Collagen-like Proteins in Horse Pathogenic *Streptococcus equi*

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2005

Acta Universitatis Agriculturae Sueciae
2005:99

ISSN 1652-6880
ISBN 91-576-6998-8

Tryck: SLU Service/Repro, Uppsala 2005

Abstract

Karlström, Å. 2005. Collagen-like proteins in horse pathogenic *Streptococcus equi* Doctoral dissertation
ISSN 1652-6880, ISBN 91-576-6998-8

Streptococcus equi subspecies *equi* is the causative agent of strangles, a respiratory disease that affects horses worldwide. Like other pathogenic bacteria, *S. equi* subspecies *equi* expresses a number of extracellular proteins important for the interaction with the host and thus for the pathogenesis.

In this thesis, one aim was to identify and characterise novel extracellular proteins in this subspecies. Signal phage display was used to screen the genome of *S. equi* subsp. *equi* strain 1866 for signal sequences thereby identifying extracellular proteins. A novel collagen-like protein, SclC, was found and further characterised. The SclC protein, similar to Scl proteins in *S. pyogenes,* has an N-terminal signal sequence, a unique region, a collagen-like region and a wall-spanning region ending with an LPXTG-motif preceding the membrane-bound region in the C-terminal end. Using bioinformatic tools, the collagen-like protein was found to be a member of a family consisting of seven collagen-like proteins, called SclC-SclI, in subspecies *equi*. In contrast to mammalian collagen, all these proteins have collagen-like regions that consist of KGD/KGE triplets, which are suggested to be of importance for the structure of the proteins.

When sera from horses previously infected with subsp. *equi* and sera from healthy horses were analysed, antibodies against all seven proteins were detected. Furthermore, antibodies against SclC recognised all members of the family of collagen-like proteins. In an immunisation study in a strangles model in mice, immunisation with SclC resulted in protection against infection by *S. equi* subsp. *equi*. The biological role of SclC was further investigated in a collagen lattice gel contraction study to investigate whether mammalian integrins would recognise SclC, which turned out not to be the case.

The biological role of the collagen-like proteins identified here is not fully comprehended. However, since the vaccination studies in mice resulted in protection against infection, these proteins have an interesting potential for further vaccine trials in horses.

Keywords: *Streptococcus equi*, strangles, collagen-like proteins, Scl, extracellular proteins, pathogenic bacteria, phage display, integrins, vaccination

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Amor vincit omnia

Abbreviations

α_2-M	α_2-macroglobulin
CL	collagen-like region
CNE	collagen-binding protein in *S. equi*
EAG	α_2-macroglobulin-, albumin-, IgG-binding protein in *S. equi* subsp. *equi*
ECM	extracellular matrix
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	endoplasmatic reticulum
EtxB	recombinant B-subunit of *E. coli* enterotoxin
FN	fibronectin
FNE	fibronectin-binding protein from *S. equi* subsp. *equi*
FNEB	fibronectin-binding protein B in *S. equi* subsp. *equi*
FNZ	fibronectin- and collagen-binding protein from *S. equi* subsp. *zooepidemicus*
GAG	glycosaminoglycan
GAS	Group A Streptococci
Hyp	hydroxyproline
i.n.	intranasally
ISCOM	immune stimulating complex
ORF	open reading frame
PDGF	platelet derived growth factor
s.c.	subcutaneously
Scl	streptococcal collagen-like protein
SclC	streptococcal collagen-like protein in Group C streptococcus
SFS	secreted fibronectin-binding protein in *S. equi* subsp. *equi*
subsp.	subspecies

Contents

Appendix

Paper I-IV

This thesis is based on the following papers, which are referred to in the text by their Roman numbers.

I. Karlström, Å., Jacobsson, K., Flock, M., Flock, J.-I. and Guss, B. 2004. Identification of a novel collagen-like protein, SclC, in *Streptococcus equi* using signal sequence phage display. Veterinary Microbiology 104 (179-188).

II. Karlström, Å., Jacobsson, K. and Guss, B.
SclC is a member of a family of collagen-like proteins in *Streptococcus equi* subspecies *equi* that are recognised by antibodies against SclC. *(submitted)*

III. Flock, M., Karlström, Å., Lannergård, J., Guss, B. and Flock, J.-I.
Protective effect of vaccination with recombinant proteins from *Streptococcus equi* subspecies *equi* in a strangles model in the mouse. *(manuscript)*

IV. Lidén, Å., Karlström, Å., Lannergård, J., Persson, L., Guss, B., Rubin, K. and Rydén, C.
An extracellular protein from *Streptococcus equi* modulates cell mediated collagen lattice contraction. *(manuscript)*

My contribution to each paper has been as follows

I. Participated in planning the project in collaboration with the co-authors. Performed major part of the laboratory work and analysis of the results. Main writer of the manuscript.

II. Major part of planning the project and performed all the laboratory work. Main writer of the manuscript.

III. Participated in planning the project. Designed and produced recombinant SclC proteins and the sub-domains used in the experiment. Participated in analysing the results and writing the manuscript.

IV. Took part in planning the study, regarding choice of proteins. Produced the recombinant SclC protein and fragments thereof used in the experiment. Performed minor part of the experiments. Participated in analysing the results and writing the manuscript, mainly the sections about the proteins used and the binding studies.

"It has long been an axiom of mine that the little things are infinitely the most important"
Sir Arthur Conan Doyle

General background

The state of health is the result of a constant ongoing battle between pathogenic microbes and the immune system of the host. Failure of the immune system most likely causes the host to turn from healthy to ill and the microbes possess the ability to break the defences in more ways than one. Obligate pathogenic bacteria are harmful and always cause disease, whereas opportunistic bacteria cause disease when the immune system is somewhat compromised. The pathogenic bacteria have several ways of deceiving the immune system and overcoming the defence mechanisms of the body. One strategy is to cover themselves in structures that look familiar to the host system and the pathogens are therefore not recognised, another is to have a fast entry phase in which they quickly disappear into the host cells, where they are not recognised and detected. The immune system, on the other hand, has its own strategies working to investigate all unknown agents that are discovered inside the body.

Vaccination is the concept of a vaccine protecting the host against a particular microbe (Casadevall & Pirofski, 2004). An antigen is introduced to the immune system, which reacts to it, forming antibodies that recognise the antigen. The purpose then is for specific antibody production to be activated the next time the immune system encounters the antigen. The main problem is to find the right antigens that give sufficient protection from the infective agent. Several vaccines consist of attenuated strains of bacteria, which are not as pathogenic as the wild type bacteria but still share many common features with the actual pathogenic agent. In this study, the horse is the host and the infectious agent is represented by *Streptococcus equi.*

Strangles

Strangles is a disease that affects horses caused by the bacterium *Streptococcus equi* subsp. e*qui*. The disease is spread worldwide and the classic description of strangles includes swelling and infection of the throat region, although the symptoms can vary from case to case (Harrington, Sutcliffe & Chanter, 2002). In uncomplicated cases the disease is localised to the upper respiratory tract together with fever, coughing, pus discharge from the nostrils and abscess formation in the lymph glands. In more severe cases, when the bacteria are found in the lower respiratory tract, pneumonia and heart muscle inflammation can

be further complications. Although strangles is not primarily a fatal disease, it can cause chronic illness and sometimes death (Timoney, 1988, Timoney, 2004). In Sweden, the number of reported cases has decreased from 116 in 2001 to 63 in 2003 (Jordbruksverket, 2005). However, it is important to remember that this is the number of stables and not actual infected individuals.

