

Studies of Interactions with *Streptococcus equi* and the Host

Extracellular Proteins of *Streptococcus equi* subspec.
equi and *Streptococcus equi* subspec. *zooepidemicus*

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(photo: Roland Thunholm)

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Studies of Interactions with *Streptococcus equi* and the host. Extracellular proteins of *Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus*

Abstract

Streptococcus equi subspecies *equi* (*S. equi*) causes strangles, a highly contagious and serious disease in the upper respiratory tract of horses, with a worldwide distribution. *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*) is regarded as an opportunistic pathogen infecting horses and other animals, and is believed to be the ancestor of *S. equi*. Pathogenic bacteria have evolved excellent ways of escaping the immune system and other defence mechanisms or have adapted to efficiently colonise specific areas of the host.

The overall objective of this thesis was to study the interaction between *S. equi* and *S. zooepidemicus* and the host. An important feature for establishment of an infection and survival of the microorganism inside the host is the interaction with, and adherence to, host tissues. In one of the studies, a group of extracellular proteins with sequence similarities with the fibronectin- and collagen-binding protein FNE, were identified and characterised. All of the proteins bound to collagen to different degrees, and one of the proteins, FNEE, was shown to bind both collagen and fibronectin, and mediate a collagen contraction in presence of PDGF-BB. In another study, an additional IgG endopeptidase of *S. equi* (and *S. zooepidemicus*), called IdeE2 (IdeZ2) was described. IdeE2 efficiently degrades horse IgG4, and together with another endopeptidase, this enzyme induced protection in a mouse infection model. In a continuation of the IdeE2/IdeZ2 study, on screening a panel of *S. zooepidemicus* isolates, sequence variation within the *ideZ2* gene was discovered, resulting in three major gene variations. However, the variation in this gene did not reflect the origin of the isolates or the MLST group of the strain. Extended computer alignment with IdeS from *S. pyogenes*, revealed similarities to structures known to be important for endopeptidase activity. The recombinant proteins IdeZ2₁₂, IdeZ2₁₆ and IdeZ2₂₁ all degraded horse IgG highly efficient despite amino acid differences in between the proteins, which argues for the importance of these proteins in the infection process.

Keywords: *Streptococcus equi*, *Streptococcus zooepidemicus*, strangles, IgG-endopeptidases, IdeE2, IdeZ2, extracellular matrix, fibronectin, collagen, FNE-like proteins

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PhD = Phinally Done

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Hulting, G., Flock, M., Frykberg, L., Lannergård, J., Flock, J-I. and Guss, B. (2009). Two novel IgG endopeptidases of *Streptococcus equi*. *FEMS Microbiology Letters* 298, 44-50.
- II Lannergård, J., Hulting, G., Reyhani, V., Copperfeldt, H., Löfgren, M., Hörnaeus, K., Rubin, K. and Guss, B. A family of fibronectin binding and collagen binding proteins of *Streptococcus equi* subspecies *equi* (manuscript).
- III Hulting, G., Sandgren, M., Robinson, C., Waller, A., Steward, K. and Guss, B. The IgG endopeptidase IdeZ2 of *Streptococcus equi* subspecies *zooepidemicus* displays sequence variation (manuscript).

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The contribution of the author to the papers included in this thesis was as follows:

- I Participated in planning the study. Performed the majority of the lab work. Shared the responsibility of writing the manuscript.
- II Took part in planning and performing the study. Shared the responsibility of writing the manuscript.
- III Participated in planning the study. Performed main part of lab work. Took part in writing the manuscript.

Abbreviations

Cn	Collagen
CNE	Collagen-binding protein of <i>S. equi</i> subspecies <i>equi</i>
ECM	Extracellular matrix
Fab	Antigen-binding fragment of immunoglobulin
Fc	Crystallisable fragment of immunoglobulin
Fn	Fibronectin
FNE (B-F)	Fibronectin-binding protein of <i>S. equi</i> subspecies <i>equi</i>
GBD	Gelatine binding domain
GST	Glutathione S transferase
HRP	Horse-radish peroxidase
IdeE	Endopeptidase of <i>S. equi</i> subspecies <i>equi</i>
IdeE2	Endopeptidase of <i>S. equi</i> subspecies <i>equi</i>
IdeS	Endopeptidase of <i>S. pyogenes</i>
IdeZ	Endopeptidase of <i>S. equi</i> subspecies <i>zooepidemicus</i>
IdeZ	Endopeptidase of <i>S. equi</i> subspecies <i>zooepidemicus</i>
IdeZ2	Endopeptidase of <i>S. equi</i> subspecies <i>zooepidemicus</i>
IFP	Interstitial fluid pressure
Ig	Immunoglobulin
kDa	Kilo Dalton
Mbp	Mega base pair
MLST	Multi-locus sequence typing
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
SFS	Fibronectin-binding protein of <i>S. equi</i> subspecies <i>equi</i>
Subsp.	Subspecies

1 Introduction

1.1 General background

A vast proportion of the Earth - soil, water, the digestive tract of organisms etc. - is inhabited by microorganisms, each adapted to its specific ecological habitat. The vast majority of these bacteria are harmless or even essential for human survival, providing nutrients and cofactors, but there are also bacteria that are harmful and sometimes cause infection or disease when the immune system of the host is compromised. These pathogenic bacteria cause many globally distributed diseases that can affect humans and animals. Over the course of evolution, the pathogenic bacteria have evolved different ways of evading the immune system or have various ways of colonising specific areas of the host. These bacteria have several strategies for escaping the host immune system, thereby disabling the defence mechanisms of the body. One such strategy is to enter the host cells quickly or imitate structures that are familiar to the host immune system, thereby escaping recognition and detection. The host immune system has its own strategies for recognising and targeting all foreign agents, viruses and bacteria detected inside the body.

One group of bacteria commonly recognised as pathogenic are the different species within the genus *Streptococcus*, which belongs to the phylum Firmicutes and contains a wide variety of homofermentative species. *Streptococcus equi* subspecies *equi* (in this thesis referred to as *S. equi*) is responsible for strangles, a severe upper respiratory tract infection in horses and other equines. Outbreaks of strangles are common and the disease is spread all around the world. At present, there is no safe and efficient vaccine on the market.

Streptococcus equi subspecies *zooepidemicus* (in this thesis referred to as *S. zooepidemicus*) is believed to be the ancestor of *S. equi* and a broadly adapted pathogen. As the name suggests, it can be found in a variety of animals such as dogs, cats, horses and humans. It can cause infection often in connection with

some kind of stress, *e.g.* severe tissue injury or viral infection and is regarded as an opportunistic pathogen.

This thesis is based on studies on the interaction between *Streptococcus equi* subspecies *equi* and *Streptococcus equi* subspecies *zoepidemicus* and their host.

1.2 The immune system

The role of the immune system is to protect the host from potentially harmful pathogens such as parasites, fungi, bacteria and viruses. The system is based on complex reaction pathways involving both cellular and biochemical signalling and reactions in complicated networks. The immune system is basically composed of two major components, innate and adaptive (acquired) immunity. A simplified description of the immune system will be further described based on the following books, *Cellular and Molecular Immunology* and *Grundläggande immunologi* (Abbas, 2000; Brändén, 1998).

1.2.1 Innate immunity

The first line of early defence against microorganisms before actual infection in basically all mammals is a physical barrier such as skin, saliva, tears and mucosal layers. The physical barrier makes it difficult to enter the mammalian body, but also provides effective protection through mucus production, sneezing, urine and diarrhoea that enables the body to eradicate invading microbes. If a pathogen manages to enter this first physical barrier, a second line of defence is activated. The innate immune response recognises different components that are conserved among a broad group of microorganisms, but are not present in mammalian cells. These structures are often essential in the survival of the microbe. Recognition of these structures can activate different pathways of a complement system, resulting in early opsonisation and enhanced phagocytosis of the pathogen. The activation of complement produces cytokines that contribute to inflammatory responses and thereby recruitment and activation of leukocytes. The innate immune system responds to repeated infection in a similar way regardless of previous exposure, *i.e.* lacking a memory of previous infections.

