

**Potentially Virulence-Related  
Extracellular Proteins of  
*Streptococcus equi***

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## Abstract

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Equine strangles, a disease of the upper respiratory tract caused by the bacterium *Streptococcus equi* subspecies *equi*, is one of the most commonly diagnosed and serious diseases in horses. However, the molecular basis of *S. equi* subsp. *equi* infection is poorly understood and there are no safe and effective vaccines on the market. The main objective of this study was to identify and characterise extracellular proteins used by *S. equi* subsp. *equi* to initiate infection and cause disease.

Extracellular proteins, which can be secreted or cell surface-located, play an important role in the initiation of infection and in continued bacterial survival inside the host. Adhesins are a specific class of virulence-related proteins that are used by the bacteria to attach to host tissues. This study focused on a number of cell-surface anchored proteins that specifically adhere to collagen and fibronectin, two major extracellular matrix proteins of vertebrates. The binding characteristics of the fibronectin-binding protein FNEB were compared to two previously studied fibronectin-binding proteins, FNE and SFS. A follow-up investigation showed that FNE and FNEB are part of a family of six similar proteins encoded by *S. equi* subsp. *equi*. One of the four novel proteins identified (FNEE) was shown to bind to fibronectin and all four to collagen. In another study, a secreted immunoglobulin-specific protease was characterised. This enzyme could be used by the bacteria to interfere with the immune response of the infected horse. A useful tool in this research was the public *S. equi* subsp. *equi* genome database, which can be used to identify homologues to virulence-related proteins of other pathogens.

An applied objective of the present study was to identify potential components for a future vaccine against strangles. One of the collagen-binding proteins characterised (CNE) is currently being used in vaccination trials as a component of a protein subunit vaccine for horses.

**Keywords:** *Streptococcus equi*, *Streptococcus zooepidemicus*, strangles, virulence, fibronectin, collagen, extracellular matrix, adhesin, immunoglobulin

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## Abbreviations

$\alpha_2$ M	$\alpha_2$ -macroglobulin
aa	Amino acids
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
CNA	Collagen-binding protein of <i>S. aureus</i>
CNE	Collagen-binding protein of <i>S. equi</i> subsp. <i>equi</i>
C-terminal	Carboxy-terminal
EAG	$\alpha_2$ -macroglobulin-, albumin- and immunoglobulin G-binding protein of <i>S. equi</i> subsp. <i>equi</i>
ECM	Extracellular matrix
Fab	Fragment antigen binding of immunoglobulin
Fc	Fragment crystallisable of immunoglobulin
Fg	Fibrinogen
Fn	Fibronectin
FNE (B, C, D, E, F)	Fibronectin-binding protein of <i>S. equi</i> subsp. <i>equi</i>
F1, F2, F3	Fibronectin modules type 1, 2 and 3
FNZ	Fibronectin-binding protein of <i>S. equi</i> subsp. <i>zooepidemicus</i>
IdeE, IdeS, IdeZ	Immunoglobulin-degrading enzymes of <i>S. equi</i> subsp. <i>equi</i> , <i>S. pyogenes</i> and <i>S. equi</i> subsp. <i>zooepidemicus</i>
IgG	Immunoglobulin G
IS element	Insertion sequence element
kDa	KiloDalton
LTA	Lipoteichoic acid
mRNA	Messenger RNA
NMR	Nuclear magnetic resonance
N-terminal	Amine-terminal
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMN	Polymorphonuclear leukocyte
rRNA	Ribosomal RNA
RT-PCR	Reverse-transcript PCR
Scl (C-I)	Streptococcal collagen-like protein
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SeM	<i>S. equi</i> subsp. <i>equi</i> M-like protein
SFS	Secreted fibronectin-binding protein of <i>S. equi</i>
SS	Signal sequence (signal peptide)
Subsp.	Subspecies
TA	Teichoic acid
TLR	Toll-like receptor

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# Appendix

## Papers I-IV

The present thesis is based on the following papers, which are referred to in the text by their Roman numerals:

- I.**     **Lannergård J.**, Frykberg L. & Guss B. 2003. CNE, a collagen-binding protein of *Streptococcus equi*. *FEMS Microbiology Letters* 222: 69-74.
- II.**    **Lannergård J.**, Flock M., Johansson S., Flock J-I. & Guss B. 2005. Studies of fibronectin-binding proteins of *Streptococcus equi*. *Infection and Immunity* 73: 7243-51.
- III.**   **Lannergård J.** & Guss B. 2006. IdeE, an IgG-endopeptidase of *Streptococcus equi* ssp. *equi*. *FEMS Microbiology Letters* 262: 230-235.
- IV.**    **Lannergård J.** & Guss B. FNE belongs to a novel family of fibronectin-binding and collagen-binding proteins of *Streptococcus equi* subsp. *equi*. (Manuscript)

Papers **I**, **II** and **III** are reproduced with the kind permission of the journals concerned.

My contributions to the papers were as follows:

- I.**     Took part in planning the study and analysing the results. Performed the majority of the laboratory work. Wrote the paper together with B. Guss.
- II.**    Planned the study together with B. Guss and J-I. Flock. Performed the majority of the laboratory work, excluding the antibody titre measurements. Main writer of the paper.
- III.**   Major part in planning the study, performing the laboratory work and analysing the results. Wrote the paper.
- IV.**    Major part in planning the study and analysing the results. Performed all laboratory work. Wrote the manuscript.

## General background

The Earth is inhabited by a massive array of bacterial species, each adapted to their particular ecological niche through the struggle for existence. Many bacterial species colonise higher organisms, and the vast majority of these have adapted to peacefully coexist with their hosts. In many cases this partnership can be described as symbiotic, for example when the bacteria provide essential nutrients and co-factors. Other bacteria have evolved a pathogenic lifestyle, when the colonisation of the host results in disease. Generally speaking, a pathogen multiplies and persists more successfully in nature when it is transmitted between healthy and mobile hosts. For that reason, a successful pathogen cannot afford to cause too much injury during the course of infection. The more severe bacterial diseases observed in nature are often the result of opportunistic infections in compromised individuals, or ‘accidental’ infections of species that are irrelevant for the survival of the bacteria. However, some bacterial species are specifically adapted to colonise privileged sites of their hosts, such as the lymphatic system, which often results in a strong inflammatory response and severe disease symptoms.

A bacterial infection usually involves several steps, such as the initial attachment and sometimes entry into the body, local or general dissemination, multiplication, and finally shedding from the body and transmission to new hosts. To prevent infections, vertebrates are equipped with exterior defences, such as the skin as a physical barrier and the pH of the stomach, but also innate and acquired immunity. During the evolutionary arms race between pathogens and their hosts, the bacteria have acquired a wide arsenal of cell surface structures, enzymes and toxins that specifically interfere with this immune response.

This thesis is based on studies of the important horse pathogen *Streptococcus equi* subspecies *equi*. The main objective was to identify and characterise extracellular proteins used by *S. equi* subsp. *equi* to initiate infection and cause disease. Papers **I**, **II** and **IV** present studies of cell surface-anchored proteins that specifically adhere to collagen and fibronectin, two major extracellular matrix proteins of vertebrates. In **III** a secreted immunoglobulin-specific protease is characterised. As a general background to these studies, the subject of *S. equi* subsp. *equi* infection is discussed, followed by a review of streptococcal virulence factors in general and the virulence factors of *S. equi* subsp. *equi* in particular.

### Streptococci and streptococcal infections

Bacteria belonging to the genus *Streptococcus* are Gram-positive and catalase-negative cocci, growing in pairs or chains. They are facultatively anaerobic, homofermentative and with complex nutritional requirements. The temperature optimum varies between streptococcal species, but is usually around 37 °C. Some species form a hyaluronic acid capsule during the early phase of growth (Hardie, 1986). The cell wall is composed mainly of cross-linked peptidoglycan, with carbohydrates, teichoic acid and proteins attached and exposed to the surface

(Fischetti, 2000). Streptococci comprise a large part of the commensal flora of man and animals, where they mainly colonise the mucus membranes of the mouth, respiratory, alimentary and genitourinary tract (Hardie & Whiley, 1995). Most species can act as opportunistic pathogens and some are highly virulent.

One of the first characters used to differentiate isolates was the ability of certain clinically important streptococci to cause complete ( $\beta$ -) haemolysis when grown on solid medium containing blood (Hardie & Whiley, 1995). In 1933, Lancefield introduced a method to further differentiate the  $\beta$ -haemolytic streptococci based on carbohydrate 'group' antigens (Lancefield, 1933). The main limitation of Lancefield's system is that no streptococcal species, with the exception of *S. agalactiae*, possesses a unique group antigen that can be used for identification to species level. In 1937, Sherman made the next major contribution to streptococcal taxonomy by dividing species into four major groups based on different phenotypic properties (Sherman, 1937). Sherman's groups were the pyogenic group, the viridans (oral) group, the lactic group and the enterococci. Today, only the two first groups remain in the genus *Streptococcus*, the lactic group and the enterococci having been reclassified as separate genera (Hardie & Whiley, 1995). Since the publication of the second volume of Bergey's Manual in 1986, increased genotypic and phenotypic information has led to further changes in the genera. Several groups of Gram-positive cocci have split off the *Streptococcus* genus and new species of streptococci have been discovered, as reviewed by Facklam (2002). With the progress in bacterial genomics, the use of biochemical and serological tests to classify streptococcal isolates has largely been replaced by DNA-based molecular methods.