Streptococcus equi subsp. *equi* enters the horse via the mouth or nose and the bacteria are transported to the lymphoid glands through the mucosal layer. In the glands the bacteria form long chains of cocci. The incubation time for the disease varies from 3 to 14 days. The normal temperature of a horse ranges between 37.5°C and 38.5°C but during the disease the temperature may increase to above 39°C. During the incubation time, the infected horse is highly contagious, which is one of the reasons why stables in Sweden where an infected horse is found are placed in quarantine for at least 30 days. With the increased temperature comes formation of abscesses in lymph glands, followed by a slight cough and difficulties in swallowing. As the disease progresses the abscesses can grow hard and painful and the pressure on the retropharyngeal lymph nodes can cause respiratory difficulties, as the name of the disease indicates. In severe cases, the bacteria move into the lower respiratory tract and may cause pneumonia. In horses with the most severe form of strangles, any part of the body or viscera may be affected, including the heart, and then the disease is called bastard strangles (Timoney, 1993, Timoney, 2004).

The disease is most commonly transmitted through infective secretions. Once the disease establishes itself on a farm or ranch, it often becomes a persistent and recurring problem, even though there may be long periods when no resident horse has typical signs of the disease. Once the organism establishes itself in a susceptible population, the percentage of animals affected is generally quite high and often approaches 100%. However, the mortality rate in most uncomplicated cases is low. If the disease is discovered early, it can be treated with penicillin although often even that is not necessary as long as the horse has an appetite and not too high a fever.

The genus *Streptococcus*

Streptococcus is described in Bergey's Manual (Hardie, 1986) as spherical or sometimes ovoid shaped cells, Gram-positive cocci, catalase negative bacteria that usually are facultative anaerobes. They grow in pairs or chains and their temperature optimum is usually 37°C, even though minimum and maximum temperatures vary within the genus. Not all species form capsules, but it is not uncommon. Streptococci are responsible for a large number of important diseases in man and in animals. They are found on mucosal membranes of the mouth, respiratory and genitourinary tracts, as well as on the skin. The pyogenic group (Table 1) are regarded as pathogenic or opportunistic pathogens of mammals (Hardie & Whiley, 1995). The most studied member of the pyogenic group is *Streptococcus pyogenes*, belonging to the Lancefield serological group A streptococci. It is the causative agent of various diseases in humans, ranging from mild infections of the skin area to more severe diseases such as scarlet fever. The streptococci can be divided into groups in different ways, *e.g.* the Lancefield

grouping based on differences in serological types devised by Lancefield (Lancefield, 1933), their type of haemolysis or their genetic similarity. The genus has gone through several revisions regarding taxonomy and nomenclature through the last twenty years, as reviewed by Facklam (Facklam, 2002).

Table 1. The pyogenic group in streptococci

Species	Lancefield group	Haemolysis	Host
S. pyogenes	A	beta	humans
S. agalactiae	B	beta	humans, cattle
S. dysgalactiae			
subsp. *equisimilis*	A/C/G/L	alpha/beta	humans
subsp. *dysgalactiae*	C	none	cattle
S. equi			
subsp. e*qui*	C	beta	horses
subsp. *ruminatorum*	C	beta	sheep
subsp. *zooepdemicus*	C	beta	mammals
S. canis	G	beta	dogs
S. iniae	none	alpha/beta	dolphins
S. parauberis	E/P	alpha	cattle
S. porcinus	E/P/U/V	beta	pigs
S. uberis	E/P/G	alpha/gamma	cattle

Streptococcus equi

Three subspecies of Streptococcus equi

Streptococcus equi is β-haemolytic, belongs to the Lancefield group C streptococci and comprises three subspecies, subsp. *equi*, subsp. *zooepidemicus* and subsp. *ruminatorum* (Hardie & Whiley, 1995, Fernandez *et al.*, 2004). The cells are 0.6-1.0 μm in diameter and capsules are demonstrated in some strains either when young cultures are examined or when serum is added to the growth medium.

S. equi subsp. *zooepidemicus* is a mucosal commensal in horses and does not normally cause disease in healthy horses, although the bacterium is regarded as an important opportunistic pathogen (Timoney, 2004). The organism is found in the respiratory region, the uterus and wounds, where it occasionally may give rise to an infection. This subspecies has also been isolated from humans, as well as from a number of mammals including rats, pigs, cats, cattle and mice (Ruoff, 1992).

In contrast, *S. equi* subsp. *equi* is an obligate pathogen and is considered to be a parasite in the upper respiratory tract of horses (*equimilius*). Since donkeys and mules belong to the same taxonomic group (*Equidae*), they can also be infected (Harrington, Sutcliffe & Chanter, 2002). The bacteria cause strangles, a worldwide disease that gives rise to a pathogenic colonisation in the upper respiratory tract and affects the animal in various ways. The genome is approximately 2.3 Mb with a G+C content of approximately 41%.

Subsp. *rumatorium* was only recently described and has been isolated from sheep (Fernandez *et al.*, 2004). Even though it has not been defined as a horse pathogen, investigations have revealed genetic similarities, placing it as a *Streptococcus equi* subspecies. Since this subspecies has not been identified as a horse pathogen, it is not further discussed in this thesis.

Potential virulence factors of Streptococcus equi

The pathogenicity of different streptococci varies between different species and subspecies. Most of the streptococci belonging to the *pyogenic* group are considered pathogens. Pathogenic streptococci express a number of different components in order to establish infections in their host. Of these, some are secreted into the growth medium whereas others are associated with the cell wall and include factors such as a capsule, various cell surface-anchored proteins, secreted enzymes and toxins. In certain species of streptococci, several of these types of components have been identified as virulence factors (Lukomski *et al.*, 1997, Hytonen *et al.*, 2001, Beckmann *et al.*, 2002, Chhatwal, 2002, Batzloff *et al.*, 2004). In particular, extracellular enzymes causing disruption of tissue and cleavage of host proteins have been noted to have an effect on virulence. Since *S. equi* has been less studied than some of the pyogenic species that affect humans, all extracellular components identified have not been confirmed as virulence factors. However, many of these proteins share features with the previously reported components in *e.g. S. pyogenes*, which makes further analysis to determine their role in virulence of *S. equi* interesting.

Capsule

The capsule, consisting of the polysaccharide hyaluron (formerly called hyaluronic acid), may not be present in all pathogenic species (Ruoff, 1992). The capsule in itself can be considered to hide the bacteria from discovery by the immune system of the host. The capsule does not elicit an immune response in the host due to the fact that it is comprised of hyaluron, found naturally in mammals. Furthermore the capsule makes it harder for the macrophages to adhere to the bacteria, thus creating a bacterium that is more virulent than non-encapsulated bacteria (Wibawan *et al.*, 1999). Expression of the capsule has been identified in most of the strains investigated in subsp. *equi* but has been found to vary in the less virulent subsp. *zooepidemicus*.