1.2.2 Adaptive (acquired) immunity

Adaptive immunity is the third line of defence that allows for stronger immune responses and also immunological memory for the specific pathogen. The adaptive immune responses develop later during the infection and take days or weeks to be completely effective. The adaptive system is composed of **humoral immunity** and the **cell-mediated immune response**. The main function of humoral immunity is defence against extracellular microbes and their products, where antibodies or receptors on B lymphocytes (B cell receptors) detect cell wall antigens and secreted or cell wall bound toxins and initiate neutralisation, opsonisation and phagocytosis, and activation of the complement system. The cell-mediated immune response is the response to intracellular microbes such as some bacteria and viruses that survive and proliferate inside phagocytes. The cell-mediated immune response is initiated by the T lymphocytes, which detect antigens of the intracellular microbes or an infected cell and mediate killing of the infected cell by apoptosis.

1.2.3 Immunoglobulins (Antibodies)

Antibodies are part of the adaptive immune response and are large glycoproteins that have the ability to bind foreign substances. Antibodies, also called immunoglobulins, play an important role in the defence against bacteria and other microorganisms in mammals during an infection by complement activation and phagocyte signalling. The Y-shape structure of an antibody comprises two identical heavy chains and two identical light chains which are linked together by disulphide bonds. Both chains consist of an N-terminal variable part creating the antigen-binding property of the antibody molecule. The C-terminal parts of the chains of an antibody comprise the constant part. Depending on the structure of the constant domains, the antibodies can be grouped into different classes, *e.g.* human immunoglobulin (Ig) A, IgD, IgE, IgG and IgM. The function of the variable part of an antibody is to bind to the foreign structure of an invading organism, while the constant part of antibodies mediates the recruitment of specific host cells or activation of the complement system.

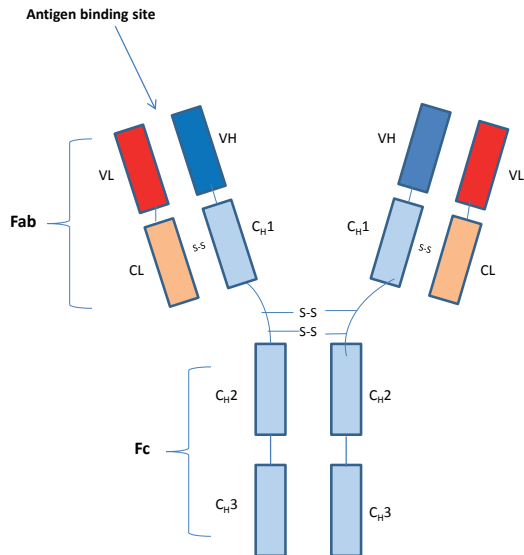


Figure 1. Schematic description of immunoglobulin (Ig) G. An antibody is composed of two chains, the heavy chain and the light chain. Each heavy chain has three constant domains (C_{H1} - C_{H3}) and a variable domain (VH). The light chain contains a constant domain (CL) and a variable domain (VL). The Fab (antigen-binding fragment) region recognises and binds to foreign objects, antigens. The Fc (crystallisation fragment) region is the base for the antibody but is also involved in opsonisation and complement activation.

1.3 Strangles

Strangles is a severe upper respiratory disease of horses caused by the bacterium *S. equi*. The disease has a worldwide distribution and is one of the most feared horse diseases due to its severity and frequency, but also due to economic losses when isolation restrictions are imposed on stables. The disease was first described and reported in 1251 by Jordanus Rufus and is one of the most commonly diagnosed equine diseases (Timoney, 1993). Strangles is not in principle a fatal disease, although it can cause chronic illness and in severe cases also death (Sweeney *et al.*, 2005; Timoney, 2004; Harrington *et al.*, 2002). Disease symptoms and severity are often correlated to immune status and age of the horse, with older horses displaying a milder version and young horses often developing a more severe form (Sweeney *et al.*, 2005).



Figure 2. Horse suffering from strangles, with ruptured abscess. Photo used with kind permission of Vet. Nick Parkinson.

1.3.1 Clinical signs and pathogenesis

The classical description of strangles includes swelling of lymph nodes, abscess formation and high fever. The disease is characterised by a rapid increase in body temperature within the range 39-41°C that continues throughout the disease, followed by coughing and a rich yellowish nasal discharge. The infection is initiated when *S. equi* invades the horse via the mouth or nose, attaches to the tonsils and is transported through the mucosal layers in the upper respiratory tract. The pathogen quickly enters the host and spreads via the lymphatic channels to the lymph nodes, resulting in abscess formation. A couple of hours after the infection, *S. equi* is difficult to detect on the mucosal surface and has moved to the lymph nodes in the pharyngeal region (Timoney & Kumar, 2008). In some cases the pathogen gains access to the circulatory system and the internal lymph nodes, thereby enabling its spread to other parts of the host body. This severe condition is called “bastard strangles” and can give rise to abscess formation in the kidneys, liver or even the brain. Another complication that can occur in infected horses is *Purpura Haemorrhagica*, an immune-mediated condition that causes small bleeds from

oedema in the limbs, eye-lids, gums and internal organs. This may cause a stoppage in the circulatory system and thus death (Harrington *et al.*, 2002).

1.3.2 Transmission of bacteria and persistent carriage

S. equi can be detected in nasal discharge after 24-48 hours from the onset of the infection and usually persists in nasal shedding for a few weeks (Sweeney *et al.*, 2005; Timoney, 2004; Timoney, 1993). Transmission of *S. equi* is thought to be *via* contact with nasal secretions, aerosols or abscess material, either from infected horses or from infected horses without clinical signs (Sweeney *et al.*, 2005). Strangles is transmitted through direct horse-to-horse contact, as well as *via* humans, clothes, shared equipment, feed or contaminated water (Waller *et al.*, 2007b; Sweeney *et al.*, 2005). Some horses that have recovered from strangles still acquire an asymptomatic subclinical infection in the guttural pouches. Remaining pus in the guttural pouches can in some cases form so-called condroids, which contain viable *S. equi*. These persistent carriers shed *S. equi* via normal nasal secretion. The ability of *S. equi* to persist and adapt in healthy horses facilitates transmission of the bacteria around the world, creating sources of new outbreaks (Harris *et al.*, 2015; Newton *et al.*, 2000; Newton *et al.*, 1997).

1.3.3 Treatment of strangles

The main focus in treating the vast majority of horses with uncomplicated strangles is primarily on providing the horse with good care and keeping good hygiene conditions, which helps the horse to recover from the infection on its own. In severe cases, antibiotic treatment can be suggested, although it may not be effective (Harrington *et al.*, 2002). *Streptococcus equi* is sensitive to most antibiotics, with penicillin as the first choice. Some horses develop high fever and swollen lymph nodes, resulting in anorexia, in which case anti-inflammatories such as phenylbutazone may be administered (Mallicote, 2015; Harrington *et al.*, 2002).

1.4 The genus *Streptococcus*

The genus *Streptococcus* in the phylum Firmicutes contains a wide variety of homofermentative, chain-forming species of microorganisms. These bacteria were initially found in wounds in 1874 by Billroth who named them *Streptococcus*, based on the Greek *streptos*, meaning “twisted chain”.

Streptococci are Gram-positive bacteria with spherical or ovoid cells with a diameter of 0.5-2.0 μm that grow in pairs or chains. They are catalase-negative and most commonly facultative anaerobes that require a rich medium and occasionally 5% CO_2 for growth. The temperature optimum for the bacteria is around 37 $^\circ\text{C}$, although the minimum and maximum temperature varies within the genus. During growth on blood agar, pathogenic streptococci produce three distinct types of haemolytic patterns, with streptococci that produce streptolysin O or S generating red blood cell haemolysis called β haemolysis. Many streptococci do not produce these haemolysins and instead cause a discoloration of the red blood cells that creates a greenish or brownish zone around the colonies, called α haemolysis, or no haemolysis, called γ haemolysis (Madigan, 2003). Genetically, streptococci are grouped into species, but traditionally streptococci and other related cocci are also serologically divided into groups based on the presence of a specific carbohydrate antigen, the Lancefield serological groups A-O (Lancefield, 1933).

Streptococci are responsible for a large number of diseases in both humans and animals worldwide and are found on mucus membranes of the mouth, respiratory and genitourinary tracts, as well as on the skin. To distinguish between pathogenic streptococci, they are divided into two groups, the oral and the pyogenic groups. The pyogenic group (Table 1) is regarded as pathogenic or opportunistic pathogens, the most well-studied example being the human serological group A bacteria streptococcus (*Streptococcus pyogenes*), which is responsible for various diseases ranging from mild infections of the skin to potential life-threatening infections such as bacteraemia or toxic shock.