A majority of the most pathogenic streptococci belong to the pyogenic (pus-forming) group (Table 1), which mainly includes the  $\beta$ -haemolytic species with defined group antigens (Hardie & Whiley, 1995; Facklam, 2002). *S. pyogenes*, commonly referred to as Group A Streptococci (GAS), is the most common cause of streptococcal infection in man. Primary infection often occurs in the form of sore throat or skin lesions, which may become invasive and lead to potentially life-threatening conditions such as bacteraemia, septicaemia or necrotizing fasciitis. *S. agalactiae* (Group B Streptococci), another potent pathogen in the pyogenic group, is an important cause of neonatal sepsis and meningitis. The most important human pathogen outside of the pyogenic group, *S. pneumoniae*, is the major cause of community-acquired pneumonia. (Hardie & Whiley, 1995)

Table 1. *The pyogenic group of streptococci*

Species	Lancefield group	$\beta$ -haemolysis	Origin
<i>S. pyogenes</i>	A	+	Human
<i>S. agalactiae</i>	B	+	Human, bovine
<i>S. dysgalactiae</i>			
subsp. <i>equisimilis</i>	A/C/G/L	-/+	Human, mammals
subsp. <i>dysgalactiae</i>	C	-	Bovine
<i>S. equi</i>			
subsp. <i>zooepidemicus</i>	C	+	Mammals, human
subsp. <i>ruminantium</i>	C	+	Sheep, goat
subsp. <i>equi</i>	C	+	Horse
<i>S. canis</i>	G	+	Dog, human
<i>S. iniae</i>	-	-/+	Dolphin, fish, human
<i>S. parauberis</i>	E/P	-	Bovine
<i>S. porcicus</i>	E/P/U/V/-	+	Swine, human
<i>S. uberis</i>	E/P/G	-	Bovine

### The three subspecies of *Streptococcus equi*

*S. equi* subsp. *equi* (for simplicity here referred to as *S. equi*) is a group C streptococcus, which typically is highly capsulated, giving large mucoid colonies with clear haemolytic zones when grown on blood agar. In phenotypic tests, *S. equi* is separated from related group C streptococci by its inability to ferment lactose, sorbitol and trehalose (Grant *et al.*, 1993). Different isolates show very little serologic or antigenic variation (Galan & Timoney, 1988), and are generally considered as clonal. The genome is approximately 2.25 Mbp in length, with a G+C content of 41% (Sanger Institute, 2006). *S. equi* is an obligate parasite in the upper respiratory tract of *Equidae* (horses, donkeys and mules) where it causes a severe disease called strangles (Timoney, 1993). Unlike its related subspecies, *S. equi* is highly host-adapted, a recently reported case of strangles in dog being a rare exception (Ladlow *et al.*, 2006). At least one human infection with *S. equi* has been reported, where it was isolated from the cerebrospinal fluid of a child with meningitis (Elsayed *et al.*, 2003). No molecular tests were used in the identification though, so it cannot be ruled out that the infection was due to a related species with a fermentation pattern similar to *S. equi*.

*S. equi* subsp. *zooepidemicus* (*S. zooepidemicus*) is, as the name suggests, not strictly adapted to horses and can be found on the mucus membranes of a wide range of other animals, including humans. *S. zooepidemicus* exist as an opportunistic commensal, causing disease when the host is subjected to stress, for example due to virus infection or tissue injury (Timoney, 2004). In horses the most common complications include pyogenic infections of the joints, lymph nodes, nasal cavities, uterus and the respiratory tract. It has also been associated with bovine mastitis (Sharp *et al.*, 1995). *S. zooepidemicus* has been associated with serious infections in man, including septicaemia, meningitis, pneumonia, septic arthritis, endocarditis and poststreptococcal acute glomerulonephritis (Nicholson *et al.*, 2000; Kuusi *et al.*, 2006). Infections have mainly been reported in patients over the age of 70 and can often be traced to consumption of dairy products, such as unpasteurized cheese, which caused two recent outbreaks in Gran

Canaria (Bordes-Benitez *et al.*, 2006) and Finland (Kuusi *et al.*, 2006). The novel subspecies *S. equi* subsp. *ruminatorium* was recently isolated and identified from milk samples from sheep and goats with mastitis (Fernandez *et al.*, 2004). Phenotypic and genomic assays confirmed that these new isolates were clearly divergent from subspecies *equi* and *zooepidemicus*, and should be given separate subspecies status.

On the basis on DNA hybridisation, *S. equi* has formally been regarded as the archetype of *S. zooepidemicus* (Farrow & Collins, 1984). Later examinations of multilocus enzyme electrophoresis (Jorm *et al.*, 1994) and the composition of the interspacer sequences for the 16S and 23S rRNA genes (Chanter *et al.*, 1997) have indicated that *S. equi* actually evolved from an *S. zooepidemicus* ancestor. These studies and more recent analyses of the bacterial genomes (Artiushin *et al.*, 2006; Mitchell *et al.*, 2006) suggest that *S. equi* should be reclassified as a biovar of *S. zooepidemicus*. The ongoing annotation of the genomes of these subspecies, which are both accessible online (<http://www.sanger.ac.uk/Projects/Microbes/>), will give a more complete insight into their evolutionary relationship. This subject is discussed further in the section *S. equi* subsp. *equi* evolved through gene acquisition and gene loss.

## Strangles

Equine strangles, the disease caused by *S. equi*, is one of the most commonly diagnosed and serious diseases in horses. The disease and its transmission have been discussed in the medical literature since the late eighteenth century, and the first description of strangles goes back to 1251 (Timoney, 1993). Today the disease is spread worldwide, leading to both severe suffering in the affected animals and large economic losses. The following description is based on reviews written by Timoney (1993), Sweeney *et al.* (2005) and Waller & Jolly (2006).

### *Clinical signs and conditions*

Early signs of *S. equi* infection are a rapid onset of fever, followed by nasal discharge, coughing, loss of appetite and swelling of the lymph nodes of the head and neck. As the disease progresses, the inflamed lymph nodes become hard and painful and may restrict the airway, hence the name of the disease. Abscesses in infected lymph nodes usually rupture within 7 to 14 days after onset of disease, resulting in mucopurulent drainage through the nostrils of the horse. The majority of animals are fully recovered from infection within a 4-6 week period. Severe cases of strangles are more common in younger horses, while older animals with residual immunity to *S. equi* often develop a clinically milder form of the disease. In severe cases, bacteria disseminate and form abscesses in other parts of the body, such as the lungs, brain or abdominal lymph nodes. This condition, called 'bastard strangles', often leads to death or euthanasia. *Streptococcus equi* infection may also lead to the potentially fatal condition purpura haemorrhagica, an immune system-mediated acute inflammation of peripheral blood vessels characterised by subcutaneous oedema.

### *Pathogenesis*

The infection is initiated via the mouth or nose, where bacteria attach to the epithelium of the tonsils and adjacent lymphoid nodules. Within a few hours the bacteria have been translocated below the mucosa, reaching the deeper tissues of the tonsils. Bacteria can be detected in the lymph nodes that drain the pharyngeal and tonsillar region 18 hours after experimental infection (Kumar *et al.*, 2004; Timoney *et al.*, 2006). Once inside the lymph nodes, bacterial multiplication leads to the release of chemotactic factors, attracting large numbers of neutrophils. High fever develops 3 to 12 days after experimental infection, possibly associated with release of pyrogenic exotoxins. By that time chains of *S. equi* cells, most of which are dead, can be observed inside the infected lymph nodes and tonsils (Kumar *et al.*, 2004; Timoney *et al.*, 2006).



*Fig. 1.* Nasal discharge from a horse with strangles. Photo used with the kind permission of Prof. Derek C. Knottenbelt.

### *Transmission and persistent carriage*

Nasal shedding of *S. equi* usually begins within 2 to 3 days after onset of fever and persists for 2 to 3 weeks. In many cases, however, the horse continues to harbour *S. equi* for several weeks after recovery from strangles. During this period, transmission of infection can continue either by direct horse-horse contact, or via indirect routes such as contaminated water buckets. Isolation of recovering animals is therefore critical to avoid spread of the disease. Furthermore, in up to 10% of affected animals a long-term subclinical infection is established, most commonly in the guttural pouches (Newton *et al.*, 1997). This asymptomatic carrier state can persist for months or years, and is often difficult to diagnose and treat (Newton *et al.*, 2000; Verheyen *et al.*, 2000). Some animals in the carrier state sporadically shed *S. equi* through normal nasal secretions, in that way

providing a source of new outbreaks. Identification of asymptomatic carriers is therefore critical to prevent transmission of disease.

#### *Antibiotic treatment*

*Streptococcus equi* is sensitive to the majority of antibiotics *in vitro*, but the use of antibiotics to treat animals with strangles remains controversial. Immediate delivery of antibiotics during the early acute phase of strangles usually aborts the infection. However, the treated animal is unlikely to develop protective immunity and is susceptible to reinfection after the treatment is withdrawn. At later stages of infection the use of antibiotics is usually not effective. This is probably due to a lack of sufficient vascularity in the abscess to enable effective antibiotic penetration (Harrington *et al.*, 2002).

#### **Vaccination against *S. equi***

The majority of animals recovering from strangles acquire a solid immunity to reinfection that persists for 5 years or longer (Sweeney *et al.*, 2005; Waller & Jolley, 2006). This observation raised hopes for the development of an effective vaccine against strangles, but in spite of intensive research the progress in vaccine development has been slow. Early vaccines based on whole inactivated cells or extracts failed to provide adequate protection and often induced adverse reactions (Sweeney *et al.*, 2005; Waller & Jolley, 2006). An attenuated, unencapsulated strain, (Pinnacle I.N.) has been on the market in the USA since 1998 (Timoney, 2004). The Pinnacle strain has a proven protective effect, but unwanted strangles-like symptoms have been reported following intranasal vaccination and it has not been licensed for sale in Europe for this reason. Another drawback with the Pinnacle strain is that it was developed by random chemical mutagenesis, which means it is prone to back-mutations that would revert the strain into an encapsulated phenotype. To prevent this, an improved unencapsulated mutant (Pinnacle HasNeg) has been generated by deleting a defined part of the *has* operon (Walker & Timoney, 2002). Another live attenuated vaccine (Equilis StrepE) has recently been introduced on the European market (Jacobs *et al.*, 2000; Newton *et al.*, 2005; Intervet, 2006). In order to minimise adverse reactions, the vaccine is injected submucosally under the upper lip of the horse. The vaccine appears to be safe, but the immunity is limited to a three-month period and only about 50% of vaccinates receive full protection following experimental infection. For this reason, the vaccine is only recommended for horses that are at high or moderate risk of infection.

Protein subunit vaccines could offer an alternative to vaccines based on killed or attenuated bacteria. Acquired immunity appears to be primarily due to antibodies directed to the SeM protein and other unidentified antigens (Timoney, 2004). However, vaccines based on purified or recombinant SeM offer little or no protection (Sheoran *et al.*, 2002; Timoney, 2004). A combination of different antigens is therefore likely to be necessary for a protective subunit vaccine. The protective immunity in horses recovered from strangles is believed to depend on a

combination of mucosal and serum immunoglobulins, and the failure of M protein-based vaccines could in part be due to the lack of an adequate mucosal response (Timoney & Eggers, 1985; Timoney & Trachman, 1985; Hamlen *et al.*, 1994; Sheoran *et al.*, 1997). Vaccination trials have also been performed with a combination of three cell surface proteins (CNE, EAG and ScIC) which showed promising results in a mouse model (Flock *et al.*, 2004; Flock *et al.*, 2006). Follow-up trials in horses vaccinated with these antigens combined with different adjuvants are currently being performed and will hopefully be evaluated soon.