Extracellular proteins

Many of the cell surface-anchored proteins in Gram-positive bacteria such as streptococci display a common structural organisation and features that are important in binding the protein to the cell wall (Navarre & Schneewind, 1999). The motifs are: an N-terminal signal sequence, which directs the secretion through the cell membrane, and a hydrophobic C-terminal domain preceded by an LPXTG motif. All these motifs are essential in the protein/cell wall linkage process. Examples of host proteins that streptococcal cells can bind via their cell surface proteins to their surface include IgG, α_2-macroglobulin, serum albumin, fibronectin, fibrinogen and collagen (Table 2). In addition the bacteria can secret

proteins that have other activities and of which some are considered to affect the virulence, *e.g.* haemolysins (Flanagan *et al.*, 1998), hyaluronidase (Woischnik, Buttaro & Podbielski, 2000), proteinases (Rasmussen, Muller & Bjorck, 1999, von Pawel-Rammingen, Johansson & Bjorck, 2002) and streptolysins (Kehoe *et al.*, 1987, Okumura *et al.*, 1994, Navarre & Schneewind, 1999, Sierig *et al.*, 2003). The relative importance of the various potential virulence factors between species and sites is likely to vary.

The first reported cell surface protein from *S. equi* was the M-protein (SeM), which was described in the 1980s by Srivastava *et al.* (Srivastava, Barnum & Prescott, 1985). The M-protein is a fibrinogen-binding protein and most importantly it is opsonogenic when introduced into a host. Binding of fibrinogen has been reported to have an antiphagocytic property and the binding of fibrinogen to the *S. pyogenes* cell surface leads to resistance to phagocytosis by neutrophils (Boschwitz & Timoney, 1994). It has also been shown that binding of fibrinogen to the subsp. *equi* surface is partly due to the protein SeM.

The horse pathogenic subspecies of *S. equi* differ in expression of M-like proteins, as subsp. *equi* have been found to express two (SeM and SzPSe) whereas subsp. *zooepidemicus* only have the homologue to SzPSe (SzP) (Timoney *et al.*, 1995, Timoney, Artiushin & Boschwitz, 1997, Meehan, Nowlan & Owen, 1998). The SeM protein is only opsonogenic in subsp. *equi* and not in subsp. *zooepidemicus*. Furthermore, M-like proteins have been reported in other streptococci (Schnitzler *et al.*, 1995, Cedervall, Johansson & Åkerström, 1997, Geyera *et al.*, 1999, Vasi *et al.*, 2000) and especially in *S. pyogenes* (Fagan *et al.*, 2001). In these species there are several different variants of M-like proteins that vary between the strains, thus making them important for serotype and strain classification.

After the report on the M-protein, several more cell surface and secreted proteins from subsp. *equi* have been identified. Another large group of these extracellular proteins are the fibronectin-binding proteins. These proteins consist of not only cell wall-bound proteins, FNEB (Lannergard *et al.*, in press), but also secreted proteins, SFS (Lindmark & Guss, 1999) and FNE (Lindmark, Nilsson & Guss, 2001), which have been reported in both the horse pathogenic subspecies of *S. equi*. Notably the FNE protein is called FNZ in subsp. *zooepidemics* (Lindmark *et al.*, 1996) and is cell wall-anchored, whereas it is a truncated, secreted protein called FNE in subsp. *equi.* In *S. pyogenes*, binding to fibronectin is important since it has been reported to have an impact on the internalisation of the bacteria into host epithelial cells (Talay *et al.*, 1992, Molinari *et al.*, 1997). Fibronectin is one component in the extracellular matrix (see section *Extracellular matrix*) and is an abundant protein in the mammalian body also present in soluble form in plasma.

Table 2. Extracellular proteins previously reported in S. equi subsp. equi and subsp. zooepidemicus

Protein	Feature	Anchored / Secreted	Reference	in subsp. *zooepidemicus*
CNE	collagen-binding	anchored	Lannergård *et al.* 2003	CNE
EAG	α_2-macroglobulin-, albumin-, IgG-binding	anchored	Jonsson *et al.* 1995 Lindmark *et al.* 1999 Flock *et al.* 2004	ZAG
FNE	fibronectin-binding	secreted	Lindmark *et al.* 2001 Lindmark *et al.* 1996	FNZ
FNEB	fibronectin-binding	anchored	Lannergård *et al.* in press; Hong *et al.* 2005	FNZ2
SeM	fibrinogen-binding (M-protein)	anchored	Timoney *et al.* 1985; Srivastava *et al.* 1985	not found
SePE-H (SeeH)	exotoxin	secreted	Artushin *et al.* 2002	not found
SePE-I (SeeI)	exotoxin	secreted	Artushin *et al.* 2002	not found
SeeL (SPE-Lse)	exotoxin	secreted	Proft *et al.* 2003	SZEL
SeeM (SPE-Mse)	exotoxin	secreted	Proft *et al.* 2003	SZEM
SFS	fibronectin-binding	secreted	Lindmark *et al* . 1999	SFS
SzPSe	fibrinogen-binding (M-like protein)	secreted	Timoney *et al.* 1997	SzP

S. equi also expresses a collagen-binding protein, CNE (Lannergard, Frykberg & Guss, 2003) that is similar to the previously reported virulence factor CNA in *Staphylococcus aureus*. A multi-binding protein called EAG/ZAG (Jonsson, Lindmark & Guss, 1995, Lindmark, Nilsson & Guss, 2001) is also present in both subspecies. EAG is a multi-binding protein with an N-terminal α_2-macroglobulin-binding domain, followed by an serum albumin-binding domain and the C-terminal IgG-binding domain.

Toxins

S. equi subsp. *equi* expresses four superantigens called SePE-H, SePE-I (Artiushin *et al.*, 2002), SeeL and SeeM (Proft *et al.*, 2003) (Table 2). These proteins are homologous to the pyrogenic exotoxins previously reported in the more investigated species *S. pyogenes* (Smoot *et al.*, 2002). These toxins have an immuno-modulating capacity and bind to variants of class II MHC molecules and to the beta-chain in the T-cells, resulting in a T-cell proliferation, which leads to

an enhancement of susceptibility to endotoxic shock. SeeL and SeeM have homologues in subsp. *zooepidemicus* (Proft *et al.*, 2003, Alber *et al.*, 2005) whereas this does not seem to be the case with SePE-H and SePE-I. There have been reports of differences in expression of toxins between the two subspecies when comparing culture supernatants of various isolates from *S. equi*. The subsp. *zooepidemicus* does not express the SePE-H and SePE-I exotoxins, whereas subsp. *equi* does, indicating they could be important in explanation of the difference in virulence between the two subspecies (Artiushin *et al.*, 2002).

Vaccination against S. equi *infection*

There are three vaccines against strangles available today. None of these gives full protection from the disease and both the degree of protection and the safety of the animals immunised differ between all three.

Pinnacle™ I.N. (Pinnacle™) was introduced in the US in the late 1990s. It is a vaccine consisting of a live attenuated strain of subsp. *equi* lacking the hyaluron capsule. The vaccine is distributed intranasally to the horse as a spray, thus giving local nasopharyngeal antibodies believed to be important in protection. However, since a horse immunised with the Pinnacle strain has been exposed to a strain of bacteria without any genetic markers, it cannot be distinguished from a horse that has simply been infected and has strangles (Walker & Timoney, 2002). Since the Pinnacle bacteria can sometimes revert to their original state, produce a capsule and become aggressive, this is a concern because the vaccine can then cause the disease instead of protecting from it.