Table 1. The pyogenic group of streptococci

Species	Lancefield group	Haemolysis	Host
<i>S. pyogenes</i>	A	β	Humans
<i>S. agalactiae</i>	B	α/β	Humans, cattle
<i>S. dysgalactiae</i>			
subsp. <i>equisimilis</i>	A, C, G, L	β	Humans, mammals
subsp. <i>dysgalactiae</i>	C		Cattle
<i>S. equi</i>			
subsp. <i>equi</i>	C	β	Horses
subsp. <i>zooepidemicus</i>	C	β	Humans, mammals
subsp. <i>ruminatorium</i>	C	β	Sheep, goat
<i>S. canis</i>	G	β	Dogs
<i>S. iniae</i>	-	α/β	Fish, dolphins
<i>S. parauberis</i>	E, P	$\alpha/-$	Cattle
<i>S. porcinus</i>	E, P, U, V	β	Pigs
<i>S. uberis</i>	E, P, G	$\alpha/-$	Cattle

1.4.1 *Streptococcus equi*

Streptococcus equi is a Gram-positive, catalase-negative, facultative anaerobic bacterium that belongs to the Lancefield group C streptococci (GCS). Three subspecies are included in this group, subspecies *equi*, subspecies *zooepidemicus* and subspecies *ruminatorium*.

Streptococcus equi which causes strangles is host-restricted to members of Equidae (Timoney, 1993). It displays β - haemolysis (Table 1) and is unable to ferment lactose, trehalose and sorbitol. It can therefore be biochemically distinguished from the other *S. equi* subspecies (Fernandez *et al.*, 2004; Grant *et al.*, 1993). Another way to identify streptococcal isolates is by DNA-based molecular methods, for example PCR (Webb *et al.*, 2013; Lindahl *et al.*, 2011; Heather *et al.*, 2008; Baverud *et al.*, 2007). The genome is fully sequenced and available at Sanger Institute online

(<http://www.Sanger.ac.uk/projects/Microbes>). The genome sequence has a GC content of 41% and is about 2.3 Mbp in length (Holden *et al.*, 2009). Genetically, different strains of *S. equi* are quite conserved and it is suggested that *S. equi* originated from an ancestral strain of *S. zooepidemicus* through gene gains and losses and should therefore be considered a biovar or clone of *S. zooepidemicus* (Waller & Robinson, 2013; Holden *et al.*, 2009; Webb *et al.*, 2008).

Subspecies *zooepidemicus*, in contrast to *S. equi*, is not restricted to causing infection in equines only and can be found in a variety of animals, such as dogs, cats, horses and humans (Pelkonen *et al.*, 2013; Timoney, 2004). It can cause infection, often in connection with some kind of stress, *e.g.* severe tissue injury or a viral infection, and is therefore regarded as an opportunistic pathogen (Timoney, 2004). In fact, *S. zooepidemicus* is the most frequently isolated opportunistic pathogen in young horses with upper respiratory infections (Lindahl *et al.*, 2013). In horses, *S. zooepidemicus* can cause different kinds of infections such as uterine, wound, and upper respiratory infections, to name a few. The genome of *S. zooepidemicus* is also fully sequenced (Sanger Institute) and displays a GC content of 41.5% and is 2.15 Mbp in length (Holden *et al.*, 2009).

Streptococcus equi subspecies *ruminatorium* (*S. ruminatorium*) was isolated from sheep and goats with mastitis quite recently (Fernandez *et al.*, 2004). Both its genotypic and phenotypic characteristics are different from those of the other related *S. equi* subspecies and it has therefore been debated whether it belongs to the *S. equi* group or not.



Figure 3. *S. equi* when grown on a blood agar plate. Photo used with kind permission from Jonas Lannergård.

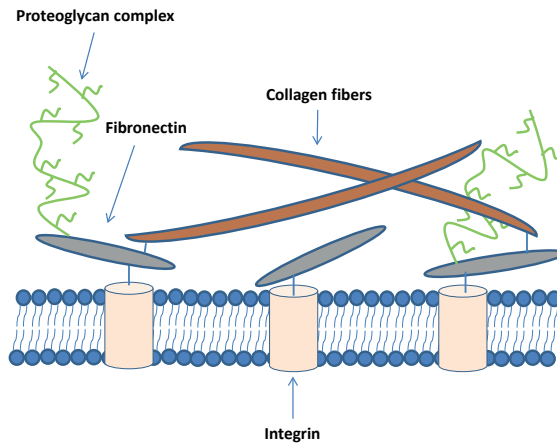


Figure 4. Cartoon showing a simplified representation of the extracellular matrix (ECM) and its major components e.g. proteoglycan complexes, fibronectin, collagen and integrin.

1.5 Extracellular matrix (ECM)

The extracellular matrix (ECM) is a complex system of polysaccharides and proteins that contributes to the structure and function of a tissue (Becker, 2000; Lodish, 2000). It is responsible for cell adhesion, cell-to-cell communication and differentiation of cells. The ECM shows a remarkable variety of forms in different tissues, but the ECM is basically composed of three major classes of molecules. The first of these are the protein-polysaccharide complexes, called the **proteoglycans** which are space filling molecules that have a shockwave absorbing function in mammalian tissues. The second major class of molecules are the **structural proteins**, such as collagen and elastin, which give the ECM its strength and flexibility. The **specialised proteins**, the adhesive glycoproteins fibronectin and laminin, attach the ECM to cells and are the third major class of molecules.

The most common cell type in the ECM is fibroblasts, which maintain and provide a structural framework. Chondrocytes and osteoblasts are other cell types in the ECM and are found in cartilage and bone formation respectively. Certain eukaryotic cell-surface receptors, including integrins, bind to components of the ECM, thereby mediating adherence of the cells to each

other through their interaction with the matrix. The matrix is also a reservoir for extracellular signalling molecules that controls differentiation and cell growth.

Adherence of pathogenic bacteria to host cells and tissues via the ECM is considered an important step in the early bacterial infection process. The bacterial components interacting with the ECM are either secreted proteins or proteins located on the bacterial cell surface. Two major ECM components that are important in this study, *fibronectin* and *collagen*, are described below.

1.5.1 Fibronectin

Fibronectin is a large glycoprotein in the ECM. The function of the protein is reflected in its name, which originates from the Latin word *fibra*, meaning fibre, and *necto*, meaning attach or connect. As the name suggests, the role of fibronectin is to act as a connector molecule that attaches cells to various receptors and cells in the ECM. Fibronectin can be found in insoluble form in the ECM (cellular fibronectin) or in soluble form in blood or other body fluids (plasma fibronectin). The protein is involved in many important processes including cell migration in the embryo, morphology, thrombosis, growth and differentiation (Henderson *et al.*, 2011; Pankov & Yamada, 2002; Lodish, 2000). Fibronectin can bind various host substances such as fibrin, collagen/gelatine, heparin and cellular integrins.

A fibronectin molecule consists of two almost identical peptides (250-280 kDa) that are coupled together by two disulphide bridges near the C-terminal end of the molecules (Becker, 2000; Lodish, 2000). Each peptide is built up of a series of globular domains, mainly three types of homologous repeats, which are linked by short flexible segments. The domains comprise 12 type I modules, two type II modules and 15-17 type III modules. The cell-surface binding domain contains a RGD-motif.

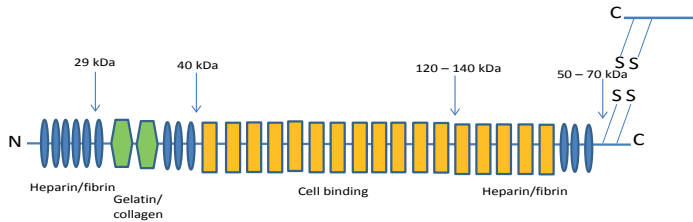


Figure 5. Schematic figure of a fibronectin chain. The chain is composed of 12 FI modules (blue), two FII modules (green) and 15-17 FIII modules (yellow). Major proteolytic cleavage sites are indicated with arrows and its respective ligand indicated.