### ***S. equi* subsp. *equi* evolved through gene acquisition and gene loss**

Comparisons between the genomes of *S. equi* and *S. zooepidemicus* have shown about 97% sequence identity. When analysing the genomes of these closely related bacteria, two major factors seem to be associated with the evolution of *S. equi*; a large number of gene disruptions caused by mobile genetic elements and four prophage-like insertions, containing a number of potentially virulence-related genes. So far no genome-scale study of *S. equi* has been published, and for that reason the following discussion is largely based on conference abstracts from two independent groups, Artiushin *et al.* (2006) and Mitchell *et al.* (2006).

At present, 71 IS elements have been identified in the genome of *S. equi* and the number is likely to be somewhat higher in the final annotation of the genome (Matt Holden, unpublished results). These mobile genetic elements have caused numerous lesions in genes that are predicted to be important for the ability of the bacteria to adapt to changing environments, including genes involved in transcription regulation, quorum sensing and two component regulatory systems (Artiushin *et al.*, 2006). Genetic interruptions responsible for various defects in nutrient transport and metabolism – such as the inability of the bacteria to ferment lactose, sorbitol and ribose – have also been identified (Artiushin *et al.*, 2006; Mitchell *et al.*, 2006). In this respect clear parallels can be seen in studies of the evolution of the genus *Bordetella* (Preston *et al.*, 2004). The three bacterial species *Bordetella pertussis*, *B. parapertussis* and *B. bronchiseptica* are all closely related, but have different host ranges and cause different diseases. *Bordetella bronchiseptica* is closest to the evolutionary ancestor of these bordetellae and is asymptotically carried by a wide range of mammals. The primarily human pathogens *B. parapertussis* and *B. pertussis* are independent derivatives of *B. bronchiseptica*, and genome analysis suggests that they evolved primarily due to loss of gene function caused by insertion of a large number of IS elements. Another more closely related species that evolved mainly through loss-of-function events is *Streptococcus thermophilus* (Bolotin *et al.*, 2004), the only ‘Generally Recognized as Safe’ species within the genus *Streptococcus*. In this species the adaptation to a constant milk environment has led to the inactivation of most of the streptococcal virulence-related genes.

It has long been known that phage-mediated gene transfer plays an important role in the evolution and virulence of pathogens (Brussow *et al.*, 2004). With the

advent of bacterial genome sequencing, a large number of new prophages have been discovered, which has led to increased interest in this area of research. In the genome of *S. equi*, the majority of the genes that have no homologues in *S. zooepidemicus* have been located in four prophage-like insertions. These genes include previously reported virulence factors such as the pyrogenic mitogens SeeH, SeeI, SpeL, SpeM, but also a putative phospholipase with high similarity to the Sla toxin of *S. pyogenes* M3, a potential mitogen with similarity to the streptodornase of *S. pyogenes* M3 and a hyaluronidase that is required for phage penetration of the bacterial capsule (Mitchell *et al.*, 2006; Tiwari *et al.*, 2006). Interestingly, the Sla and streptodornase homologues are located in two separate prophages in both *S. equi* and *S. pyogenes* M3, highlighting the importance of gene transfer between these closely related species. It has been proposed that the recent acquisition of Sla by *S. pyogenes* M3 (Beres *et al.*, 2002) may have occurred via phage-mediated transfer from *S. equi* (Mitchell *et al.*, 2006).

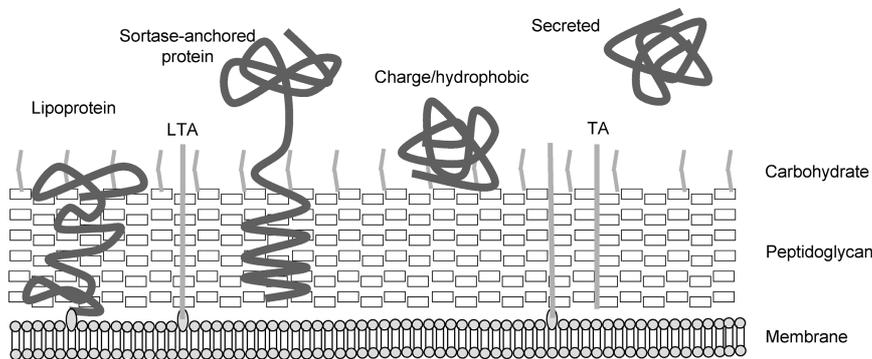
To summarise, the rapid IS element expansion in the genome of *S. equi* has led to numerous interruptions of genes with important functions for the ability of the bacteria to adapt to changing environments. Loss of gene function therefore seems largely accountable for the inability of *S. equi* to colonise mucosal surfaces. The acquisition of virulence-related genes through phage-mediated transfer, on the other hand, has most likely been essential for the bacteria in its adaptation to a new and unique ecological niche.

## **Extracellular proteins of streptococci**

Extracellular proteins, which here collectively refers to secreted and cell surface-located proteins, are believed to play an important role in the process of infection and in bacterial survival inside the host. This section describes the general characteristics of streptococcal cell surface-anchored proteins, while the next section discusses the importance of some of these proteins in more detail.

### *The streptococcal cell wall*

The streptococci, like other Gram-positive bacteria, are surrounded by a rigid cell envelope made from cross-linked peptidoglycan (Navarre & Schneewind, 1999). This large macromolecule works as a physical barrier, protecting the bacteria against both mechanical and osmotic lysis. The cell wall also serves as a scaffold for the attachment of proteins, teichoic acids and polysaccharides that are exposed from the bacterial surface. Three different mechanisms of anchoring proteins to the cell surface are common among the streptococci (Ton-That *et al.*, 2004) (Fig. 2): (i) Sortase-mediated attachment of proteins with LPxTG-type motifs to the cell wall; (ii) N-terminal linkage to lipids of the underlying cell membrane (lipoproteins); and (iii) binding by charge or hydrophobic interaction. In *S. equi*, as in the other streptococci, the majority of all cell surface proteins studied are predicted to be sortase-attached.



*Fig. 2.* Schematic presentation of the major surface structures of the streptococcal cell wall (modified from Fischetti, 2000). Surface proteins can be linked to the cytoplasmic membrane through a cysteine in the N-terminus (lipoproteins), to the peptidoglycan through a C-terminal LPxTG motif (sortase-anchored proteins) or to the cell surface by charge or hydrophobic interactions. Other proteins are secreted to the growth medium. Linked to the outer surface of the streptococcal cell wall are polysaccharide structures that are used in some cases for their immunological classification. Teichoic acids (TA) span through the cell wall and can be linked to the peptidoglycan, or as the lipoteichoic acids (LTA) to fatty acids of the cytoplasmic membrane.

### *Export of extracellular proteins*

Proteins that are destined to the outside of bacterial cells are labelled with an N-terminal signal peptide (signal sequence) that is proteolytically removed upon translocation across the cell membrane (Fig. 3). The signal peptide is characterised by short sequence of positively charged residues in the N-terminal end, followed by a central hydrophobic region and a C-terminal cleavage site with small, uncharged residues in the -3 and -1 positions relative to the cleavage site (von Heijne & Abrahmsen, 1989). The signal peptides of Gram-positive bacteria are usually longer than those of Gram-negatives, with a mean length of around 29-31 aa. Cell surface proteins require an additional topogenic sequence to be correctly located to the surface, such as a lipobox for lipoproteins or a C-terminal sorting signal for sortase-anchored proteins. Proteins with signal peptides that lack this additional motif are normally released into the extracellular milieu. A number of enzymes with important functions in streptococcal virulence are secreted in this manner.

### *Sortase-anchored proteins*

Sortase-anchored proteins are characterised by a C-terminal cell wall sorting signal, spanning approximately 30 to 40 residues (Marraffini *et al.*, 2006). This sequence starts with an LPxTG motif, followed by a stretch of hydrophobic residues and a mostly positively charged tail in the C-terminal end (Fig. 3). The hydrophobic domain and the charged tail are thought to arrest the transport of the protein from the cytoplasm, placing the LPxTG motif at the outer surface of the cell membrane. The LPxTG motif is then recognised by the membrane-associated enzyme sortase, which cleaves it between the threonine and the glycine residues

and covalently links the carboxy-end of the threonine to the peptidoglycan (Navarre & Schneewind, 1999). The cell wall sorting signal, including the LPxTG motif, is useful in the bioinformatic identification of genes for cell wall-bound proteins. In many bacterial species, however, the sortase substrate motifs tend to deviate from the LPxTG pattern (Boekhorst *et al.*, 2005). Among the predicted cell surface proteins of *S. equi*, LPxTN appears to be a common variant (IV).

A recently discovered function of sortases that has received great attention is their participation in the assembly of pili in Gram-positive bacteria (Ton-That & Schneewind, 2004; Marraffini *et al.*, 2006). In this process, the sortase is believed to catalyse the polymerisation of proteins with LPxTG motifs into an extended filament, often with an adhesive component at the tip. Pili of this type have been identified in a number of streptococcal species, including *S. pyogenes* and *S. agalactiae* (Mora *et al.*, 2005; Dramsi *et al.*, 2006).

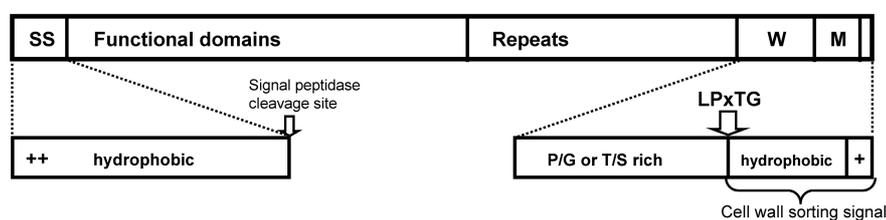


Fig. 3. Domain organisation of a characteristic sortase-anchored surface protein. SS, signal sequence; W, wall spanning region; M, membrane spanning region.