Strepguard (STREPGUARD™) is another vaccine, consisting of an adjuvanted extract from treated bacterial cells mainly consisting of M-protein. This vaccine is injected intramuscularly. It has been found that the M-proteins elicit antibodies that give weak, if any, protection (Meehan, Nowlan & Owen, 1998). Furthermore, the animal tends to suffer from a small infection and inflammation at the site of injection. This in combination with the fact that the vaccine does not result in local, nasopharyngeal antibodies gives a vaccine more likely to reduce the severity of the disease rather than one that gives complete protection.

Recently a new vaccine has been introduced, Equilis StrepE (Equilis), which consists of a live attenuated strain of subsp. *equi*, different from the Pinnacle strain. The vaccine is injected into the lip of the horse and the injection is likely to cause a swelling and inflammation at the injection site (Newton, Waller & King, 2005). Some horses express clinical signs of strangles after the vaccination, and, furthermore, this vaccine is not effective for more than three months, which also is a problem for a fully protective vaccination.

In conclusion, none of the three vaccines currently available on the market is as effective as required for an efficient large-scale vaccination of horses. This makes the need for a more effective vaccine high and desirable.

Extracellular matrix

The extracellular matrix (ECM) is a complex structural assembly surrounding and supporting cells within mammalian tissues. Often the ECM is referred to as the connective tissue and can be said to be composed of three major classes of bio molecules. These are:
Structural proteins (*i.e.* collagen and elastin) involved in maintaining the structure of connective tissue and the rigidity of cell structure
Specialised proteins acting as link proteins in adhesion and cell-cell interactions (*e.g*. fibronectin, and laminin)
Proteoglycans acting as space filling molecules which function as shock absorbing material in the tissue.

Mammalian collagen

The word collagen comes from the Greek *kólla* meaning glue and *géneo* meaning produced/created and explains rather nicely what most collagens do. Collagen is the most abundant protein in the mammalian body and the collagens constitute a family of extra-cellular proteins, which have a structural role as their primary function (Ricard-Blum & Ruggiero, 2005). The structure consists of a glycine backbone which forms an alpha helix. Glycine (G) is the first amino acid in a repetitive triplet, called Gly-Xaa-Yaa or GXY, which is the core of the protein. Usually the amino acids in the second (X) and third (Y) positions are proline (P) and hydroxyproline (O) (Fig. 1).

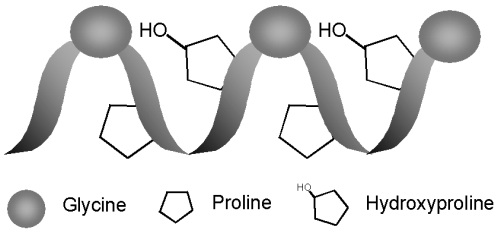

Figure 1. Schematic diagram of mammalian collagen.

The collagen protein is assembled in the endoplasmatic reticulum (ER) and the Golgi apparatus and the proline is hydroxylated after passing through the membrane, thus making the collagen stable. The stability has been proven to be linked to the formation of triple helices, with three alpha-chains linked together as a superhelix (right-handed). The collagen triple helix is one of the most stable structures found in nature. Collagen is divided into several subgroups depending on the secondary structure and most collagens are able to form supramolecular aggregates. This also means the each collagen member of a certain subclass shares a common chain structure. The first part of the superfamily to be discovered, and therefore also called classical collagens, were the collagens that are responsible for the formation of fibrils. The fibrils are relatively simple in structure, a large triple helix formation flanked by two globular extensions, and are made up of minor and major fibrillar collagens in mixtures; in connective tissue collagen types I, III and

V and in cartilage collagen types II and XI (Fichard, Kleman & Ruggiero, 1995). Collagen XXIII may contribute to cell adhesion via its RGD site and KGD motifs (Nykvist *et al.*, 2001, Banyard, Bao & Zetter, 2003), whereas collagens XV and XVIII are major components in the epithelial and endothelial basement membrane zones in a variety of tissues (Myers *et al.*, 1996, Ortega & Werb, 2002).

Fibronectin

The role of fibronectin is to attach cells to several various types of extracellular matrices, thus working as an adhesion molecule. This is reflected in the Latin origin of the name, *fibra* meaning fibre and *necto* meaning to attach or put together. Fibronectins are dimers of two similar peptides, each with at least six tightly folded domains with different affinity for various substrates, *i.e.* heparin, fibrin, collagen, cell surface receptors. The latter binding domain contains a consensus amino acid sequence, RGD, which is of importance when connecting to cellular integrins in signalling and other response mechanisms. This domain is also present in vitronectin, another major adhesion protein in the ECM which promotes cell attachment, proliferation and spreading (Schwarz-Linek, Höök & Potts, 2004) and RGD can also be found in bacterial proteins (Isberg & Tran Van Nhieu, 1995, Nilsson *et al.*, 2004). Fibronectin that is bound to a streptococcal cell can act as a bridging molecule towards the host cell integrins that initialise the uptake process leading to internalisation of the bacterial cells, which has been shown to occur in Group A streptococci (GAS) (Kreikemeyer, Klenk & Podbielski, 2004).

Proteoglycans

Proteoglycans are large carbohydrates that consist of repeating disaccharide units called glycosaminoglycans (GAGs) with a protein core forming complex high molecular weight components of the ECM. These function as stabilisers and water absorbance material when water from certain tissues leaks into the extracellular matrix. The GAGs differ in their physiological significance and even though their dominant component is disaccharide, a heterogeneity exists in the sugars when composing the different classes of GAG. For example, hyaluron (former called hyaluronic acid) is unique among the GAGs since it does not contain any sulphate and is not covalently attached to the proteins like the other proteoglycans. However, it forms non-covalently formed complexes with proteoglycans in the ECM and can displace large volumes of water in the tissue.

Collagen-like proteins in bacteria

Since the stability of mammalian collagen is based on hydroxylation of the proline into hydroxyproline and since bacteria lack the ability to form this amino acid, it was not believed that bacteria could form stable collagen-like structures. However, early in the 21st century, three different research groups showed that the bacterium *S. pyogenes* expressed collagen-like proteins called Scl1/sclA (Lukomski *et al.*, 2000, Rasmussen, Eden & Bjorck, 2000, Xu *et al.*, 2002) and Scl2/SclB (streptococcal collagen-like proteins A/1 and B/2) (Lukomski *et al.*, 2001, Rasmussen & Bjorck, 2001, Whatmore, 2001). It was established that the proteins were stable in structure and expressed during early log phase in cultures grown *in*

vitro. Furthermore, the stability of the protein was found to be due to a number of repeats present in the collagen-like domain where the important triplet G-P-O (Gly-Pro-Hyp) in mammalian collagen is replaced by the triplets KGD (Lys–Gly-Asp) and KGE (Lys–Gly-Glu). These triplets contribute to stability in the same way as the imino acids proline (P) and hydroxyproline (O) (Berisio R *et al.*, 2004). It has recently also been reported that some of these collagen-like proteins can bind to certain integrin motifs in mammalian cells, thus being involved in the bacterial-host cell interaction (Humtsoe *et al.*, 2005).