1.5.2 Collagen

The most abundant glycoprotein in the ECM of most mammalian cells is collagen. This protein maintains the structure of tissues and organs and accounts for about 25% of all proteins in the human body (Ricard-Blum & Ruggiero, 2005). The collagen proteins are a large family with up to 28 different types presenting diverse functions and tissue distributions identified to date. Collagens are divided into two main classes depending on the polymers formed; the fibril-forming collagens (I, II, III, V and XI), which are characterised by a rather homogeneous group of proteins, and the nonfibrillar collagens, which comprise a more heterogeneous group (Bosman & Stamenkovic, 2003; Becker, 2000; Lodish, 2000). Collagen is a super helix formed by three parallel extended right-handed helices in a characteristic rope-like structure. The protein is synthesised as a procollagen, a longer precursor, and undergoes hydrolysis and other modifications before forming the triple chain molecules. A distinguishing feature of all types of collagen backbone is a repetitive triplet with a glycine (G) as the first amino acid, followed by Xaa and Yaa where X is usually a proline (P) and Y is usually a hydroxyproline (O) (Zeltz & Gullberg, 2016; Gelse *et al.*, 2003).

1.6 Extracellular proteins and other potential virulence factors of *S. equi*

An important feature for establishment of an infection and survival of the microorganism inside the host is the interaction with, and adherence to, host tissues (Schwarz-Linek *et al.*, 2006; Nyberg *et al.*, 2004; Kreikemeyer *et al.*, 2003; Harrington *et al.*, 2002; McElroy *et al.*, 2002). Some pathogenic streptococci express different molecules in order to interact with host tissues and cells, or to escape the host immune defence mechanisms. Some of these molecules have been identified as virulence factors (Waller *et al.*, 2011; Batzloff *et al.*, 2004; Beckmann *et al.*, 2002; Chhatwal, 2002; Hytonen *et al.*, 2001). Virulence factors are either expressed on the bacterial surface or secreted into the surroundings by the bacteria. *Streptococcus equi* possesses several different potential virulence factors including a hyaluronic capsule, exotoxins, proteases, various surface-anchored proteins and the antiphagocytic SeM protein.

1.6.1 Capsule

The capsule is regarded as an important virulence factor for many streptococci (Waller *et al.*, 2011; Anzai *et al.*, 1999). A carbohydrate capsule composed of hyaluronic acid surrounds the bacteria and hides it from the host immune system. The reason for evading the immune system is because hyaluronic acid is also naturally occurring in mammals. The capsule also complicates attachment of macrophages to the bacteria and is greatly influenced by the age of the culture *in vitro*, where young cultures are highly encapsulated and cultures after 24 h barely have detectable capsules, suggesting increased virulence of young cultures (Harrington *et al.*, 2002). The capsule could also have an important function for the surface-exposed hydrophobic proteins by covering the active sites of the proteins, thereby maintaining their structure and function, as well as hindering aggregation (Srivastava & Barnum, 1983).

The presence of a capsule varies among the less virulent *S. zooepidemicus* but has been found in almost all *S. equi* strains investigated (Wibawan *et al.*, 1999). In a recent study, Steward *et al.* (2016) showed that a reduced capsule phenotype is not due to genetic variation, but is instead supported by differences in gene transcription (Steward *et al.*, 2016).

1.6.2 Surface proteins

Many of the extracellular proteins situated on the bacterial cell surface share a common structural organisation, enabling identification of certain domains and motifs. Many of the cell wall-anchored proteins are attached to the cell by the C-terminal sorting signal, starting with an amino acid sequence motif LPxTG.

Other cell wall-anchoring mechanisms are the N-terminal linkage displayed by lipoproteins or hydrophobic interactions.

M-proteins were first described in streptococci in the late 1920s (Lancefield, 1928). M-proteins or M-like proteins are described as cell surface proteins that bind fibrinogen and have antiphagocytic activity, and this is one of the most widely studied group of bacterial virulence factors (Staali *et al.*, 2003). The antiphagocytic effect of M-proteins depends on inhibition of C3b (a complement factor) deposition on the bacterial surface or binding to fibrinogen (Carlsson *et al.*, 2005) but also on capsule formation (Timoney *et al.*, 2014). *Streptococcus equi* possesses two homologues of an M-like protein, SzPSe and SeM (also known as FgBP) (Timoney *et al.*, 1997; Timoney *et al.*, 1995; Srivastava & Barnum, 1983). The SeM and SzPse proteins show low homology and are only distantly related (Timoney *et al.*, 1997; Timoney *et al.*, 1995). The less virulent *S. zooepidemicus* also has two homologues of M-like proteins, SzP and SzM (Velineni & Timoney, 2013; Kelly *et al.*, 2006; Timoney *et al.*, 1997). Studies have shown that the SeM protein of *S. equi* is an important virulence factor where *S. equi* mutants lacking the SeM gene were unable to colonize infected horses (Timoney *et al.*, 2014).

One example of a cell wall-anchored protein regarded as a virulence factor is CNE from *S. equi* (Lannergard *et al.*, 2003). CNE binds to native fibrillary collagen types I, II and III with high affinity and is believed to be important in general adhesion to host tissues (van Wieringen *et al.*, 2010). It has also been suggested that CNE could be the accessory pilin of *S. equi* (Waller *et al.*, 2011). To analyse the protective effect of CNE as an antigen in a vaccine Flock *et al.* (2006) used the recombinant protein in an experimental mouse model resembling *S. equi* infection in the horse and found that antibodies to CNE were able to partially block (75%) the binding of *S. equi* to immobilised collagen. Moreover, recombinant CNE was included in a trial of a multi component vaccine that conferred protection against *S. equi* in horses (Guss *et al.*, 2009).

Another cell wall-anchored protein in *S. equi* is EAG (ZAG in *S. zooepidemicus*). EAG is a multi-binding protein that binds IgG, α_2 -macroglobulin and albumin (Lindmark *et al.*, 2001; Jonsson *et al.*, 1995). EAG binds to α_2 -macroglobulin in the N-terminal part of the protein, followed by an albumin-binding region and the IgG-binding part in the C-terminal end. EAG was also included in the multi-component vaccine that protected horses from strangles in the trials by Guss *et al.* (2009).

Seven different collagen-like proteins (SclC-SclI) have been reported in *S. equi*. Each of these seven proteins displays a unique N-terminal domain with as yet unknown functions in *S. equi* (Karlstrom *et al.*, 2006; Karlstrom *et al.*, 2004). One of the collagen-like proteins, SclC, was included in a vaccination experiment together with six other potential virulence factors of *S. equi* that resulted in good protection of the horses in a strangles challenge (Guss *et al.*, 2009; Waller *et al.*, 2007a).

In a study by Lannergård *et al.* (2005), FNEB a cell wall-anchored protein that binds fibronectin was described. FNEB specifically binds to the 29-kDa fragment on fibronectin and the binding activity on FNEB is located on the C-terminal part of the protein. It has been suggested that FNEB mediates binding of fibronectin to *S. equi* cells grown *in vitro* (Lannergard *et al.*, 2005).

1.6.3 Secreted proteins and toxins

Streptococcus equi is a β -haemolytic bacterium that produces a secreted protein called streptolysin S-like toxin (SLS) that is responsible for the characteristic cleared zone on blood agar (Waller *et al.*, 2011; Flanagan *et al.*, 1998). The activity of streptolysin S-like protein requires a carrier molecule, *e.g.* albumin or double-stranded RNA. The streptolysin complex binds to neutrophils and macrophages, forming transmembrane pores and thereby permitting osmotic lysis of the cells (Timoney, 2004). The exact role of this toxin is not fully understood, but the lysis of cells is intended to supply the bacteria with essential nutrients (Harrington *et al.*, 2002).

Streptokinase is another secreted protein of *S. equi* suggested as a virulence factor. This protein activates equine plasminogen which hydrolyses fibrin clots and thereby facilitates dispersion of the bacteria in the tissue (McCoy *et al.*, 1991).

Evasion of phagocytosis is an important feature in virulence. *Streptococcus equi* possesses a couple of proteins predicted to be secreted in order to inhibit phagocytosis.

Protein Se18.9 inhibits phagocytosis through binding factor H, which regulates the alternative complement pathway by reducing the C3 deposition on *S. equi* (Tiwari *et al.*, 2007). All *S. equi* strains tested to date produce Se18.9 but only one of 140 strains of *S. zooepidemicus* suggesting, for *S. equi*, an evolutionary important gene (Waller *et al.*, 2011; Holden *et al.*, 2009).