### *Common structures and functions of cell wall-anchored proteins*

Streptococcal cell wall-anchored proteins display a wide variety of different structures and functions. A common domain in almost all cell wall-anchored proteins is a 50-125 aa long proline/glycine or threonine/serine-rich sequence, located next to the LPxTG motif (Fischetti, 2000). This part of the protein is positioned within the cell wall. The part of the protein that is exposed outside the cell contains the functional domain(s) and is therefore typically unique. In many proteins, however, the C-terminal part contains a set of repeated sequence blocks of varying lengths (Navarre & Schneewind, 1999). In some proteins this repetitive region displays binding activity, but in others it is believed to function merely as a spacer region, exposing the functional domain from the cell.

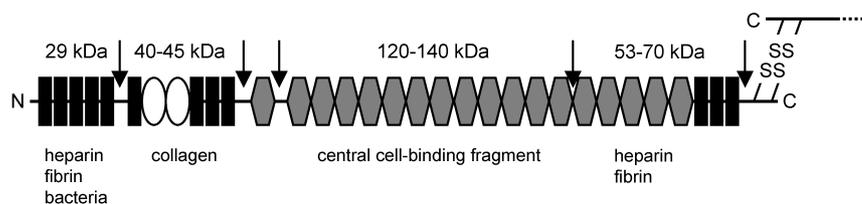
The most commonly studied functions of cell wall-anchored proteins are the binding to host tissues, plasma proteins and specific immune system components. In this respect they stand in contrast to lipoproteins, which are more commonly involved in transport systems, often involving uptake of nutrients (Sutcliffe & Russell, 1995). Bacterial proteins, cell-bound or soluble, that specifically interact with host tissue or plasma components are generally called receptors (Kronvall & Jonsson, 1999). The commonly used term adhesin is more narrow, specifically referring to proteins that the bacteria use to adhere to host tissues. Other cell wall-anchored proteins function as enzymes, often proteases, with the catalytic site exposed to the surrounding environment.

## Bacterial binding to components of the extracellular matrix

Adherence to host tissues is the first step in bacterial infection, and in Gram-positive pathogens this initial attachment is to a large extent mediated by cell surface proteins. The majority of the known bacterial adhesins specifically interact with components of the extracellular matrix (ECM) and are therefore commonly referred to as MSCRAMMs (microbial surface components recognising adhesive matrix molecules) (Patti *et al.*, 1994). Common ligands for MSCRAMMs include fibronectin, collagen, laminin and elastin. This section describes two major ECM components that are important in the present study, fibronectin and collagen, and how the attachment to these ligands is exploited by Gram-positive pathogens.

### *Fibronectin*

Fibronectin (Fn) is a large and complex glycoprotein, which usually exists as a dimer of two nearly identical subunits (250-280 kDa) linked near their C-termini by a pair of disulphide bonds. Fibronectin is involved in several important biological processes in vertebrates, including cell adhesion, migration, growth and differentiation (Pankov & Yamada, 2002). These biological interactions are mediated by a number of functional domains that interact with cellular integrins, collagen/gelatin, heparin, fibrin and other ligands. Fibronectin is present both in soluble form in plasma and other body fluids (termed plasma Fn), and in insoluble form as part of the extracellular matrix (cellular Fn). Cellular Fn constitutes a larger and more heterogeneous group of different isoforms, resulting from cell- and species-specific mRNA splicing patterns (Pankov & Yamada, 2002). Approximately 90% of the Fn sequence consists of three repetitive sequences that form distinct modules: 12 F1 modules, two F2 modules and 15-17 F3 modules (Fig. 4). Fibronectin is only cleaved at specific non-folded, unprotected sites when subjected to limited proteolytic digestion, and the resulting fragments are commonly used to locate the different active sites of the molecule.

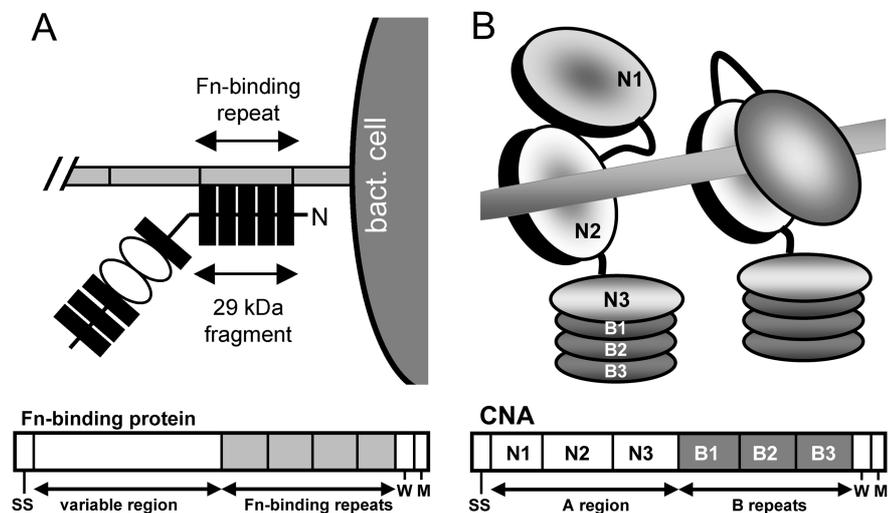


*Fig. 4.* Modular representation of one Fn chain. F1 modules are in black, F2 modules in white and F3 modules in grey. The number of F3 modules varies due to differences in RNA splicing. Arrows mark the major proteolytic digestion sites. The molecular weights of the resulting fragments, and their major ligand interactions, are indicated.

### *Collagens*

Collagens, the most abundant structural components of the ECM, are a family of proteins that form a characteristic rope-like right-handed helix of three polypeptide chains. Up to now, 26 different collagen types have been identified, most of which show diverse functions and tissue distributions, but all with at least one triple-

helical domain formed by a number of Gly-x-y repeats (Gelse *et al.*, 2003; Ricard-Blum & Ruggiero, 2005). Collagens can be assembled by three identical peptide chains (homotrimers) or by a combination of different chains (heterotrimers). The collagen superfamily serves a number of different biological functions, but most noticeably they function by forming highly organised polymers that contribute to the structural integrity of the extracellular matrix. Collagens can be grouped in two main classes based on the type of polymers formed; the fibril-forming collagens (collagens I, II, III, V and XI), which represent a rather homogeneous group, and the more heterogeneous group of nonfibrillar collagens (Aumailley & Gayraud, 1998). Collagen fibrils are supramolecular aggregates that give tissues such as tendons, bone, cartilage and skin the ability to resist tear, tensile stress and pressure (Bosman & Stamenkovic, 2003). A major nonfibrillar sub-group is the network-forming collagens (IV, VIII and X), which form a large part of the basement membrane (Bosman & Stamenkovic, 2003).



*Fig. 5.* Cartoon representations of two common models of bacterial binding to components of the extracellular matrix. A: In the  $\beta$ -zipper model, the Fn-binding repeat forms an additional antiparallel strand to the F1 modules of the 29-kDa fragment of Fn. Modified from Schwarz-Linek *et al.*, (2004). B: In the collagen hug model, the collagen triple helix is initially associated with the N2 domain of CNA, and then wrapped by the N1-N2 linker and the N1 domain. Modified from Zong *et al.* (2005).

#### *Bacterial proteins with fibronectin-binding repeats*

Fibronectin-binding adhesins are common among streptococci, staphylococci and other Gram-positive pathogens. The majority of these proteins specifically bind to the N-terminal 29-kDa fragment of Fn with a sequence of conserved repeats: ED(T/S)(X<sub>9,10</sub>)GG(X<sub>3,4</sub>)(I/V)DF (Table 2). Some proteins in this family also display an additional binding region that interacts with the adjacent 40-kDa fragment of Fn. The Fn-binding repeats are typically located in the C-terminal end, next to the cell wall-spanning region. The N-terminal part of these proteins often contains additional binding sites, in some cases with enzymatic properties, which

gives them mosaic-like structures of different functional domains. The properties of this family of Fn-binding proteins have been discussed in a number of recent reviews (Schwarz-Linek *et al.*, 2004; Hauck & Ohlsen, 2006; Schwarz-Linek *et al.*, 2006). The structure of typical Fn-binding repeats in complex with the 29-kDa Fn domain has been determined using NMR spectroscopy (Schwarz-Linek *et al.*, 2003; Pilka *et al.*, 2006). An unusual feature of this model is that the Fn-binding repetitive part of the protein has a free, linear conformation in solution, which upon binding to Fn forms an additional antiparallel strand to the  $\beta$ -sheet structure of the N-terminal F1 modules (Fig. 5).

Interestingly, Fn-binding proteins of *S. pyogenes* and *Staphylococcus aureus* have both been confirmed to mediate internalisation in several non-professional phagocytes, including epithelial and endothelial cells (Ozeri *et al.*, 1998; Sinha *et al.*, 1999). This process starts with the recruitment of plasma Fn to the bacterial surface. Upon binding, a conformational change is triggered in Fn, leading to the exposure of the integrin-binding RGD motif, which is cryptic in soluble Fn (Tomasini-Johansson *et al.*, 2001). Fn-integrin interactions lead to integrin clustering on the cell surface, which triggers signalling pathways leading to cytoskeleton rearrangements and bacterial uptake through membrane invaginations (Schwarz-Linek *et al.*, 2004; Hauck & Ohlsen, 2006). This mechanism of invasion might help the bacteria evade host immune responses and has been linked to persistent bacterial infections. It has also been suggested that intracellular survival of *S. pyogenes* contributes to penicillin treatment failures.

Studies to evaluate the role of Fn-binding proteins in experimental infections have led to unexpected and seemingly conflicting results. In two separate experiments, *S. pyogenes* strains lacking Fn-binding proteins (SOF and FbaB, respectively) were used in experimental mouse infections, both resulting in decreased mouse mortality (Courtney *et al.*, 1999; Terao *et al.*, 2002). However, the observed effect could not be conclusively linked to a lack of cellular binding to Fn in either of these studies. In a more recent study, the gene for protein F1 was introduced into a *S. pyogenes* strain lacking this gene (Nyberg *et al.*, 2004). Surprisingly, these F1-expressing bacteria were less virulent than the F1-negative strain in a mouse model. The virulence of these bacteria was partially restored when the bacteria were used to infect mice lacking plasma Fn, confirming that the decrease in virulence could be attributed to the Fn-binding activity of F1. Furthermore, infections with F1-negative bacteria caused an increased bacterial dissemination to the spleen, compared to the more localised infections caused by F1-expressing bacteria. A similar enhancement of virulence has been observed in a FnBP-negative strain of *S. aureus* when used in a rat model of pneumonia (McElroy *et al.*, 2002). Together, these experiments indicate that the Fn-binding activity of *S. pyogenes* and *S. aureus* leads to less virulent infections, as the bacteria are being immobilised by host cells and tissues.