Integrins

The integrins are part of a family of adhesion receptors on the cell surface that are involved in cell-cell, cell-ECM and cell-bacterial interactions. The receptors are described as transmembrane glycoproteins consisting of two non-covalently linked subunits acting as a heterodimer. The subunits, one α- and one β-subunit can be combined in several different combinations, giving a large variety of integrins. To date, 18 α-chains and 8 β-chains together combined into 24 different heterodimers have been found in mammals (Fig. 2) (Hynes, 2000).

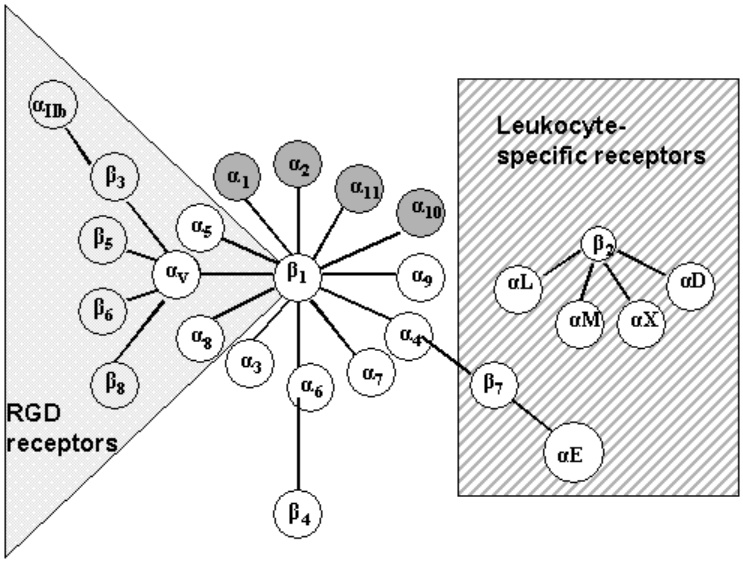

Figure 2. The integrin receptor family. The different α- and β- heterodimer combinations with indicated subfamilies and ligand-binding. The grey circles(α_1, α_2, α_{11}, α_{10},) are collagen-binding integrins together with β_1 and have I-domains. RGD-receptors are found inside the triangle and the leukocyte-specific receptors inside the striped box. Entities α_3, α_6, α_7 and α_4 are laminin-binding receptors as are $\alpha_1\beta_1$ and $\alpha_2\beta_1$. All the β_2 integrins (striped box) have I-domains. (Modified from Hynes, 2000)

Both subunits are involved in binding the ligand and the integrin structure forms a globular head in the N-terminal end and a long stalk region that is connected to the smaller intracellular C-terminal cytoplasmic bound tail. The ligand-binding region is determined to the N-terminal region of the heterodimer (Fig. 3).

The α subunits form a so-called sevenfold β-propeller where seven homologous repeats bind together in the N-terminal end, similarly to previously reported nucleotide binding pockets (Springer, 1997) (see Fig. 3). There are also other previously reported binding sites, *e.g.* binding motifs for various cations, and for four of the α-subunits a von Willebrand factor A-domain (I) is found in the propeller region. This domain is involved in direct recognition and binding of the ligand in question(Kern & Marcantonio, 1998). The propeller heads of the α-subunit show homologies to each other between the α-subunits.

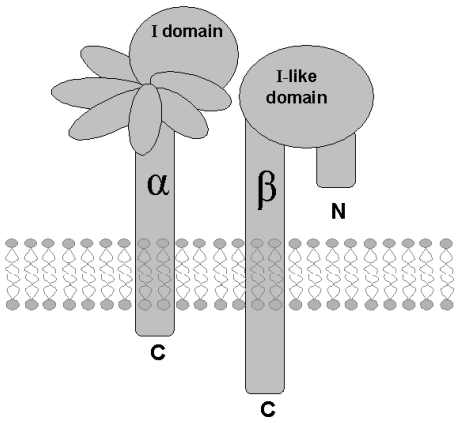

Figure 3. Schematic figure of the integrin structure with an I-domain present. The α-subunit form an β-propeller in the N-terminal where the I-domain will be located if an I-domain is present. The α- and β-chains are non-covalently linked and transmembrane.

The β-subunit has a cysteine-rich N-terminal end involved in the association with the α-subunit and is dependent on the propeller shape in order to get correct folding. The cytoplasmic tails of the β-subunits have similarities in that 6 of the 8 subunits described have a high degree of similarity in that region.

The integrins as heterodimers can be divided into several subgroups, *e.g.* the collagen-receptor subgroup where the $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$. Of this subgroup, $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$have been shown to recognise the GFOGER-motif, and other GFOGER-like motifs, found in certain collagens (Zhang *et al.*, 2003). The collagen-binding of these four integrins is not due to RGD-motif recognition,. This in contrast to the fibronectin-binding integrins $\alpha_5\beta_1$ and $\alpha_V\beta_3$ where the α_v integrins bind to the RGD-motif found in fibronectin (Nykvist *et al.*, 2001) and this motif is also present in several bacterial proteins (Isberg & Tran Van Nhieu, 1995, Nilsson *et al.*, 2004). α_V integrins could play essential roles in cell growth, survival and migration (Sheppard, 2004), since knock out mice have certain mutations with 100% mortality even though other tissues and organs develop as normal. α_V-integrins combine with five different β-subunits (β_1, β_3, β_5, $\beta6$, β_8) (Fig. 2). The $\alpha_V\beta_3$ form a heterodimer with a structure similar to a 'stopping figure' bending down when inactive, and most likely this is true for all α_V-integrins. This structure is inactive in contracted form, due to steric inhibition of binding to the binding site, and when activated the head 'opens up' and makes ligand-binding possible. $\alpha_V\beta_3$ are activated when inflammation occurs and they act as a signal receptor in order to activate the immune response (Wilder, 2002).

"One never notices what has been done, one only can only see what remains to be done"
Marie Curie

Present study

Aim

When this study was initiated the genome of subsp. *equi* had not been annotated but was available only as shorter contigs without annotation, and information about extracellular proteins in this subspecies was scarce. Therefore, to identify extracellular proteins in *S. equi* subsp. *equi*, a signal sequence phage display was used. A novel collagen-like protein called SclC was found and characterised. Using bioinformatics, this protein was defined as a member of a novel family of collagen-like proteins in subspecies *equi*. One of the aims was to investigate the collagen-like protein SclC as a vaccine component against strangles in a mouse model and finally study its biological role in host cell-bacterial interactions in a collagen lattice contraction system.

Phage display

Identification of potential virulence factors

In order to identify potential virulence factors, molecules involved in adhesion and infection have to be identified and analysed. The processes of investigating such factors include gene cloning and different strategies to express the genes in another host, usually *E. coli*. Phage display is a technique developed for expressing proteins in fusion with the phages' own coat proteins (Smith, 1985). The filamentous phage M13 has a capsid consisting of approximately 2700 copies of the major coat protein pVIII and much lower numbers of proteins pIII, pVI, pVII and pIX (Fig. 4). The phages infect *E. coli* and their progeny are secreted without killing the bacteria. When foreign DNA is inserted into one of the genes encoding a coat protein, fusion proteins are then expressed on the surface of the phage. The phage display technique was improved by Bass *et al*. (Bass, Greene & Wells, 1990) who instead of a phage used phagemid-vectors.

