection is rather general cytotoxicity of both *S. uberis* strains, while at 3 hours of co-incubation of MAC-T cells with bacteria, a differential expression of ezrin mRNA could still be detected.

A high level of expression of ezrin was associated with the development of metastatic cancer (Chen, 2001) and is necessary for metastasis in the murine osteosarcoma model (Khanna, 2004). Members of the ERM family of proteins were demonstrated to mediate contact between a Na⁺/H⁺ cell membrane pump and cell actin cytoskeleton to regulate cell shape, adhesion and motility, factors that are probably related to metastases formation and spread. Aggregation of Su-1- and SuM13-infected MAC-T cells was observed during prolonged (24 hours) co-culturing with bacteria (Fig. 4.6.2.5, B); however, we did not address the role of regulation of the expression of ezrin in this process.

Aside from a cell shape formation due to cross-linking of actin and outer cellular membrane proteins, ezrin was implicated in signal transduction and in regulation of the cell cycle. It was suggested that ezrin transduces a survival signal through the activation of the PI3K/Akt pathway (Gautreau, 1999). When apoptotic stress is applied to the cells, a Na⁺/H⁺ pump physically associates with phosphorylated ERM and the actin cytoskeleton, which results in activation of the antiapoptotic kinase Akt and in cell survival (Wu, 2004). Reduced activity of Akt was observed when expression of ezrin protein was suppressed (Khanna, 2004). Treatment of ovarian epithelial carcinoma cells with endothelial growth factor (EGF) or IL-1-α increased ezrin tyrosine
phosphorylation, ezrin translocation and cell growth and also induced an invasive phenotype (Chen, 2001). This is consistent with the data indicating that no apoptosis was induced in MAC-T cells infected with S. uberis (Fig. 4.6.2.4, 4.6.2.5 and 4.6.2.6). In contrast to the above data, which suggests an antiapoptotic function of ezrin, it was demonstrated that CD95-mediated apoptosis depends on direct binding of ezrin to CD95, connecting it to actin (Lozupone, 2004), while the other two proteins of the ERM family, radixin and moesin, do not bind to CD95. In MAC-T cells infected with S. uberis Su-1, along with ezrin, the other host cell genes related to the regulation of cytoskeleton organization were down-regulated (Table 4.6.4.1). This suggests a possible compensation for a pro-apoptotic activity of ezrin by regulation of other components potentially involved in inducing of apoptosis.

All three members of the ERM family can also localize to the nucleus (Batchelor, 2004). Although the significance of the ERM proteins binding to the nucleus is unknown, it was suggested that cytoskeletal components can directly link the plasma membrane with nuclear events. This indicates that down-regulation of ezrin by S. uberis expressing Lbp might be involved in interfering with the host cell signal transduction.

The above data suggest that the decrease in villin 2 (ezrin) expression, as well as the decrease in annexin I expression, interfere with host cell morphogenesis and membrane-to-nucleus signalling. The downregulation of villin2 by Lbp may be related to modifying host cell plasticity. Indeed, S. dysgalactiae surface protein MIG, also an M-related protein, reduced the invasion of the bacteria into cultured epithelial cells (Song, 2004). Invasion of S. dysgalactiae is inhibited by cytochalasin D (Almeida,
and villin-induced growth of microvilli can be inhibited by cytochalasin D (Friederich, 1993), suggesting a possible involvement of the villin-dependent cell morphogenesis in internalization of streptococci.