Table 2. *Proteins with Fn-binding repeats recognising the 29-kDa fragment of Fn. Most of these proteins are multi-functional with additional binding or enzymatic properties*

Species	Name	Additional function	Reference
<i>S. aureus</i>	FnBPA	Elastin- and Fg-binding	(Signas <i>et al.</i> , 1989; Wann <i>et al.</i> , 2000; Roche <i>et al.</i> , 2004)
	FnBPB	Elastin-binding	(Jonsson <i>et al.</i> , 1991; Roche <i>et al.</i> , 2004)
<i>S. pyogenes</i>	SfbI / F1	Fibrinogen-binding	(Hanski & Caparon, 1992; Talay <i>et al.</i> , 1992; Katerov <i>et al.</i> , 1998)
	Pfbp / F2		(Jaffe <i>et al.</i> , 1996; Rocha & Fischetti, 1999; Ramachandran <i>et al.</i> , 2004)
	SOF/SfbII	Lipoproteinase	(Rakonjac <i>et al.</i> , 1995; Kreikemeyer <i>et al.</i> , 1999)
	FbaB / F2		(Jaffe <i>et al.</i> , 1996; Ramachandran <i>et al.</i> , 2004)
<i>S. dysgalactiae</i>	SfbX		(Jeng <i>et al.</i> , 2003)
	SOF/FnBA	Lipoproteinase	(Lindgren <i>et al.</i> , 1992; Katerov <i>et al.</i> , 2000)
<i>S. zooepidemicus</i>	FnBB		(Lindgren <i>et al.</i> , 1992)
	FNZ	Collagen-binding	(Lindmark <i>et al.</i> , 1996)
	FNZ2	Collagen-binding	(Hong, 2005)
<i>S. equi</i>	FNEB		(II)
<i>Borrelia burgdorferi</i>	BBK032		(Probert <i>et al.</i> , 2001)

### *Collagen-binding bacterial proteins*

The best studied collagen-binding adhesin of Gram-positive cocci is CNA of *S. aureus* (Patti *et al.*, 1992), and the crystal structure of the binding region of this protein was recently published (Zong *et al.*, 2005). In contrast to the Fn-binding proteins described above, the binding region of CNA forms two separate subdomains with fixed three-dimensional structures. These two domains attach to, and wrap around, the collagen monomer in a binding model called ‘collagen hug’ (Fig. 5). The proposed model explains why CNA does not bind to gelatine or denatured collagen. Similar collagen-binding proteins have been found in a number of Gram-positive pathogens, forming what appears to be a family of evolutionary related homologues (Table 3). Typically, the N-terminal half of these proteins contains the collagen-binding domain (A-domain), which is followed by a number of repeats (B-repeats) and a cell wall-anchor domain in the C-terminus. Unlike the Fn-binding proteins previously described, these proteins have no mosaic structures of different functional domains. The B-repeats have no known function other than to expose the active A-domain away from the cell. No CNA-homologue has been found in *S. pyogenes*, but some strains express an unrelated collagen-binding adhesin called CPA (Podbielski *et al.*, 1999). Interestingly, the predicted three-dimensional structure of the CPA collagen-binding site resembles the active site of CNA, suggesting that these proteins have acquired a similar structure through convergent evolution (Kreikemeyer *et al.*, 2005). CNA-deficient isogenic mutants have been used to confirm that CNA is a virulence factor in animal models of staphylococcal keratitis, endocarditis, osteomyelitis and septic arthritis (Hienz *et al.*, 1996; Rhem *et al.*, 2000; Elasri *et al.*, 2002; Xu *et al.*, 2004),

which indicates that the ability to bind collagen is advantageous for the bacteria in several different types of infection.

Table 3. *The CNA family of collagen-binding proteins*

Species	Name	Reference
<i>S. aureus</i>	CNA	(Patti <i>et al.</i> , 1992)
<i>S. equi</i>	CNE	(I)
<i>Streptococcus mutans</i>	CNM	(Sato <i>et al.</i> , 2004)
<i>Enterococcus faecalis</i>	ACE	(Rich <i>et al.</i> , 1999)
<i>Enterococcus faecium</i>	ACM	(Nallapareddy <i>et al.</i> , 2003)
<i>Bacillus anthracis</i>	BA0871	(Xu <i>et al.</i> , 2004)
	BA5258	(Xu <i>et al.</i> , 2004)

### *Fibronectin- and collagen-binding proteins as vaccine components*

The important functions of bacterial adhesins in the early phase of bacterial infections make them promising as components in subunit vaccines (Wizemann *et al.*, 1999). The proposed strategy has been to block the initial bacterial attachment with adhesin-specific antibodies at the site of infection. However, the level of success for adhesin-based vaccines or for subunit vaccines in general has been very limited to date (Moxon & Rappuoli, 2002; Grandi, 2003; Scarselli *et al.*, 2005; Serruto & Rappuoli, 2006). A common difficulty in vaccination trials is to induce a strong and lasting immune response at the mucosal level. Furthermore, most bacteria express a large number of adhesins that can compensate for each other during infection. An immune response against a specific combination of surface antigens might therefore be necessary to successfully block bacterial binding. To complicate matters further, several bacterial pathogens display strain-specific antigenic variations in their surface proteins. A broad-range vaccine must therefore induce an immune response directed to conserved epitopes of proteins that are common among different strains of the bacteria in question. This has been a great obstacle in the development of vaccines against *S. pyogenes* and other pathogens with great antigenic variation, but will probably not be as much of a problem with more homologous bacterial species such as *S. equi*.

Proteins with Fn-binding repeats have shown potential as vaccine components in several animal studies, including a rat model for *S. aureus* endocarditis and a mouse model for *S. pyogenes* infection (Rennermalm *et al.*, 2001; Schulze *et al.*, 2003). In contrast, no protection was observed in a mouse model for *S. equi* infection (Flock *et al.*, 2006). The observed protection is however most likely due to an antibody-mediated bacterial clearance by phagocytes (opsonisation). It has been reported that these antibodies actually recognise the Fn-binding proteins in complex with Fn and therefore do not prevent, but could actually enhance, the binding (Speziale *et al.*, 1996; Casolini *et al.*, 1998). The reason for this failure to generate neutralising antibodies could be that the 'free' Fn-binding region is poorly immunogenic due to the lack a secondary structure, or simply that Fn-binding proteins quickly form complexes with plasma Fn before they are recognised by the immune system.

A recombinant fragment of CNA has been shown to be effective in protecting mice against *S. aureus*-induced septic death (Nilsson *et al.*, 1998). Antibodies against CNA have also been shown to effectively interfere with the attachment of *S. aureus* to a collagen substrate (Visai *et al.*, 2000). Likewise, the use of CNE in a stranglers model in mice gave encouraging results, and the same study showed that serum antibodies against CNE were able to block the binding of *S. equi* to immobilised collagen (Flock *et al.*, 2006).

## **Potential virulence factors of *Streptococcus equi* subsp. *equi***

The exact definition of the term virulence factor has been a matter of debate, but it is generally defined as a component of a pathogen that fulfils molecular Koch's postulate, i.e. the deletion of the gene associated with the trait specifically impairs virulence (Casadevall & Pirofski, 1999; Falkow, 2004). To separate virulence factors from fitness factors, it is often implied that the deletion should not lead to a decrease in general viability. Factors that are believed to be important in the virulence of *S. equi* include the carbohydrate capsule, several surface proteins and a number of toxins. To date, however, only a few *S. equi* deletion mutants have been made and used in infection models. For that reason these factors are here collectively referred to as potential virulence factors.

### *Capsule*

*S. equi* display an highly encapsulated phenotype when grown on blood agar, giving the colonies a 'honeydew' appearance. This hyaluronic acid capsule is non-antigenic and has in several studies been associated with virulence and resistance to phagocytosis. The encapsulation in liquid growth media is greatly influenced by culture age, as young cultures (4-6 h) are highly encapsulated, whereas the encapsulation of overnight cultures is barely detectable (Harrington *et al.*, 2002). Infection experiments in mice and foals have both shown that young cultures are significantly more virulent and also more resistant to phagocytosis (Harrington *et al.*, 2002). A study by Anzai *et al.* (1999) confirmed that resistance to phagocytosis is also correlated with levels of encapsulation when strains with different expression of capsule are compared. Mutants completely lacking a capsule are unable to colonise the lymph nodes of experimentally infected horses (Timoney *et al.*, 2006). Hyaluronidase treatment of *S. equi* cells results in a slight increase in adherence to tongue cells of ponies, suggesting that heavy encapsulation partially covers the active sites of adhesins that are expressed on the cell surface (Srivastava & Barnum, 1983).

### *M-like proteins*

The M protein family of *S. pyogenes* is an extensively studied class of cell surface-anchored proteins that extend from the surface as alpha-helical coiled-coil dimers (Cunningham, 2000). The N-terminal region is often hypervariable and induces opsonic antibodies that normally do not cross-react with other M types. An important function of the M proteins is to enable the bacteria to resist

phagocytosis, or even facilitate intracellular survival inside neutrophils (Staal *et al.*, 2003). The antiphagocytic properties of the M proteins depend on binding to fibrinogen (Fg) or to human C4b-binding protein, which in turn prevents the deposition of complement factors on the bacterial surface (Carlsson *et al.*, 2005). The ‘M-like proteins’ of related streptococci often refer to Fg-binding proteins with antiphagocytic activity, and these proteins may or may not be structurally related to the M proteins of *S. pyogenes*.

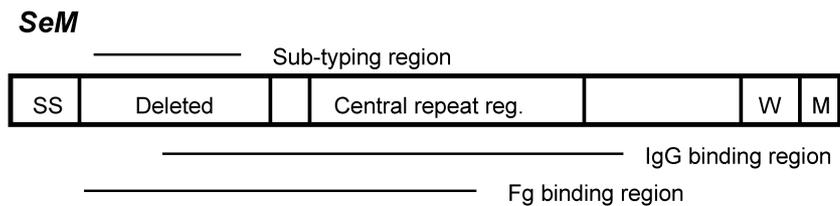


Fig. 6. Schematic representation of the SeM protein, adapted from Kelly *et al.* (2006). The region found deleted in 24% of *S. equi* strains isolated from subclinical carriers, the variable region used in subtyping of strains and the Fg- and IgG-binding regions are indicated.