Another secreted protein with anti-phagocytic properties is *S. equi* glycosyl hydrolase EndoSe (Flock *et al.*, 2012). EndoSe inhibits IgG binding to Fc receptors by hydrolysing the sugar substituents of IgG, thereby damaging the antibacterial effect on IgG (Flock *et al.*, 2012).

Streptococcus equi encodes two IgG-degrading enzymes called IdeE and IdeE2 (Se44.2) (IdeZ and IdeZ2 in *S. zooepidemicus*) (Hulting *et al.*, 2009; Lannergård & Guss, 2006). IdeE cleaves horse IgG, thereby reducing IgG recognition by the host immune system (Lannergård 2006). IdeE has also been suggested to have anti-phagocytic properties by blocking phagocytosis *in vitro* (Timoney *et al.*, 2008), although the role of the endopeptidase in *S. equi* infection is currently uncertain (Liu & Lei, 2010). IdeE together with IdeE2 significantly improved the efficiency of the multi-component subunit vaccine tested by Guss *et al.* (2009). IdeE2 also degrades IgG from human and cleaves equine IgG highly efficiently (Hulting *et al.*, 2009). IdeE2 is discussed further in Chapter 2 of this thesis.

Superantigens are powerful immune-stimulatory disease-causing toxins produced by certain bacteria such as *S. pyogenes* and *Staphylococcus aureus*. The superantigens cause activation of T-cells, which produces a release of enormous amounts of cytokines, leading to an obsessive inflammatory response that overloads the host body, resulting in fever and multi-organ failure (Alouf & Muller-Alouf, 2003). *Streptococcus equi* has acquired four mitogenic exotoxins/ superantigens; SePE-H, SePE-I, SePE-Lse and SePE-Mse (Paillot *et al.*, 2010a; Paillot *et al.*, 2010b; Proft *et al.*, 2003; Artiushin *et al.*, 2002). These superantigens contribute to many of the severe symptoms characteristic of strangles (Paillot *et al.*, 2010b; Timoney, 2004; Anzai *et al.*, 1999). Three of the superantigens, SePE-I, SePE-Lse and SePE-Mse, stimulate proliferation of equine PBMCs (peripheral blood mononuclear cells) *in vitro* (Paillot *et al.*, 2010b; Proft *et al.*, 2003; Artiushin *et al.*, 2002).

Table 2. *Extracellular proteins of S. equi and S. zooepidemicus. Only published proteins are included.*

Protein	Anchored / secreted	Function	<i>S. zooepidemicus</i>	Reference
CNE	Anchored	Cn-binding	CNE	Lannergård <i>et al.</i> 2003
EAG	Anchored	α_2 M-, albumin-, IgG-binding	ZAG	Jonsson <i>et al.</i> 1995
EndoSe	Secreted	Evasion of immune system	EndoSz	Flock <i>et al.</i> 2012

FNE	Secreted	Fn- and Cn-binding	FNZ	Lindmark <i>et al.</i> 1996
FNEB	Anchored	Fn-binding	FNZ2	Lannergård <i>et al.</i> 2005
IdeE	Secreted	IgG-endopeptidase	IdeZ	Lannergård <i>et al.</i> 2006
IdeE2	Secreted	IgG-endopeptidase	IdeZ2	Hulting <i>et al.</i> 2009
ScIC-ScII	Anchored	Unknown	-	Karlström <i>et al.</i> 2006
Se18.9	Secreted	Factor H-binding	-	Tiwari <i>et al.</i> 2007
SeM	Anchored	Fg- and IgG-binding	SzM	Timoney <i>et al.</i> 1985
SePE-H	Secreted	Exotoxin	-	Artushin <i>et al.</i> 2002
SePE-I	Secreted	Exotoxin	-	Artushin <i>et al.</i> 2002
SePE-Lse	Secreted	Exotoxin	SZEL	Proft <i>et al.</i> 2003
SePE-Mse	Secreted	Exotoxin	SZEM	Proft <i>et al.</i> 2003
SFS	Secreted	Fn-binding	SFS	Lindmark <i>et al.</i> 1999
SzPSe	Secreted	Fg-binding	SzP	Timoney <i>et al.</i> 1997

Some of the secreted proteins of *S. equi* bind to soluble components in body fluids or to components in the ECM. One example of this protein is the fibronectin binding protein SFS. Due to its non-exciting cell-wall anchoring motif, SFS is predicted to be secreted, but the protein has not been detected in growth cultures and expression of SFS *in vitro* is very low (Lindmark & Guss, 1999). SFS contains a GEXGE amino acid motif known to be conserved in other fibronectin-binding proteins such as FNZ from *S. zooepidemicus*. Studies have suggested that SFS forms a stable complex with fibronectin in a two-step binding process (Ma *et al.*, 2015).

Another secreted protein of *S. equi* is FNE (FNZ in *S. zooepidemicus*). FNE binds to both fibronectin and collagen and lacks a cell-wall anchoring motif due to a deletion in the *fne* gene resulting in a stop codon in the open reading frame (ORF) creating a truncated protein missing the C-terminal part FNZ

protein in *S. zooepidemicus* (Liden *et al.*, 2006; Lindmark *et al.*, 2001). FNE specifically binds to the 40 kDa collage/gelatine-binding domain (GBD) of fibronectin (Lindmark *et al.*, 2001; Lindmark *et al.*, 1996). Studies have shown that FNE mediates interaction between fibronectin and native collagen type I in a collagen gel contraction model, creating a fibronectin-FNE-collagen fibre complex (Liden *et al.*, 2008). Moreover, the crystal structure of FNE has been established to show similarities to a minor pilus protein of *S. pyogenes* (Tiouajni *et al.*, 2014).

In addition to FNE the genome of *S. equi* encodes four putative FNE-like proteins that bind to collagen (FNEC, FNED, FNEE and FNEF) of which one is predicted to be cell wall-anchored and binds both fibronectin and collagen. These FNE-like proteins are further discussed in Chapter 2 in this thesis.

2 Present investigation

2.1 Aims of the present investigation

The overall objective of this thesis was to study the interaction between *S. equi* and *S. zooepidemicus* and the host. Previous research has shown that both *S. equi* and *S. zooepidemicus* express, or have the genetic potential to express, a great number of extracellular proteins that interact or are suspected to interact with components present in different structures or body fluids of the host (Holden 2009). It is generally believed that the extracellular bacterial proteins contribute in various ways to the virulence of the bacteria, making it interesting to study these interactions.

In this thesis the focus was directed on two types of extracellular proteins. The first type was soluble proteins with specific IgG endopeptidase activity. Since IgG is a crucial component of the immune system, the activity of the bacterial endopeptidases should interfere with the host immune defence. The second type of protein studied was the FNE-like family of bacterial proteins, where the majority of members should be anchored to the bacterial cell surface. These types of fibronectin- and/or collagen-binding proteins are suggested to affect adhesion of the bacterium to the host or the connective tissue during an infection.

2.2 Results and Discussion

2.2.1 IgG endopeptidases of *S. equi* and *S. zooepidemicus*

Immunoglobulin G plays a central role in the host in the protection against invading bacteria and other microorganisms. Binding of IgG to bacteria facilitates opsonisation, phagocytosis and complement activation. Furthermore, the binding of IgG can also mediate inactivation of bacterial adhesins and

toxins. Antibodies (immunoglobulins) are Y-shaped large glycoproteins that are part of the adaptive immune response (see section 1.2.3).