Initially, stathmin was identified as a regulatory phosphoprotein present in almost all cell types and as being involved in cytoplasmic signal transduction from a specific membrane receptor and in the generation of a secondary messenger of a signalling pathway (Sobel, 1989). In leukemia, the increase in the cellular amount of stathmin was associated with an increase of the stathmin mRNA content, suggesting transcriptional regulation of the gene expression (Melhem, 1991), although the activity of various present isoforms of the protein may be regulated by its post-translational differential phosphorylation (Marklund, 1993). Since no differential protein phosphorylation was detected in MAC-T cells infected with S. uberis Su-1 as compared to the cells infected with SuM13, Lbp of S. uberis probably does not regulate the activity of stathmin through protein modification (Fig. 4.6.2.3, B). Intracellularly, at least some phosphoisoforms of stathmin are associated with Golgi apparatus and with microtubules (Gavet, 1998), suggesting that it may play a possible role in mitosis and in the synthetic phase of a cell cycle. Direct interaction of tubulin with stathmin has been demonstrated (Redeker, 2000) and it was observed that in the cells expressing the mutant forms of stathmin the transition to mitosis is inhibited, while the cell polyploidization is induced (Chang, 2001). Although we do not have data on polyploidisation of bovine epithelial cells infected with S. uberis, multiple nuclei in infected MAC-T cells were commonly observed (Fig. 4.6.2.4, B and C). Although the microtubule-regulatory function of stathmin is regulated predominantly by the
differential phosphorylation of this protein (Larsson, 1995), an induced polyploidization and disruption of a normal mitotic microtubule spindle formation during mitosis was also observed in cells with reduced stathmin mRNA and protein content (Rubin, 2003). The data above indicate that stathmin plays a role in intracellular signal transduction and in cell cycle regulation. The significance of the decreased content of stathmin mRNA in the mammary epithelial cells infected with *S. uberis* Su-1 expressing Lbp versus cells infected with SuM13 mutant with no Lbp expressed is not clear. However, cells containing more than one nucleus were observed both among cells infected with Su-1 and among cells infected with SuM13 (Fig. 4.6.2.4, B and C).

This data is consistent with the down-regulation of the stathmin mRNA by *S. uberis* expressing Lbp (Table 4.6.4.1) and observed polynucleated infected MAC-T cells (Fig. 4.6.2.4, B and C), suggesting that Lbp may play a role in altering host cell signalling pathways, including those regulating cell cycle.

Elfin was identified as a 36 kilodalton protein, containing LIM motif(s) at its carboxy terminus and PDZ domain at the aminoterminal region (Kotaka, 1999), characteristics of the enigma family of proteins. LIM domains, named for the three proteins in which they were first recognized (lin-11, isl-1, and mec-3), are cysteine-rich domains that contain two coordinated Zn$^{2+}$ atoms (Perez-Alvarado, 1994). Several PDZ domain-containing proteins serve as scaffolds for assembling components of large protein complexes at cell-cell junctions and for assembling proteins involved in signal transduction (Tsunoda, 1997). Therefore, the down-regulation of the expression of elfin may have contributed to the polynucleation of infected MAC-T cells (Fig. 4.6.2.4, B, C) by disrupting cell-cell junctions and interfering with the host cell signal transduction
pathways. Elfin mRNA was detected in various types of cells, including epithelial (Vallenius, 2000), but displayed the highest levels of expression in the skeletal and heart muscle cells (Kotaka, 1999). By using the yeast two-hybrid screening system, it was demonstrated that elfin binds α-actinin 2 (Kotaka, 2000) and it was suggested that elfin functions as an adapter, bringing other proteins to the cytoskeleton. For example, it was shown that the Clik1 kinase, normally located in the cell nucleus, may be recruited to actin stress fibers upon binding to elfin (Vallenius, 2002) and that such recruitment may affect the regulation of stress fiber formation. Subsequent studies showed that elfin also associates with α-actinin-1 filaments and with stress fibers that are formed during cellular shape change, contraction and spreading (Bauer, 2000), indicating the role of elfin in cell shape formation and morphogenesis. This suggests that insufficient amounts of elfin also may have contributed to the morphological changes of infected cells.

The processes that require substantial rearrangements of the host cell’s cytoskeleton appear to be influenced by M-like protein Lbp of S. uberis. Streptococcal M-like proteins were implicated in influencing phagocytosis and the invasion of non-phagocytic cells, which also involve the reorganization of the host cell’s cytoskeleton. It is tempting to conclude that down-regulation of the genes involved in cell shaping might contribute to the internalization of streptococci by non-phagocytic cells and resistance to phagocytosis by polymorphonuclear leukocytes.

Lbp appears to play a role in the down-regulation of expression of IL-13 receptor α (Table 4.6.4.1) by bovine epithelial cells, suggesting interference with cytokine signalling pathways. The role of bovine mammary gland cells in streptococcal infection has been studied and the dynamics of the selected crucial cytokines produced
in the early stages of bovine *S. uberis* mastitis have been determined (Rambeaud, 2003; Hockett, 2000). However, the role that individual cytokines play in development of intramammary infection with Gram-positive microorganisms is still not fully understood. Both IL-4 and IL-13 induce protein tyrosine phosphorylation (Smerz-Bertling, 1995), leading to the activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling cascades in cells expressing both the IL-4 receptor α and the IL-13 receptor α1 (Wang, 2004; Kelly-Welch, 2003), as well as phosphorylation of STAT6 and STAT3 (Wery-Zennaro, 1999). This suggests a role for IL-13 receptor α1 in signal transduction and JAK/STAT-dependent (Murata, 1998) modulation of gene transcription. We cannot determine the role of Lbp in the regulation of the JAK/STAT pathways in MAC-T cells since no differential phosphorylation was detected in MAC-T cells infected with *S. uberis* Su-1 in comparison to those infected with SuM13. However, there was no IL-13 added to the medium to bind the IL-13 receptor α and to trigger a phosphorylation cascade of a JAK/STAT pathway.