In *S. equi* two M-like proteins have been studied, SeM (also known as FgBP) (Fig. 6) and SzPSe. These proteins show no significant sequence similarity between each other, or to the M proteins of *S. pyogenes*, but both have extended regions of alpha-helical coiled-coils and bind strongly to equine Fg (Timoney *et al.*, 1997; Meehan *et al.*, 2002). In addition, SeM binds to IgG-Fc (Meehan *et al.*, 2001). SzPSe is the *S. equi* homologue of the *S. zooepidemicus* protein SzP, but SeM has been regarded as unique to *S. equi*, and the acquisition of the gene encoding this protein has been proposed to be an important step in the evolution of this subspecies (Timoney *et al.*, 1997). However, recent genome studies have shown that *S. zooepidemicus* indeed encodes an allelic variant of SeM, denoted SzM (Kelly *et al.*, 2006). This protein shows no significant similarity to SeM in the N-terminal region, which is necessary for the binding of SeM to Fg and IgG-Fc, so it is possible that the replacement or modification of this region, rather than the acquisition of the whole protein, represents the important evolutionary step. SeM is the major cell wall-associated protein of *S. equi* cells grown *in vitro* (Meehan *et al.*, 1998) and antibodies to this protein are strongly opsonogenic in whole blood or with purified neutrophils (Boschwitz & Timoney, 1994). Antibodies to SzPSe, on the contrary, have no opsonic effect and the protein is not believed to be as important in resistance to phagocytosis (Timoney *et al.*, 1995). The importance of SeM as a virulence factor was demonstrated in a study where a knockout mutant lacking this protein was employed in a number of experiments (Meehan *et al.*, 2001). The mutant failed to autoaggregate when it reached the stationary phase under the conditions of laboratory growth, bound no Fg or IgG, was rapidly killed in horse blood and showed decreased virulence in a mouse model. Mutants lacking SeM are also unable to colonise the lymph nodes of experimentally infected horses (Timoney *et al.*, 2006).

Studies on the cross-reactivity of convalescent horse sera and Southern blots with a *sem* probe have suggested that SeM is highly homogeneous across different

bacterial isolates (Galan & Timoney, 1988). Exceptions were found in a study of long-term carriers with subclinical infections of the guttural pouches, where 24% of the isolates harboured genes with slightly different in-frame deletions of the 5' end encoding the N-terminal part of the mature protein (Chanter *et al.*, 2000). The deletions covered a part of the protein associated with Fg-binding, located between the signal sequence and the central repeat region (Fig 6). This N-terminal part of SeM thus seems necessary for full virulence, and the deletion might represent an escape mutation from the immune response of the horse that facilitates a prolonged infection (Chanter *et al.*, 2000). Isolates with these truncations were more susceptible to phagocytosis *in vitro* than typical isolates. Following this study, two independent groups have found several minor differences in the 5' end of the gene encoding SeM, and have shown that they can be used in the subtyping of different isolates (Anzai *et al.*, 2005; Kelly *et al.*, 2006). A combination of the results of these studies led to the identification of 43 different alleles of SeM (excluding alleles with changes in the signal sequences) (Waller & Jolley, 2006). The ratio of non-synonymous to synonymous substitutions in this variable region of SeM indicates that it is under high immunological pressure.

Released M proteins of *S. pyogenes* have recently been reported to induce inflammatory responses by two different mechanisms; direct activation of monocytes by interaction with TLR2 (Pahlman *et al.*, 2006) and, when forming complexes with fibrinogen, by activation of neutrophils (Herwald *et al.*, 2004). No similar inflammatory effect by M-like proteins of *S. equi* has been reported, but if such a property could be confirmed it would provide a good explanation for the reduced clinical symptoms observed in horses infected by *S. equi* with truncated protein SeM.

Table 4. Potentially virulence-related extracellular proteins of *S. equi*. Anchoring or secretion is in most cases predicted and not experimentally verified. Only published proteins are included

Name	Anchored or secreted	Function	In <i>S. zooepidemicus</i>
CNE	Anchored	Collagen-binding	CNZ?
EAG	Anchored	$\alpha_2$ M-, albumin and IgG-binding	ZAG
FNE	Secreted	Collagen- and Fn-binding	FNZ
FNEB	Anchored	Fn-binding	FNZ2
IdeE	Secreted	IgG-peptidase	IdeZ
ScIC-ScII	Anchored	Unknown	-
SeM	Anchored	Fg- and IgG-binding	SzM
SePE-H (SeeH)	Secreted	Exotoxin	Not detected
SePE-I (SeeI)	Secreted	Exotoxin	Not detected
SpeL (SeeL)	Secreted	Exotoxin	SzeL
SpeM (SeeM)	Secreted	Exotoxin	SzeM
SFS	Secreted	Fn-binding	SFS
SzPSe	Anchored	Fg-binding	SzP

### *Other receptors and adhesins*

EAG is the *S. equi* equivalent of ZAG, a plasma protein receptor of *S. zooepidemicus* that specifically binds to albumin, IgG and the protease inhibitor  $\alpha_2$ -macroglobulin ( $\alpha_2$ M) (Jonsson *et al.*, 1995). In Northern blots no transcripts of the gene encoding EAG could be detected from cells grown in laboratory cultures (Lindmark, 1999). Consequently, the binding of *S. equi* cells grown *in vitro* to IgG is reported to be very low (Lindmark *et al.*, 1999) and mediated primarily by SeM (Meehan *et al.*, 2001). Based on these data, the importance of EAG as a virulence factor could be questioned. However, measurements of antibody titres specific to EAG in convalescent horses can provide indirect evidence for the *in vivo* expression of EAG during *S. equi* infection (Flock *et al.*, 2004).

Three different Fn-binding proteins of *S. equi* have so far been published. FNE is a homologue of FNZ of *S. zooepidemicus*. FNZ contains two different Fn-binding domains: a region with typical repeats in the C-terminal end that interacts with the 29-kDa fragment of Fn, and an hitherto uncharacterised 40-kDa fragment binding domain in the N-terminal half of the protein (Lindmark *et al.*, 1996; Lindmark *et al.*, 2001) (II). However, the gene encoding FNE is interrupted by a frameshift mutation, leading to the transcription of a truncated protein without the 29-kDa binding repeats or the C-terminal cell wall anchor domain of FNZ (Fig. 7) (Lindmark *et al.*, 2001). FNE has consequently been found released in the medium during *S. equi* cultivation. Interestingly, the N-terminal half of FNZ, which is identical to the entire protein FNE, was recently found to bind collagen in addition to Fn (Liden *et al.*, 2006). Four new putative paralogues to FNE have been found in *S. equi*, all of which bind collagen to different degrees and one that binds Fn. These are discussed in the section *Present investigation*. Another Fn-binding protein (SFS) also binds the 40-kDa fragment (Lindmark & Guss, 1999) (II). SFS lacks any putative cell-wall anchoring domains, and has for that reason been predicted to be a secreted protein. However, the *in vitro* expression of SFS is low, and no protein has been detected in growth cultures. Finally, FNEB is the only published Fn-binding protein of *S. equi* that appears to be anchored to the cell wall (II). FNEB contains typical Fn-binding repeats and specifically interacts with the 29-kDa fragment. This protein is further described in the section *Present investigation*.

Seven different proteins with collagen-like repeats (SclC-SclI) have been studied in *S. equi* (Karlstrom *et al.*, 2004; Karlstrom *et al.*, 2006). The C-terminal parts of these proteins contain similar collagen-like repeats, and in the N-terminal ends each have a unique A-domain with no significant similarities to any known proteins. Two proteins with similar collagen-like repeats have been found in *S. pyogenes*, and these have been shown to form triple helices and interact with mammalian collagen-binding integrins (Xu *et al.*, 2002; Humtsoe *et al.*, 2005). Whether the Scl-family of proteins in *S. equi* have similar structures and biological functions remains to be investigated.

### *Secreted toxins and enzymes*

*Streptococcus equi* has acquired genes for at least four mitogenic exotoxins by phage-mediated transfer. These toxins are believed to function as superantigens and misdirect the immune response by activating a large number of T cells. This activation leads to a massive release of pro-inflammatory cytokines, which is likely to contribute to the severe symptoms of strangles, such as high fever and neutrophilia (Anzai *et al.*, 1999). Two of these toxins (SePE-H and SepE-I) are located in the same phage-associated locus and are almost identical to their counterparts in *S. pyogenes*. Both proteins have been shown to induce a strong mitogenic response in horse peripheral blood monocytes and pyrexia in rabbits, but only SEPE-I is pyrogenic in ponies (Artiushin *et al.*, 2002). The two other superantigens (SPE-L and SPE-M) also share high sequence similarity to orthologues found in *S. pyogenes* (Proft *et al.*, 2003). In a recent study, the genes for these two superantigens were also found in one out of 31 strains of *S. zooepidemicus* (Alber *et al.*, 2005).

Studies of the  $\beta$ -haemolysis in *S. equi* cultures under different growth conditions have suggested that this activity is due to a Streptolysin S-like toxin (Flanagan *et al.*, 1998). Production of the haemolysin is induced during logarithmic growth and is dependent on a serum supplement. However, the gene(s) responsible for the observed activity have not yet been published. The precise role of this toxin is not known, but the lysis of neutrophils and macrophages is expected to be important in immune evasion (Harrington *et al.*, 2002). This lytic activity is also likely to make essential nutrients available to the bacteria.

*Streptococcus equi* also secretes a streptokinase that specifically activates equine plasminogen and thereby induces the hydrolysis of fibrin (McCoy *et al.*, 1991). The function of this streptokinase in infection has not been proven, but it is possible that the hydrolysis of fibrin clots could aid bacterial spread.

*“Sometimes you make the right decision,  
sometimes you make the decision right”*

Dr Phil

## **Present investigation**

The main objective of this study was to identify and characterise new potential virulence factors expressed by *S. equi*. The focus was mainly on cell surface proteins that specifically adhere to collagen and fibronectin (**I**, **II**, **IV**), two major extracellular matrix proteins of vertebrates, but a secreted immunoglobulin-specific protease was also studied (**III**). A useful tool in this research was the publicly available *S. equi* genome, which can be used to identify homologues to virulence-associated proteins of related pathogens such as *S. pyogenes* and *S. aureus*. This genome database was also useful in identifying potential paralogues to previously characterised proteins (**IV**). The present study was mainly of a basic science nature, but had also the applied objective of identifying components for a future vaccine against strangles.