Some pathogenic bacteria have evolved different mechanisms to escape the host immune response by expressing and secreting proteins that interact with antibodies. Binding to IgG with specific receptors for the Fc region (C_H2-C_H3), thereby enabling the interaction between Fc-receptors on phagocytes, is one way of interaction (exhibited by SeM and EAG of *S. equi*). Another approach is to inhibit the interaction between Fc receptors on phagocytes and IgG, either by hydrolysis of sugars on IgG (EndoSe) or by cleavage of the IgG molecule (Flock *et al.*, 2012; Collin & Olsen, 2001). In *S. pyogenes*, a cysteine protease, IdeS (Mac-1), has been shown to cleave IgG in a two-step reaction, resulting in separation of the Fab and Fc part with high specificity and efficiency, thereby preventing phagocytosis by leucocytes (Barnett *et al.*, 2015; Persson *et al.*, 2015; Vindebro *et al.*, 2013; Brezski & Jordan, 2010; Akesson *et al.*, 2006; Vincents *et al.*, 2004; von Pawel-Rammingen & Bjorck, 2003; Lei *et al.*, 2002; von Pawel-Rammingen *et al.*, 2002; Lei *et al.*, 2001). Studies have shown that IdeS (Mac-1 and Mac-2) displays both IgG degrading activity and Fc γ receptor binding (Soderberg *et al.*, 2008; Soderberg & von Pawel-Rammingen, 2008). A homologue of IdeS has also been reported in *Streptococcus suis* (*S. suis*) with endopeptidase activity with high specificity and efficiency towards IgM (Seele *et al.*, 2015; Seele *et al.*, 2013). A recent study described another cysteine protease that specifically cleaves porcine IgG at the hinge region in *S. suis* (Spoerry *et al.*, 2016).

An IdeS-like protein has previously been described in *S. equi* and *S. zooepidemicus* (IdeE and IdeZ respectively) (Lannergard & Guss, 2006). These enzymes efficiently degrade IgG molecules from a variety of mammals such as humans, dogs and guinea pigs. IdeE and IdeZ cleave IgG subclasses containing the LLGGP substrate binding site, suggesting a similar specificity as IdeS (Lannergard & Guss, 2006). A study by Timoney (2007) reported antiphagocytic activity of IdeE on equine neutrophils, where the binding was suggested to be mediated through the integrin-binding motif RGD present in the IdeE protein. However, the main antiphagocytic activity has been questioned and remains unclear (Liu & Lei, 2010).

An additional IgG endopeptidase was described in both *S. equi* and *S. zooepidemicus* (IdeE2 and IdeZ2 respectively) in Paper I of this thesis. In that study, purified IgG from a variety of animals, including humans and horses, was incubated with IdeE2 and IdeZ2. These endopeptidases showed high enzymatic activity to horse and human IgG and only weak activity to pig, goat and bovine IgG. In order to study the endopeptidase activity in a more complex system, strains of *S. equi* and *S. zooepidemicus* were incubated with THBY

(Todd-Hewitt broth supplemented with yeast extract) supplemented with horse serum (Paper I). Supernatants of *S. equi* contained a degradation product of IgG4. To analyse whether this degradation product was due to IdeE or IdeE2 enzymatic activity both enzymes were incubated, either separately or in a mix, together with horse serum. The results showed that in fact IdeE2 alone efficiently degrades IgG4. IdeE2 and IdeZ2 lack the integrin-binding motif present in IdeE, IdeZ and in IdeS and it is therefore doubtful whether IdeE2/IdeZ2 can mediate host cell binding or not. IdeE and IdeE2 were also used to immunise mice followed by experimental infection in order to evaluate these enzymes as vaccine candidates (Paper I). Both enzymes induced protection in an experimental infection and challenge model in mice. Mice intranasally vaccinated with IdeE2 showed a significant reduction in weight loss and nasal colonisation of *S. equi* compared with the control group. Since IdeE2 has no enzymatic activity against mouse IgG, the protective effect in mice was quite unexpected. One possible explanation could be that a portion of the protein is exposed on the bacterial surface for a short period before being secreted, which could lead to opsonisation of the bacteria in vaccinated animals. However, IdeE2 could have other, as yet unknown functions apart from the enzymatic activity studied here, which is inhibited by the vaccination process. IdeE and IdeE2 have also been used to vaccinate Welsh mountain ponies, together with five other recombinant proteins (Septavacc), in order to evaluate their ability to induce protection (Guss *et al.*, 2009). Vaccination with Septavacc led to 85% protection in horses when challenged with *S. equi* (Guss *et al.*, 2009). However IdeE2 failed to protect horses when combined with other proteins from *S. equi* in a vaccination trial by Timoney *et al.* (2007).

In a recent study of an isolate of *S. equi* showing a mixed phenotype lacking the mucoid capsule, IdeE and IdeE2 were upregulated in the reduced capsule variant, suggesting a broad feedback effect when *S. equi* adapts to the host environment, but also showing the contribution of IdeE and IdeE2 to virulence (Steward *et al.*, 2016). Data from other studies also indicate that IdeE2, together with SeM and Se75.3 (SEQ2190), are the proteins most differentiated in immune responses of horses with respiratory infections caused by *S. equi* (Velineni *et al.*, 2015). Another study has shown that the expression of IdeE2 in *S. equi* was effected by a peptidyl-propyl isomerase (PrsA/PrtM), a protein involved in secretion of several extracellular proteins *S. equi* (Ikolo *et al.*, 2015).

On PCR screening a panel of *S. zooepidemicus* isolates, only one of 10 isolates showed presence of the *ideZ2* gene (Paper I). Further screening of the *ideZ2* gene showed that it varies in sequence, displaying three main different

gene variations named *ideZ2*₂₁, *ideZ2*₁₂ and *ideZ2*₁₆ (Paper III). However, the variation in this gene did not reflect the origin of the isolates or the MLST group of the strain (Paper III). The IdeZ2₁₂ protein is similar to a Mac-1-like IgG endopeptidase of a human isolate of *S. zooepidemicus* previously described (Beres *et al.*, 2008), while IdeZ2₁₆ seems to be novel. To investigate the endopeptidase activity of the different IdeZ2 proteins, the recombinant proteins were incubated with a selection of antibodies from a variety of species. The proteins displayed, despite of sequence variation, the same IgG endopeptidase activity to IgG from human and horse (Paper III). In order to further investigate the degradation activity of the IdeZ2 variants in a more complex environment, *S. equi* and *S. zooepidemicus* cells were grown in THBY supplemented with either horse serum (10%) or horse blood (1%, 5%, 10%). Following 24 h incubation, all the supernatants of *S. equi* showed an IgG degradation product, whereas only *S. zooepidemicus* strains grown in the presence of horse blood displayed an IgG degradation product (Paper III). The crystal structure of the IgG cleaving enzyme IdeS from *S. pyogenes* have been determined (Agniswamy *et al.*, 2006; Wenig *et al.*, 2004). In order to analyse the structure of the IdeZ2 proteins, a computational alignment to IdeS was performed, which showed high similarities in the regions known to be important for IgG cleavage. Furthermore, the alignment showed that the IdeZ2 proteins (and IdeE2) have long insertions in the N-terminal part compared to IdeS, and that IdeZ2₁₆ has a long C-terminal extension.

2.2.2 FNE belongs to a family of fibronectin-binding and collagen-binding proteins of *Streptococcus equi*

Bacterial infection usually involves several steps such as adherence, invasion and colonisation. The initial adherence to host cells and structures is initiated by structures of the cell surface of the bacteria. *Streptococcus equi* possesses several different adhesins including CNE, which specifically binds to collagen (Lannergard *et al.*, 2003), SFS and FNEB, which bind to fibronectin (Lannergard *et al.*, 2005; Lindmark & Guss, 1999) and FNE (Lindmark *et al.*, 2001). Other studies have confirmed that FNE binds both fibronectin and collagen (Liden *et al.*, 2006; Lindmark *et al.*, 2001; Lindmark *et al.*, 1996). Previous studies of the biological implication of this interaction in a eukaryotic collagen-gel contraction model showed that FNE enhanced the binding of soluble fibronectin to immobilised collagen and *in vitro* and also lowered the interstitial fluid pressure (IFP) *in vivo* (Liden *et al.*, 2008; Liden *et al.*, 2006).

A BLAST search of the genome of *S. equi* strain 4047 showed that this strain encodes several FNE similar proteins (Paper II). Four ORFs of putative

FNE-like proteins with specific similarities to FNE and FNEB were found. These proteins are referred to hereafter as FNEC, FNED, FNEE and FNEF. Three of the proteins display the characteristic C-terminal membrane spanning region and wall-anchoring sortase site LPXTG. The *fned* gene is interrupted by a stop codon, resulting in a truncated protein and is therefore predicted to be secreted into the medium similarly to FNE. The fibronectin- and/or collagen-binding regions of the FNE-like proteins are located in the N-terminal parts of the proteins and the C-terminal parts either contain collagen-like repeats (FNEC, FNEF) or P/K- rich repeats (FNEE) or are predicted not to be transcribed (FNED). An alignment of the different FNE-like proteins showed areas of conserved residues between all proteins.