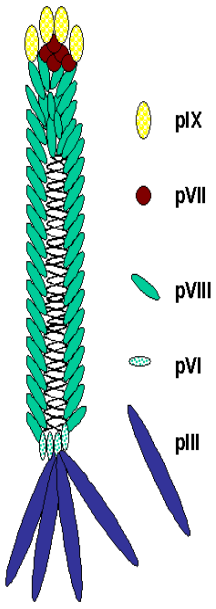

Figure 4. A schematic diagram of a phage with its coat proteins

A phagemid used for phage display is a plasmid with a phage origin of replication, the sequences required for packaging into the phage particle and a phage gene encoding a specific phage coat protein, usually pIII or pVIII. When foreign DNA with an open reading frame (ORF) is inserted into the phagemid, only one or a few fusion proteins are displayed on the surface of the phagemid particle. This technique requires a helper phage that allows all other phage proteins to co-infect the *E. coli* cells containing the phagemid in order for all other phage proteins to be expressed. This is more useful since the copy number of the expressed fusion protein decreases, thus allowing studies of weak and strong binding phages. The use of a pVIII phagemid-vector results in expression of a varying number of copies of the protein in question, compared to the pIII system where on average less than one fusion protein per phage is obtained.

In order to study interactions between bacterial proteins and ligands, shotgun phage display can be used (Jacobsson *et al.*, 2003). In shotgun phage display, the library is constructed from bacterial chromosomal DNA that is fragmented randomly and then the fragments are inserted into a phagemid vector. Theoretically this would mean that a large enough library would contain phagemid particles expressing all genes, in the genome of the organism, as polypeptides on the surface.

Signal sequence phage display

Signal sequence phage display is an application using a modified phagemid vector, pG3DSS that lacks the signal sequence required for transport of the fusion protein to the bacterial membrane, where incorporation into the phage particle occurs (Rosander *et al.*, 2002). Thus, only phages containing a vector with an insert encoding a signal sequence display a fusion protein on the phage surface. The vector also encodes an E-tag, a short peptide recognised by an antibody. By selecting the library against anti-E-tag antibodies, phages expressing a fusion protein can be isolated. Theoretically this technique allows for selection of 'all' signal sequence containing proteins encoded by a genome, secreted as well as cell membrane-anchored proteins, including adhesins, enzymes and transport proteins. The technique has previously been used in investigations of extracellular proteins in *Staphylococcus aureu*s (Rosander *et al.*, 2002), *Bradyrhizobium japonicum* (Rosander *et al.*, 2003) and *Lactobacillus reuteri* (Wall *et al.*, 2003).

*Panning of the signal sequence library **(I)***

A signal sequence phage library was made from genomic DNA from subsp. *equi* strain 1866, which was fragmented by sonication and then ligated into the pG3DSS vector lacking a signal sequence. Primarily the library was panned against anti-E-tag antibodies in order to identify fusion proteins with the E-tag peptide and then inserts of the clones were sequenced and investigated. The panning was done in microwells for two consecutive cycles to enrich the E-tag expressing clones (Fig. 5).

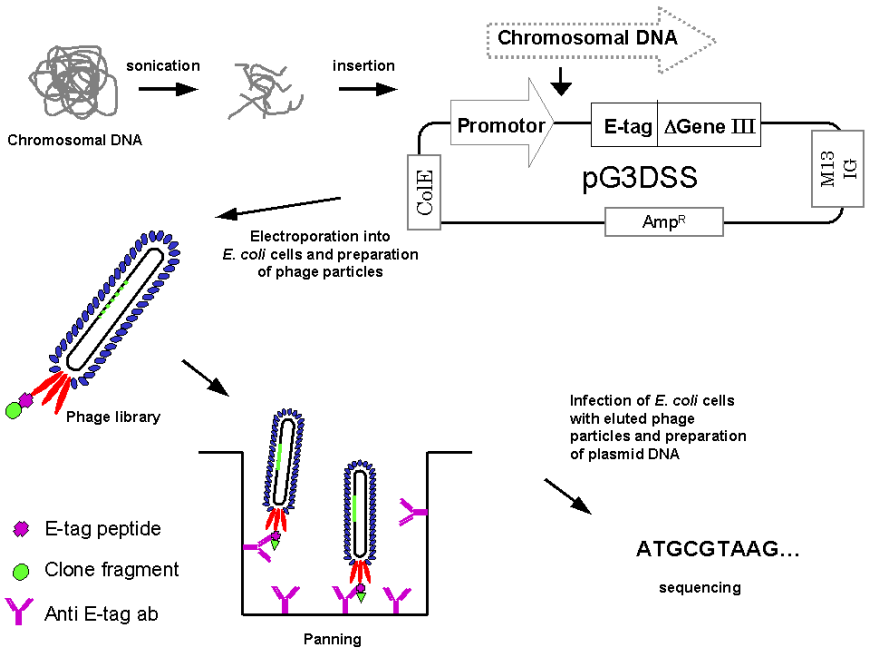

Figure 5. Construction and panning of the signal sequence phage display library. The DNA is fragmented and inserted into the vector pG3DSS. Fragments with an open reading frame are expressed in fusion with the E-tag peptide and the partial gene III protein. Panning of the library is done in microwells coated with anti E-tag antibodies. E. coli cells are infected with the eluted phagemid particles for DNA isolation, and sequencing of the foreign DNA.

Sequencing 120 clones from the first and second pannings revealed 20 new signal sequences found in 48 unique clones. The ORFs investigated showed homologies to previously described putative proteins in *S. pyogenes, S. agalactiae* and *S. dysgalactiae* (Table 1 in **I**). Notably there was a high frequency of ABC transporters among the identified putative proteins, as well as a number of homologues to predicted conserved hypothetical proteins.

The library was also panned against α_2-M as well as against horse serum. Of the clones investigated, no novel proteins with affinity for either of the ligands could be detected. This is most likely due to the size of the DNA fragments used for construction of the library (~0.3 - 3 kb with the majority of the fragments shorter than 1 kb). In order to be expressed and to bind to the ligand, the clones have to encode both the signal sequences and the binding regions, which would require fragments larger than this. Panning of a pVIII shotgun phage display library from subsp. *equi* against other ligands has resulted in findings of proteins interactive with ligands (Lindmark & Guss, 1999), supporting the previous statement.

SclC (I)

Characterisation of the collagen-like protein

One of the clones isolated from the pannings of the signal sequence phage display library harboured an ORF encoding the N-terminal part of a collagen-like protein called SclC. The protein had similarities to the previously described SclB in *S. pyogenes* (Whatmore, 2001). The complete SclC protein is approximately 30 kDa in size and consists of an N-terminal signal sequence followed by an A-region with no known homologies, a collagen-like (CL) region followed by a proline-rich wall-associated (W) region ending with an LPXTG-motif that proceeds the C-terminal membrane anchoring domain (M) (Fig. 6). This, combined with the fact that in *S. pyogenes* the collagen-like proteins have been reported to display a high degree of polymorphism in several of the serotypes investigated, made the protein in subsp. *equi* interesting to study further.