Alteration or inhibition of cytokine signalling during *S. uberis* infection may potentially interfere with the host’s response to bacterial infection, preventing the clearance of the bacteria by both innate and adaptive host immunity effectors. This alteration may also modulate the severity of an inflammatory response to the streptococcal infection, preventing the manifestation of the clinical signs of mastitis in infected animals.

The release of proinflammatory cytokines (tumor necrosis factor α and interleukin-6) by phagocytes upon adhesion of heat-killed *S. suis* (Segura, 2004) suggests a transcriptional response by the host cell to stimulation with killed bacteria,
since cytokines are not stored as pre-synthesized intracellular products and secretion of a cytokine requires transcriptional activation of the gene coding for it. We demonstrated regulation of expression of a receptor component in the host cytokine signalling network. The significance of such regulation is not clear and might be a subject of future studies. There was no observed relationship between cytokine production by the cells stimulated with bacteria and the origin or virulence of the bacterial strain, as was demonstrated for *S. suis* (Vadeboncoeur, 2003). Similarly, Lbp of *S. uberis* may be an important component interfering with mammary gland cytokine signalling, but deletion of the *lbp* gene may not affect bacterial virulence (see section 4.5).

As was demonstrated by cDNA microarray hybridization, the content of mRNA coding for several host proteins is down-regulated by *S. uberis* Su-1, which expresses Lbp on the surface. The down-regulation of villin 2, stathmin 1, the IL-13 receptor α and annexin I was verified by real time PCR, demonstrating a 1.5- to 3-fold decrease in the content of corresponding transcripts. The consistent decrease of the mRNA content in the presence of Lbp is not accompanied by a high level of protein phosphorylation or a detectable level of phosphorylated transcriptional regulator in host cells. Interestingly, stathmin 1, villin 2 and annexin I mRNAs contain putative iron responsive elements with CAGUG motifs in the stem-loop secondary structures predicted by Software for Statistical Folding and Rational Design of Nucleic Acids algorithm at http://sfold.wadsworth.org/srna.pl (Fig. 4.6.4.1). Iron, brought by the bacteria to the epithelial cell surface along with lactoferrin, if acquired by the epithelial cell may affect post-transcriptional regulation of mRNA containing IREs.

1,25-Dihydroxyvitamin D3, for the synthesis of which the Fe-S cluster-
containing mitochondrial cytochrome P450 is required (Guryev, 2003), down-regulates the expression of the transferrin receptor but does not affect the plasma membrane endocytosis rate (Tanaka, 1990). 1,25-Dihydroxyvitamin D3, however, up-regulates the expression of stathmin (Kumar, 1994), an oncoprotein p18 whose mRNA cellular content is down-regulated by Lbp and iron.

A detected altered expression of bovine genes in response to the presence of Lbp on the bacterial cell surface (Table 4.6.3.2) indicates the possibility that Lbp may play a role in regulation of the host gene expression.
CONCLUSIONS

1. A defined mutant of the *S. uberis* strain Su-1 unable to express the lactoferrin-binding protein has been isolated and characterized;

2. Lbp did not play role in the resistance of *S. uberis* to ingestion by bovine neutrophils or in the inhibition of host serum complement;

3. Both wild type *S. uberis* and its *lbp* mutant had similar abilities to adhere to and invade cultured host epithelial cells;

4. Lbp of *S. uberis* did not play a role of iron acquisition from bovine lactoferrin;

5. Lack of expression of the Lbp by *S. uberis* Su-1 did not abolish the ability of the bacteria to colonize mammary glands of lactating cows and did not significantly affect the virulence of *S. uberis*;

6. Lbp of *S. uberis* may play a role in regulation of the expression of host genes potentially involved in pathogenesis of bacterial infection.
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