The mature recombinant proteins, FNE-FNEF, were analysed using a microwell assay with radiolabelled plasma fibronectin and collagen type I (Paper II). The wells were coated with respective mature recombinant protein, labelled fibronectin or labelled collagen added and the specific binding was measured. In that assay, FNEE was the only recombinant protein, apart from FNE, found to bind both collagen and fibronectin. The remaining three FNE-like proteins bound soluble collagen (Paper II).

To further analyse the binding activity, the FNE-like proteins, including FNE and FNEB, were sub-cloned using the GST system. Using a microwell binding assay, biotinylated bovine fibronectin and collagen and were added to wells previously incubated with the GST-tagged proteins. The binding assay showed that FNE-GST, FNEB-GST and FNEE-GST were able to bind to soluble fibronectin, while no detectable binding of FNEC-GST, FNED-GST and FNEF-GST to fibronectin was observed. All GST-tagged proteins except FNEB-GST displayed binding to collagen type I although FNED-GST showed remarkably lower binding activity than the other fusion proteins (Paper II).

In order to analyse the specific binding of the GST-tagged proteins to proteolytic fragments of fibronectin, 30-kDa or 45-kDa were used in a microwell assay. The results showed that only FNEB-GST was able to bind to the 30-kDa fragment of fibronectin, which is in agreement with previous findings (Lannergard *et al.*, 2005). The results using the 45-kDa fragment showed that FNEE-GST binds to this part of fibronectin. However, no binding of FNE-GST to 45-kDa was detected, although previous studies have shown that FNE binds to the GBD domain of fibronectin (Tiouajni *et al.*, 2014; Lannergard *et al.*, 2005). One explanation could be that the proteolytic fragment was produced with different cleaving enzymes in Paper II and was therefore not identical to that used in previous studies.

Using biotinylated FNE-GST, the competition of binding to fibronectin and collagen of the GST-tagged proteins was tested in a microwell assay.

Microwells coated with fibronectin or collagen was incubated with different concentrations of recombinant fusion protein together with biotinylated FNE. The binding was detected using HRP-conjugated streptavidin. The results showed that the binding of biotinylated FNE-GST to fibronectin was inhibited by FNEE-GST and that the binding of FNE-GST to collagen was inhibited by FNEC-GST, FNEE-GST and FNEF-GST. The FNE binding site to procollagen type I has previously been reported to be located 122 nm from the C-terminus (van Wieringen *et al.*, 2010). The results from the competition assay in Paper **II** suggested that the collagen binding of FNEC-GST, FNEE-GST and FNEF-GST is located at the same site or sufficiently close for steric hindrance.

In order to further investigate the binding of FNE-GST and FNEE-GST to fibronectin, competition binding assays were performed using the fibronectin-binding protein SFS. The binding site in SFS to fibronectin are localised to the ⁸⁹FI module of the GBD domain according to recent report (Ma *et al.*, 2015). The results showed that SFS inhibits binding of both FNE-GST and FNEE-GST to fibronectin, confirming the location of binding for these proteins (Paper **II**).

The binding site of CNE to collagen has previously been reported (van Wieringen *et al.*, 2010; Lannergard *et al.*, 2003). To analyse whether CNE could inhibit binding of FNE-GST and FNEE-GST to collagen, further competition binding analysis was performed. No inhibition of binding was detected, suggesting another binding location than CNE to collagen type I (Paper **II**).

A set of deletion clones of FNE and FNEE was also produced in order of mapping the binding domains of respective protein to fibronectin and collagen. The results showed that the fibronectin binding in both FNE and FNEE was located in the C-terminal region. The collagen binding was located to the N-terminal part of the proteins (Paper **II**).

Recent studies have shown that an internal thioester bond is present in FNE (Tiouajni *et al.*, 2014) and Walden *et al.* (2015) reported that the fibronectin-binding protein Sfbl from *S. pyogenes* also contains a thioester which forms a covalent bond between Sfbl and fibrinogen. Based on the similarities of the FNE-like proteins and presence of a possible thioester bond in all of the proteins except FNED, gel-shift experiments were performed. The GST-tagged FNE-like proteins were incubated with collagen type I. After incubation prior to SDS-PAGE analysis, the samples were mixed with β -mercaptoethanol and SDS and thereafter boiled. The results demonstrated that FNE, FNEC, FNEE and FNEF displayed a gel-shift indicating a binding that resists harsh treatments, suggesting covalent binding to collagen type I (Paper **II**).

The interaction of the FNE-like proteins with fibroblasts was also investigated using a collagen gel contraction model described previously (Reyhani *et al.*, 2014). The different FNE-like proteins were added to the collagen gels and the contraction measured at different time points using an inverted light microscope. The results of these experiments showed that, as previously reported (Liden *et al.*, 2008), FNE mediates a contraction due to dual binding activity to both fibronectin and collagen. FNEE and FNED can also enhance a contraction in presence of PDGF-BB in this system. The finding that FNEE also mediates contraction is in agreement with the suggestion that, as in the case with FNE, dual binding activity is needed to enhance contraction. Surprisingly, although lacking fibronectin-binding activity and with only weak collagen-binding activity, FNED enhanced contraction. At the moment there is no explanation for this, although other as yet unknown functions of the FNED proteins cannot be ruled out (Paper **II**).

“I know one thing: that I know nothing”
Socrates

3 Concluding remarks and future perspectives

Pathogenic bacteria produce different virulence factors that contribute to their ability to adhere and colonise the host or avoid the host immune system. This thesis showed that *S. equi*, the causative agent of strangles in equines, possesses two different types of potential virulence factors that interact with components of the host ECM or interferes with the host immune system.

Paper I and paper II describes the identification and characterization of two novel IgG endopeptidases IdeE2 of *S. equi* and IdeZ2 of *S. zooepidemicus*. Compared to the previous described IgG endopeptidases from these subspecies, IdeE and IdeZ, the IgG cleavage pattern of IdeE2 and IdeZ2 is narrower. However, both IdeE2 and IdeZ2 cleave IgG from human and horse efficiently which are interesting since IdeE and IdeZ cleaves horse IgG poorly. Sequence alignment shows that the enzymes shows pairwise similarities between IdeE and IdeZ and between IdeE2 and IdeZ2 but the similarities are much lower when the two groups are aligned. Comparison of the *S. equi* and *S. zooepidemicus* IgG endopeptidases with the IgG endopeptidase IdeS reveals that the critical amino acids important for IgG cleavage in IdeS are also present in the *S. equi* and *S. zooepidemicus* enzymes and the regions in proximity of these amino acids are conserved in IdeE, IdeZ, IdeE2 and IdeZ2. While IdeE2 protein seems to be well conserved among different isolates, like the IdeE and IdeZ, the IdeZ2 protein was found to display sequence variation forming three major groups of IdeZ2 proteins. The differences in the IdeZ2 proteins could not be correlated to either type of infection, origin of the host or to MLST group of the *S. zooepidemicus* isolate. A computational model aligning IdeS and the different IdeZ2 proteins (and IdeE2) were produced showing that the main changes in the structures were in the postulated loops of the proteins and the

regions responsible for endopeptidase activity are well conserved within the three groups.

In order to confirm the structures of the IdeZ2 proteins and also IdeZ, IdeE and IdeE2 proteins, determination of the crystal structures of all proteins would give the answer. To further investigate the role of the Ide-proteins it should also be interesting to investigate if these proteins binds to certain antibodies, although no enzymatic activity have been observed, to investigate if they like e.g. streptococcal protein G or *S. aureus* protein A can inhibit other functions of the antibodies e.g. complement activation. Further analysis of functional properties of the extended N-terminal the IdeE2 and IdeZ2 proteins and the C terminal part of the IdeZ2₁₆ would also be interesting for the future research. The regulation of expression the Ide-proteins would also be an important field of research. Today we do not know if or when the expression occurs during an infection or what induce expression. However, IdeE and IdeE2 induced protection in mice when used as vaccine candidates (Paper I) and have been used in a horse vaccination trial, where they gave a good protection in horses when combined with other recombinant proteins (Guss *et al.*, 2009) results that suggests that these type of proteins are important in the infection process.