The collagen-like region (CL) is similar to the previously reported collagen-like proteins from bacteria (Lukomski *et al.*, 2000, Rasmussen, Eden & Bjorck, 2000, Lukomski *et al.*, 2001, Rasmussen & Bjorck, 2001, Whatmore, 2001, Xu *et al.*, 2002) in the aspect of G-X-Y repeats and ending in a proline-rich wall spanning part. The G-X-Y repeats are not identical in the species *S. pyogenes* and subsp. *equi* but the similarity of the organisation of the proteins indicates that they are related. SclC was found to have most similarities to the SclB protein of *S. pyogenes*. The proteins do not have exactly the same amino acid sequence yet a similarity can be observed. The total content of KGD/KGE repeats in the collagen-like region is also consistent between the proteins (Fig. 2 in **I**), indicating similarity. Comparing the various collagen-like proteins found in *S. pyogenes*, the same observation can be drawn. The different collagen-like proteins vary depending on which serotype and which M-protein is expressed in the strain. SclC also differ from previously described collagen-like proteins since there are no repetitive regions within the CL-region of the protein, which has been found in the collagen-like proteins found in *S. pyogenes* (Lukomski *et al.*, 2001, Rasmussen & Bjorck, 2001, Whatmore, 2001, Xu *et al.*, 2002). Furthermore, SclC lacks the linker region PKSAPS found in Scl1 in *S. pyogenes* (Lukomski *et al.*, 2000).

Protein production and binding studies

To study whether the SclC protein displays binding to components in plasma or the extracellular matrix, different binding experiments were performed. P24, the full-length mature protein, was expressed in *E. coli* using the IMPACT^{TM} system from New England Biolabs. This system uses a vector that produces a three-part fusion protein with a protein splicing element called intein, which when DTT is added uses self cleavage to produce an end product of a purified recombinant protein. Binding investigations were performed in microwells with different immobilised proteins (fibronectin, fibrinogen, hyaluronan, collagen I and CNE) as well as horse sera and plasma using ^{125}I-labelled P24. The ligands were coated in microwells to which the ^{125}I-labelled P24 was added. No binding could be detected to any of the ligands tested.

To further study whether SclC displays binding to component(s) that occur in low concentrations in horse plasma, the P24 protein was immobilised on an NHS-activated HiTrap column (Amersham Biosciences). Plasma was passed over the column and after washing the bound material was eluted by lowering the pH. The eluted samples were subjected to native PAGE and Western blotting. The result showed that one of the fractions contained protein fragments, which in Western blot reacted with ^{125}I-labelled P24. This fraction was then separated on a 2D-polyacrylamide gel and the spots obtained were sequenced and analysed (data not shown). However, they turned out to be the heavy and light chains of IgG and IgM molecules. This was not surprising considering the large amount of plasma passed over the column, since plasma from horses, both those previously infected with subsp. *equi* and horses with no history of infection, contain detectable levels of antibodies against the protein (see Fig. 3 and Fig. 4 in **I**). Thus, passing plasma over immobilised P24 would result in purification of these antibodies. In addition, subsp. *zooepidemicus* is likely to express SclC. This subspecies is part of the normal flora in horses and accordingly, the immune system has probably developed antibodies against the protein. However, the antibody titres against SclC are significantly higher in horses previously infected with subsp. *equi*, supporting the theory that even though the protein might be expressed in subsp. *zooepidemicus*, infected horses are exposed to the protein to a much higher degree (**I**).

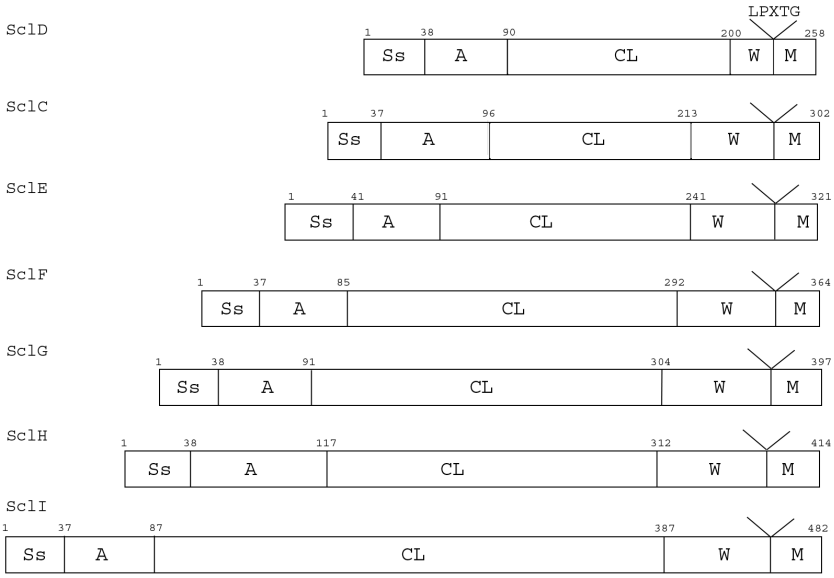

Figure 6. The collagen-like proteins found in S. equi *subsp.* equi. *Ss; signal sequence, A; the variable region, CL; the collagen-like region, W; proline rich wall spanning region, M; membrane spanning region, and the LPXTG-motif in between the two most C-terminal regions.*

SclD-SclI (II)

The family of collagen-like proteins

When further analysing the genome database found at Sanger.co.uk, six novel open reading frames similar to the SclC were discovered. The novel sequences showed homologies in both the nucleotide sequences and in the deduced amino acid sequences. The six deduced amino acid sequences showed similarities in organisation with an N-terminal signal sequence, a variable region (A) followed by a collagen-like region (CL) expressing Gly-X-Y repeats and a proline-rich region ending with an cell wall-anchoring LPXTG motif which precedes the C-terminal membrane-anchored domain (Fig. 6). Although the proteins varied in size, the total content of glycine varied between 17.2% and 22.6 % (Table 3).

Table 3. Amino acid content in the collagen-like proteins SclC – SclI. Mean values and standard deviation are shown as percentages and in percentage units. The proteins with the highest and lowest content are shown in the right-hand column.

Amino acid	Mean value	SD ±	Least	Most
A (Ala)	8.4%	0.028	SclG	SclC
R (Arg)	5.1%	0.025	SclD	SclG
D (Asp)	4.0%	0.017	SclG	SclC
Q (Gln)	8.8%	0.027	SclC	SclH
E (Glu)	9.7%	0.026	SclH	SclG
G (Gly)	19.8%	0.020	SclC	SclI
K (Lys)	11%	0.020	SclH	SclD
P (Pro)	10%	0.031	SclD	SclH

A phylogenetic tree of the nucleotide sequences shows that the three shortest sequences cluster closest together (Fig 1. in **II**). Four of the proteins (SclD, SclE, SclG and SclI) also contain a repetitive sequence GEKGD/EQGQR/K, whereas the remaining three do not. Comparisons between the KGD/KGE triplets that have been reported to stabilise the collagen-like proteins found in bacteria show that the collagen-like proteins found in subsp. *equi* contain more of these triplets than the proteins from *S. pyogenes* and collagen I (Table 4). This is true for all proteins except SclH, which clusters furthest away from the other proteins in subsp. *equi* (Fig. 1 in **II**). The SclD protein with the highest amount of KGD/E triplets has the lowest amount of proline, supporting the previous analysis of differences in mammalian collagen and prokaryote collagen-like structures (Persikov, Ramshaw & Brodsky, 2005).