### **CNE, a collagen-binding protein of *S. equi* subsp. *equi* (I)**

The collagen-binding protein CNE belongs to a putative family of structural and functional homologues of Gram-positive cocci described in a previous section. In this study, we expressed recombinant proteins corresponding to the mature protein (CNE L) and the collagen-binding ‘A domain’ (CNE S) (Fig. 7). These recombinant proteins were used in a Western ligand blot to confirm that CNE bound to <sup>125</sup>I-labelled bovine collagen type I, and that the binding activity was located in the A domain. It was possible to inhibit the binding of soluble radiolabelled collagen to immobilised CNE S in microwells with increasing concentrations of CNE S, indicating that the binding was specific. In a similar manner, it was possible to inhibit the binding between collagen and immobilised CNE S with recombinant CNA, confirming that the two proteins were competing for the same binding site in collagen.

CNE S was used to inhibit the binding of radiolabelled collagen to *S. equi* cells in solution, but the binding could only be reduced to 65%. The reason for this incomplete inhibition could be that multiple binding sites on collagen in combination with a high cellular expression of CNE make complete inhibition unattainable, or that the cellular binding to collagen is due to the combined activity of CNE and an unidentified surface structure. When *S. equi* cells were incubated in solution with radiolabelled CNE S and increasing concentrations of collagen, the radioactivity bound to the cell pellet was proportional to the amount of added collagen, indicating that CNE binds to multiple binding sites on the collagen monomer.

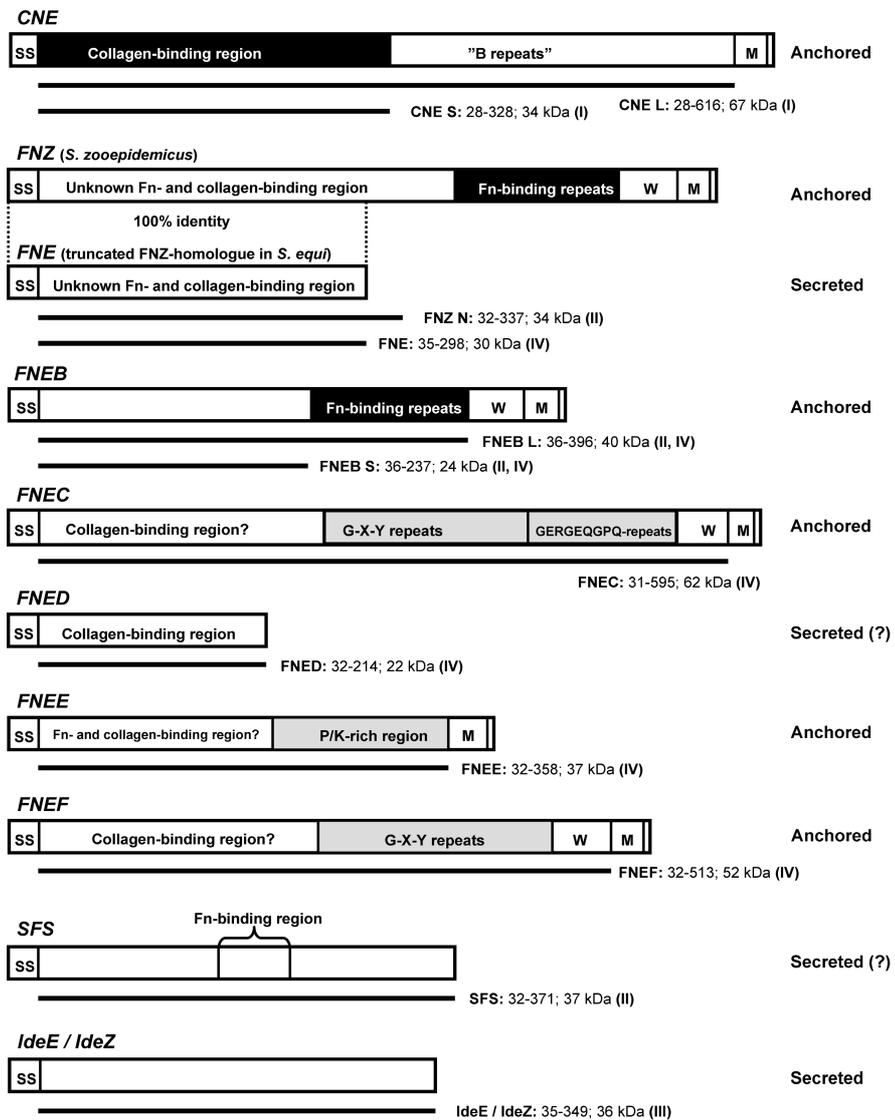


Fig. 7. Schematic representation of the extracellular proteins in the present study. The recombinant proteins used are represented as bars, with lengths in amino acids and molecular weights indicated. Roman numerals in brackets show the papers in which the recombinant proteins were used.

The gene encoding CNE was detected in all isolates of *S. equi* and *S. zooepidemicus* tested. Today, the gene encoding the CNE-homologue of *S. zooepidemicus* can be found in the publicly available *S. zooepidemicus* genome database. However, the protein encoded by this gene has not been studied.

Since the completion of this study, CNE has been evaluated as a vaccine component in a strangles model in the mouse (Flock *et al.*, 2006). Vaccination with CNE prior to experimental infection resulted in a low but statistically significant decrease in clinical symptoms, measured as weight loss and nasal shedding. Interestingly, CNE had an immunostimulatory effect on the poorly immunogenic protein EAG, and a coadministration of CNE and EAG resulted in a synergistic protective effect. The same study showed that serum antibodies against CNE were able to block the binding of *S. equi* to immobilised collagen. Vaccination trials with a protein subunit vaccine including CNE are currently being performed in horses.

## **Studies of fibronectin-binding proteins of *Streptococcus equi* (II)**

In this study, a novel Fn-binding protein of *S. equi* (FNEB) was described (Fig. 7). FNEB displays a signal sequence and a typical cell wall-anchoring domain with an LPxTG motif. FNEB can thus be predicted as cell surface-located, contrary to the two previously characterised fibronectin-binding proteins of *S. equi* FNE and SFS. Western ligand blots with two recombinant proteins, one corresponding to the mature protein (FNEB L) and one corresponding to the C-terminal half (FNEB S), confirmed that the binding activity is located near the C-terminus, and that it interacts specifically with the 29-kDa fragment of Fn. This part of the protein contains a number of characteristic Fn-binding repeats described in the section *Bacterial binding to components of the extracellular matrix*. This study also showed that the previously characterised proteins FNE and SFS both bind to the 40-kDa collagen-binding fragment of Fn. Binding-inhibition studies with iodinated Fn added to microwells with immobilised FNEB, SFS and FNE showed a cross-inhibition between FNE and SFS, indicating that these proteins bind to overlapping, or nearby located sites on the Fn molecule. As expected, FNEB could not be used to inhibit the binding activity of FNE or SFS and *vice versa*.

*Streptococcus equi* is known to bind Fn to a much lower degree than *S. zooepidemicus* (Lindmark *et al.*, 1999). By measuring the binding of iodinated Fn-fragments to *S. equi* cells, it was shown that the observable binding is primarily directed to the 29-kDa domain. Furthermore, FNEB L could be used to inhibit the binding of both radiolabelled 29-kDa fragments and whole Fn to *S. equi* cells, indicating that FNEB is the primary adhesin that mediates binding of Fn to *S. equi* cells grown *in vitro*. As described previously, experiments with *S. aureus* and *S. pyogenes* mutants have revealed that a decreased binding to Fn is associated with an increase in invasiveness and virulence. Considering the comparatively low ability of *S. equi* to bind Fn compared with the evolutionary parental *S. zooepidemicus*, we postulate that this decreased adhesion is a contributing factor to its more virulent phenotype.

Based on the low, but observable binding to Fn, it could be predicted that FNEB is expressed under laboratory conditions, albeit at a low level. To test this hypothesis, total RNA was prepared from *S. equi* cells at the logarithmic phase of growth, followed by cDNA synthesis using RT-PCR with short random primers. The relative amounts of transcripts of *fne*, *sfs*, and *fneB* were measured by gene-specific PCR, with the housekeeping gene *gyrA* used as a control. The highest expression was observed for *gyrA* (clear PCR-product after 22 cycles), followed by *fne* (24 cycles), *fneB* (27 cycles) and *sfs* (29 cycles). This pattern fits well with previous studies where transcripts of *fne*, but not *sfs*, were detected by Northern blots (Lindmark & Guss, 1999). As mentioned previously, native FNE has also been isolated from growth cultures (Lindmark *et al.*, 2001), indicating a relatively high expression. Analyses of antibody titres from mice and horses with or without *S. equi* infections were used to assess the expression of FNEB during infection. A significant increase in antibody titres in mice with experimental infection and in convalescent horses indicates that FNEB is expressed during infection and exposed to the immune system.

### **IdeE, an IgG-endopeptidase of *S. equi* subsp. *equi* (III)**

In all mammals, immunoglobulin G (IgG) has a key role in the defence against bacterial pathogens. By detecting antigens of the invading bacterium, the IgG molecule can mediate bacterial killing either by activating the classical complement system or by marking the bacterium for ingestion by phagocytes, an activity called opsonisation. Recognition by IgG can also inactivate bacterial toxins and adhesins. The IgG molecule is a glycoprotein made up of four subunits, two heavy chains and two light chains, linked by disulphide bridges. Its overall organisation is presented in Figure 8.

Bacterial pathogens have evolved several different strategies to avoid recognition by IgG. One mechanism is to bind the Fc region (C<sub>H</sub>2-C<sub>H</sub>3) of the IgG molecule with specific surface receptors, such as the previously mentioned SeM and EAG proteins, and in that way block the interaction with Fc-receptors on phagocytes. Another strategy is to interfere with the interaction between IgG and the Fc-receptors of phagocytes by releasing enzymes that either degrade the N-linked glycans of the IgG molecule, or cleave the peptide backbone of the IgG heavy chains (Collin & Olsen, 2003). IdeS, also known as Mac, is a secreted cysteine protease of *S. pyogenes* that cleaves the hinge region of human IgG with high specificity (Lei *et al.*, 2002; von Pawel-Rammingen *et al.*, 2002; von Pawel-Rammingen & Bjorck, 2003). Genes for IdeS/Mac homologues have been found in the genomes of *S. equi* and *S. zooepidemicus*. In the present study, these genes were cloned and the proteins they encode, IdeE and IdeZ, were expressed and characterised.