In paper II four FNE-like proteins in *S. equi* that bind to fibronectin and/or collagen were identified and characterized. FNE, FNEB and FNEE bind to different parts of fibronectin, whereas FNE, FNEC, FNEE and FNEF bind to collagen type I in a probable covalent manner. FNED display a weak collagen binding which most likely depends on the lack of postulated critical region in C-terminal end of the protein. Interestingly FNE, FNEE and to a minor degree FNED all enhanced contraction in a collagen gel contraction model, a model that previous been used to investigate the activity of FNE. Mice experiments have shown that FNE can normalize lowering of interstitial fluid pressure by stimulating connective tissue cell contraction (Liden *et al.*, 2008) suggesting a biological role of FNE. While FNE and most probably FNED are secreted proteins the FNEE have the characteristics of being a cellwall bound protein and the biological implication of this in relation to collagen contraction remains to be investigated. At moment the expression of the FNE-like proteins *in vivo* and their biological role and impact in the infection process remains to be investigated. Those studies should also include the FNE-like proteins in *S. zooepidemicus*. *Streptococcus equi* is a host-restricted horse pathogen, while *S. zooepidemicus* can cause infection in a variety of species and in various parts of the host. Thus it would be interesting to investigate if the FNE-like proteins in *S. zooepidemicus* have a similar binding activity to fibronectin and/or collagen.

4 Populärvetenskaplig sammanfattning

Kvarka är en anmälningspliktig mycket smittsam övre luftvägssjukdom, orsakad av bakterien *Streptococcus equi* underart *equi* (*S. equi*), som drabbar hästdjur. Sjukdomsförloppet vid kvarka inleds då bakterien infekterar slemhinnan i hästens mule, varifrån bakterierna sprider sig via det lymfatiska systemet till lymfkörtlar i hästens huvud och hals. En häst som insjuknar i kvarka får feber, svullna lymfknutor på huvudet och halsen och tjockt varigt näsflöde. Sjukdomen smittar vid kontakt mellan hästar men även vid kontakt med kontaminerat material så som vattenhinkar, grimskافت och boxinredning. Även människor kan föra smittan vidare inom och mellan stall via händer och kläder efter kontakt med infekterade hästar. Ett kvarkautbrott kan pågå i många veckor och orsakar förutom lidandet för hästarna ofta ekonomiska konsekvenser på grund av karantänbestämmelser vilka leder till inställd verksamhet såsom tävlingar och inställda ridlektioner.

Streptococcus equi underart *zoepidemicus* (*S. zoepidemicus*) betraktas vanligen som en opportunistisk patogen i luftvägarna hos häst, d.v.s. bakterien kan finnas hos friska hästar men orsaka sjukdom om hästens immunförsvar är nedsatt. *S. zoepidemicus* kan, till skillnad mot *S. equi*, orsaka andra typer an infektioner hos häst så som sårinfektioner, ledinfektioner och infektioner i livmodern. Dessutom kan *S. zoepidemicus* orsaka infektion hos andra mammalier, i sällsynta fall även hos människa.

Kunskapen om *S. equi* och *S. zoepidemicus* förmåga att orsaka sjukdom är inte fullständigt klarlagt. Bakterierna kan producera flera olika virulens faktorer vilka interagerar med värdjuret. Dessa virulens faktorer kan tex. vara extracellulära proteiner vilka är förankrade i cellväggen på bakterien eller extracellulära proteiner som transporteras ut ur cellen till den omgivande miljön. Att identifiera och förstå funktionen hos dessa proteiner är en mycket viktig del i förståelsen i infektionsprocessen.

Syftet med projektet var att identifiera och studera extracellulära cellytebundna och sekreterade (potentiella virulens faktorer) hos *S. equi* och *S. zooepidemicus* vilka har betydelse i interaktionsprocessen mellan bakterien och värden.

S. equi producerar ett okänt antal virulens faktorer som på olika sätt interagerar med värdjuret, hästen. En viss grupp av virulens faktorer, kallade adhesiner eller receptiner, har visat sig viktiga när bakterien ska binda till strukturer hos värden vilket underlättar och möjliggör kolonisation. Adhesion till komponenter i värdens extracellulära matrix (ECM) har visat sig vanligt hos *S. equi* och *S. zooepidemicus*. ECM är ett olösligt nätverk som separerar och stöttar organ och vävnad i en organism. Två vanligt förekommande proteiner i ECM är fibronectin (Fn) och kollagen (Cn). Tidigare studier har identifierat att *S. equi* och *S. zooepidemicus* producerar flera kollagen- och fibronectin bindande proteiner (Lindmark 1996, Lindmark 2001, Lannergård 2005). I artikel **II** har vi identifierat en familj av gener som kodar för fibronectin- och/eller kollagen bindande proteiner, kallade FNE-liknande proteiner som inkluderar FNE, FNEB, FNEC, FNED, FNEE och FNEF. Genom att producera dessa som rekombinanta proteiner har förmågan att binda till fibronectin och kollagen studerats. För att ytterligare kartlägga bindningen till respektive värdprotein har ett antal trunkeringar, eller förkortningar, av de rekombinanta proteinerna framställts. För att identifiera de bindande domänerna i respektive bakterieprotein samt hos de strukturella värdproteinerna Fn och Cn har flera olika tekniker används så som jod-in märkning av proteiner, mikrotiterplatta analyser samt cellbiologiska försök för studier av proteinernas funktion *in vivo*. Fyra av proteinerna tros vara cell väggs bundna proteiner medan två skickas ut i omgivningen. Bindningsstudier visade att två av proteinerna, FNE och FNEE, binder både Cn och Fn medan tre, FNEC, FNED och FNEF, endast binder till Cn. FNEB binder endast till Fn. *In vivo* försök med eukaryota fibroblaster visade att, inte bara FNE ger upphov till kontraktioner av celler i en kollagen-kontraktions modell, utan även FNEE har liknande inverkan.

Vissa patogena bakterier har utvecklat olika mekanismer för att undgå värdens immunförsvar, t.ex. genom att producera proteiner som interagerar med immunförsvaret. Immunoglobulin G (IgG) har en central roll i immunförsvaret för att skydda värden mot bakterier och andra mikroorganismer. När IgG binder till bakterier fungerar det som en signalflagga för immunförsvaret att en främmande bakterie finns i kroppen som måste oskadliggöras genom fagocytos eller genom komplement aktivering. Tidigare studier har visat att både *S. equi* och *S. zooepidemicus*

producerar extracellulära enzymer vilka specifikt klyver IgG. Enzymerna kallade IdeE (*S. equi*) och IdeZ (*S. zooepidemicus*) bryter ner/ klipper sönder IgG molekyler från olika däggdjur mycket effektivt (Lannergård 2006). En annan studie har visat IdeE även kan interagera med neutrofiler från häst genom att binda in via en integrin bindande del hos IdeE (Timoney 2008).

I artikel I visade vi på att *S. equi* och *S. zooepidemicus* har minst ytterligare två IgG klyvande enzym kallade IdeE2 och IdeZ2. Dessa enzymer klyver IgG från människa och häst mycket effektivt men inte IgG från andra mammalier. I studien visade vi att IdeE2 klyver en speciell grupp av häst IgG, IgG4 (IgGb) som finns närvarande i häst serum. För att ta reda på om IdeE och IdeE2 passar som komponenter i ett framtida vaccin mot kvarka immuniserades möss med enzymerna varefter de blev utsatta för *S. equi*. Vaccinationsstudien visade att båda enzymerna ger ett bra skydd mot *S. equi* i en musmodell av kvarka. Eftersom IdeE2 inte i klyvnings studier av IgG visat aktivitet mot mus IgG var skyddseffekten av detta enzym i mus modellen lite överraskande men det kan inte uteslutas att IdeE2 har andra funktioner hos bakterien. I studien upptäcktes att genen för IdeZ2 proteinet varierade i gensekvensen och att det finns tre olika varianter av genen (artikel III). Variationen kunde inte härledas till vilken typ av infektion den orsakat, alltså om det var från djur med luftvägsinfektion, infektion i livmodern eller en sårinfektion, eller från vilken typ av streptokock stam bakterien tillhörde. De olika IdeZ2 proteinerna hade fortfarande samma klyvningsförmåga dvs. att alla varianterna klyver häst IgG väldigt effektivt.

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