Purified horse IgG was incubated with recombinant IdeE and a cleavage product was visible when analysed by SDS-PAGE. The N-terminal end of the cleavage product was identified as GPSVFIFPP[K/N]PK by tandem mass spectrometry sequencing, confirming that IdeE and IdeS/Mac cleaves IgG at the same site. A

study of the proteolytic activity of recombinant IdeE and IdeZ on purified IgG from a selection of mammals shows that only antibodies containing the substrate site of IdeS/Mac are cleaved, indicating that the specificities of the two enzymes are very similar. Interestingly, horse IgG was cleaved less effectively than the IgG of e.g. dogs or humans. A likely explanation for this partial proteolysis would be that the hinge region of the dominating IgG isotype in horse serum (IgG4) is resistant to cleavage due to the lack of a distinct substrate site for IdeE/IdeZ. A Western blot with IgG4-specific antibodies confirmed that the heavy chain of this isotype was indeed intact after incubation with recombinant IdeE.

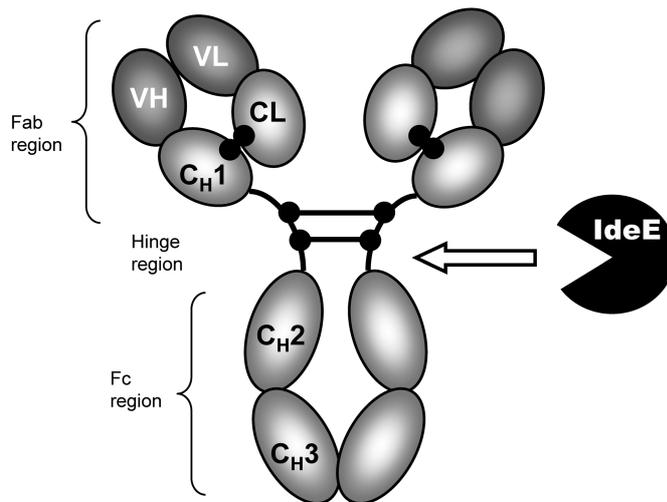


Fig. 8. Schematic presentation of the IgG molecule. The N-terminal end of the heavy chains contain the variable, antigen-binding domain, VH (variable heavy), followed by three constant domains, C<sub>H</sub>1-C<sub>H</sub>3 (constant heavy 1-3). The heavy chains are connected in the hinge region by two disulphide bridges. The light chains contain a variable domain, VL (variable light) and a constant domain, CL (constant light), which is connected to the C<sub>H</sub>1 domain by a single disulphide bridge. IdeE and IdeZ cleave the heavy chains at the same site as IdeS, in the hinge region on the C-terminal side of the disulphide bridges.

The ability of *S. equi* and *S. zooepidemicus* to degrade antibodies was studied by determining the proteolysis of IgG in cell cultures. IgG-degradation was observed when *S. equi* was grown in the presence of horse serum, but not when grown with purified IgG (Fig. 9). In contrast, the growth of *S. zooepidemicus* in the presence of serum did not result in proteolysis of IgG. The fraction of *S. equi*-degraded IgG was cleaved at the same site as from the activity of IdeE. Together, these results suggest that the growth of *S. equi* in the presence of serum induces the expression of IdeE, but that no expression of IdeZ is induced when *S. zooepidemicus* is grown under the same conditions. Surprisingly, however, a Western blot confirmed that the IgG4 fraction in the horse serum had been completely cleaved by *S. equi*, indicating that the observed activity is due to the expression of an unknown enzyme rather than IdeE. Another possible explanation for this result could be that the native IdeE is not structurally and functionally equivalent to the recombinant

enzyme, or that an unknown factor produced by *S. equi* is necessary for its full proteolytic activity.

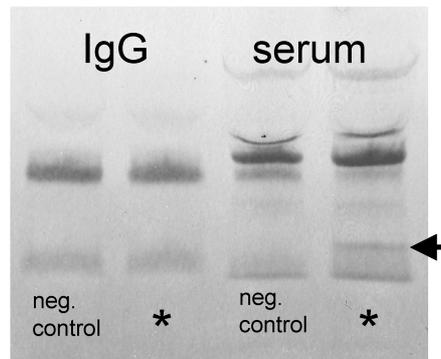


Fig. 9. Medium with horse IgG (1 mg/mL) or 10% horse serum was incubated overnight. The supernatant was analysed by SDS-PAGE. \* = Medium inoculated with *S. equi*. The IgG-degradation product is indicated (arrow).

#### **FNE belongs to novel a family of fibronectin-binding and collagen-binding proteins of *Streptococcus equi* (IV)**

The recent discovery that FNE binds both Fn and collagen and that no homologues of this protein have been identified in any related bacterial species led us to investigate whether any genes for putative paralogues to this protein could be found in the *S. equi* genome. Four ORFs encoding proteins with similarities to FNE and FNEB were found by a BLAST search in the publicly accessible genome at Sanger Center (<http://www.sanger.ac.uk/Projects/Microbes/>). Of these four novel proteins, FNEC, FNEE and FNEF have characteristic C-terminal cell wall- and membrane-spanning regions with putative sortase-substrate sites, and can thus be predicted as cell wall-anchored (Fig. 7). However, the gene encoding FNED is interrupted by a stop codon approximately halfway into the gene. As a result, the ORF encodes a truncated protein, and without a C-terminal cell wall sorting signal it is likely to be secreted in the growth medium in a similar way to FNE. The FNE-like regions of these proteins are located in the N-terminal ends, and the C-terminal parts are either not transcribed (FNED) or contain unrelated collagen-like repeats (FNEC, FNEF) or P/K-rich repeats (FNEE).

To determine the binding activity of the FNE-like proteins, a microwell assay was used. Radiolabelled plasma Fn and collagen type I in PBS-T were added to wells coated with the same molar concentration of each recombinant protein and the specific binding was measured. FNEE was found to be the only protein of this family apart from FNE with binding activity to both Fn and collagen. However, the remaining three novel proteins (FNEC, FNED and FNEF) bound different amounts of soluble collagen. The FNEB S construct (Fig. 7) representing the N-terminal part of FNEB was the only recombinant protein in the study with no

binding activity to either ligand. An alignment of the FNE-like regions showed patches of conserved residues among all proteins, and did not give any obvious clues to the observed differences in binding specificities or the location of the binding sites.

## Future perspectives

As shown in Lindmark *et al* (1999) and in **II**, a clear difference in the adhesive properties of *S. equi* and *S. zooepidemicus* is that the former binds considerably less Fn, and especially the 40-kDa fragment of Fn, when grown under standard laboratory conditions. In **II**, we postulated that this could be an important factor behind the invasive nature of *S. equi* infection. The finding that *S. equi* harbours at least two Fn-binding proteins that appear to be cell-wall anchored (FNEB and FNEE) merits further study of the expression of these proteins, and of the Fn-binding activity of *S. equi* cells, under different growth conditions and growth phases. Expression and binding studies could, for example, be performed with cells grown in the presence of blood, under anaerobic conditions, in iron-depleted medium or – even better – with cells isolated from infected horses. Studies with *S. pyogenes* have shown that growth in plasma or blood increases the expression of several secreted and cell surface-bound proteins (Graham *et al.*, 2005; Johansson *et al.*, 2005), and our own cell culture experiments in **III** indicate that the presence of blood components also influences the expression of virulence factors in *S. equi*. In a study by Meehan *et al.* (1998), FnBP/SeM was identified as the major cell wall-associated protein of *S. equi*. However, this work, like most ligand-binding studies with *S. equi*, was performed with cells from overnight cultures grown in standard laboratory culture medium. A large number of novel *S. equi* proteins with cell wall-anchoring motifs have been characterised since then, and genes for many more can be found in the genome. Given this seemingly abundant arsenal of adhesins, it is likely that different cell surface proteins are expressed during different phases of infection – such as the initial attachment to the tonsils, the proliferation inside the lymph node and during the establishment of persistent infection in the guttural pouches.

The Fn- and collagen-binding proteins FNE/FNZ have several putative paralogues in *S. equi* and *S. zooepidemicus*, but no known homologues in other bacterial species. As we show in **IV**, the binding sites for Fn and collagen cannot easily be pinpointed by aligning the sequences of binders and non-binders within the FNE-family of proteins. With truncated recombinants of FNE, FNEC, FNED, FNEE and FNEF, this could be elucidated. In addition, it would be interesting to search for homologues to these proteins in related streptococci, especially since recent studies have suggested that FNE could alter fluid homeostasis in infected tissues (Lidén *et al.*, 2006 and unpublished results).

The binding of Fn to the proteins SfbI and FnBPA of *S. pyogenes* and *S. aureus* has been shown to trigger integrin-mediated internalisation of these bacteria by epithelial and endothelial cells (Schwarz-Linek *et al.*, 2004; Hauck & Ohlsen, 2006). As we speculate in **II**, it is possible that FNEB serves a similar function in *S. equi*. It would therefore be interesting to evaluate whether FNEB contributes to the rapid internalisation of *S. equi* during the initial infection of the epithelium of the tonsil and adjacent lymphoid nodules (Timoney *et al.*, 2006).

The cell culture experiments performed in **III** suggest that an unidentified IgG-cleaving protease, rather than IdeE, is expressed when *S. equi* is grown in the presence of serum. An isogenic mutant lacking the *ideE* gene could be used to elucidate this. An alternative strategy would be to search the *S. equi* genome for genes encoding other extracellular proteases that could be expressed and characterised.

In recent years, pili have been found on the surface of a number of streptococci including *S. pyogenes*, *S. agalactiae* and *S. pneumoniae* (Ton-That & Schneewind, 2004; Mora *et al.*, 2005; Dramsi *et al.*, 2006; Marraffini *et al.*, 2006). These proteinaceous filaments are assembled from proteins with LPxTG-type motifs by a specific sortase, and the genes for the building blocks and the sortase are often found within a single operon. Interestingly, the *cne*-gene in *S. equi* is located in the upstream end of a putative operon with two other genes; just downstream is a gene for a protein with (weak) similarities to several fimbrial subunit proteins in different Gram-positive bacteria, followed by a gene for a sortase-like protein. It can thus be postulated that CNE is incorporated to the end of a pilus structure with the putative fimbrial subunit proteins as the main building block (which would explain the lack of a typical cell wall-spanning region in CNE). The best way to test this hypothesis would probably be with electron microscopy, using cells coated with gold particles conjugated to antibodies recognising CNE and/or the putative fimbrial subunit protein.

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