In *S. pyogenes,* two types of collagen-like proteins, Scl1/A and Scl2/B, have been reported and investigated (Lukomski *et al.*, 2000, Rasmussen & Bjorck, 2001, Whatmore, 2001, Akesson *et al.*, 2004). The various serotypes of *S. pyogenes* express different subtypes of M-like proteins and also different subtypes of the two collagen-like proteins (Rasmussen, Jacobsson & Bjorck, 2003). Since these proteins are found to vary, it has been concluded that there is a serotype variation in the collagen-like proteins, since there are only two proteins present in each strain/serotype. In contrast, subsp. *equi* seems to have seven collagen-like

proteins since all of these are found in both strain 1866 and in the different strain sequenced and presented at Sanger.co.uk .

Table 4. Content of imino acids and KGD/E triplets in the collagen-like region of the proteins. Proline is found in the second and/or third positions of the GXY-triplets in the CL-region

	Imino acids		KGE + KGD	
Protein	**Total #**	**% of X + Y**	**Total #**	**% of triplets**
SclC	16	22	6 + 7	35
SclD	7	7.8	11 + 7	40
SclE	10	10	13 + 1	28
SclF	30	22	7 + 12	28
SclG	16	11	16 + 2	25
SclH	39	30	0 + 10	15
SclI	30	15	14 + 11	25
Scl1*	15	15	6 + 4	20
col1A1**	236	35	6 + 6	3.6

**Streptococcal protein Scl1 (Persikov et al., 2005)*
***Mammalian collagen type 1 (Persikov et al., 2005)*

The Scl proteins Scl1/A and Scl2/B in *S. pyogenes* have previously been reported to be able to form a collagen-like triple-helical structure (Rasmussen, Eden & Bjorck, 2000, Xu *et al.*, 2002). Using electron microscopy, the authors demonstrated that the Scl proteins are organised into 'lollipop'-structures similar to those seen in human proteins containing collagenous domains. Whether the Scl proteins in subsp. *equi* can also form this type of structure remains to be determined but analysis of putative protein structure (including tests such as Hopp/Woods hydrophobicity and von Heijne transmembrane) using the software Macvector 8.02 (Accelrys, Madison, WI) indicates that it is likely that SclC - SclI can also form this type of structure.

Antibodies against SclC recognise SclD - SclI

The six novel collagen-like proteins SclD- SclI were expressed, both as mature proteins and as only the A-regions, to investigate whether they are recognised by antibodies against SclC. Microwells were coated with the mature proteins and the A-regions, respectively and the binding of antibodies against SclC was tested using ELISA. Only the mature proteins containing the CL regions were recognised by the antibodies (Fig. 2 in **II**). This indicates that the previous results in the investigation of horse sera and recognition of SclC (**I**) could be the result of recognition of antibodies against all the collagen-like proteins in subsp. *equi* rather than the SclC protein alone. Thus all CL-regions are recognised by purified antibodies against SclC and the antibodies used are not only directed to the unique A-region of SclC.

In order to establish whether horses previously infected with subsp. *equi* had antibodies recognising all collagen-like proteins, an ELISA with only the A-regions from each protein was performed. This was intended to avoid detection of

antibody binding to the similar CL-region in all seven proteins. The results showed that all sera from horses previously infected by subsp. *equi* contained antibodies against all seven A-regions, but in varying amounts. The results indicate that the proteins are expressed during subsp. *equi* infection. This was further confirmed when horse sera from both infected and uninfected horses was investigated against the A-region from SclC, where there was a significantly higher antibody titre in horses previously infected with subsp. *equi* (Fig. 4 in **III**). The cross-reaction between the antibodies against SclC and the other six proteins, SclD – SclI, cannot account for all the results in the previous investigation of horse sera against SclC (in **I**) since the results from the ELISA investigation of each A-region had a variation in amounts of antibodies within each serum. However, to measure antibody response against an individual Scl protein it has become obvious that antibodies that only react with the respective A-regions are needed.

The antibodies that recognise the CL-region in SclC in **I** are most likely a mixture of antibodies that can react with all seven collagen-like proteins. This raises the question whether an immunisation with SclC gives antibodies directed against the collagen-like region, which could then react with horse collagen. Immunisation of rabbits with SclC gave antibodies recognising the A-regions, CL-regions and mature protein (**II**), showing that antibodies are directed to different regions of the protein and indicating that this might not be a main concern. However, unspecific recognition of horse collagen by the antibodies needs to be further investigated for certainty.

Expression under different conditions

To investigate whether the collagen-like proteins are expressed *in vitro,* subspecies *equi* strain 1866 was grown in Todd Hewitt Broth supplemented with 0.6 % yeast extract (THB) combined with additional supplement of 10 % horse sera under aerobic, anaerobic and microaerobic (10 % CO_2) conditions. After harvest, the intact bacterial cells were dot blotted onto nitrocellulose (NC)-filters. The supernatant from lysed cells, separated through centrifugation, was run on a SDS-PAGE gel and then blotted onto NC filters. All the NC-filters were treated with human IgG in order to block binding to the EAG protein, which binds the Fc-part of antibodies. Affinity purified anti SclC rabbit antibodies were incubated with the pre-blocked filters. It was found that the bacterial cells grown under microaerobic and anaerobic conditions showed positive reactions, indicating that the collagen-like proteins were expressed under these conditions (data not shown). Similar results were obtained when the lysates from the anaerobic and microaerobic conditions were tested. In the addition, when the 10 % horse sera was added to the THB medium, the antibody binding was found to be stronger. These unpublished data are preliminary, and in the future it is suggested that another method like Real Time PCR should be used to study the expression of the *scl*-genes. Real Time PCR using primers specific for the specific proteins would probably give a more clear result. Still, the preliminary results suggest that the proteins could be expressed during less aerobic conditions.

Using SclC in a vaccination study in a strangles model in mice (III)

Immunity and vaccines

It has previously been shown that horses that have suffered from strangles and then recovered are protected from a re-infection with subsp. *equi* for at least three months (Sweeney *et al.*, 2005). This indicates that formation of protective antibodies has occurred and that it should therefore be possible to vaccinate against the disease if the right components are used as antigens. It has also been debated whether intranasal vaccination would result in IgA antibodies rather than the IgG antibodies that are more often produced during subcutaneous vaccination. When infection occurs in the mucosal layer in the nose of the horse, it is likely that IgA antibodies provide better protection than the IgG antibodies usually occurring in the bloodstream. Meehan *et al.* (Meehan, Nowlan & Owen, 1998) showed that M-like protein complexes gave protection in mice against lethal challenge with subsp. *equi*. However, when the M-protein was used as an antigen against a challenge with subsp. *equi* in horses, the protection was small and not useful (Sheoran, Artiushin & Timoney, 2002).

Adjuvants are important in order to enhance the immune response, *i.e.* production of antibodies, to a particular antigen. Therefore the antigen is mixed with an adjuvant when introduced to the immune system, *i.e.* by vaccination. It is important that the adjuvant in itself is not toxic. Different adjuvants can be used to stimulate production of different antibody classes. The most powerful mucosal adjuvants today are cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxin (Rappuoli *et al.*, 1999). These toxins occur naturally in the diseases cholera and traveller's diarrhoea, respectively. However, they are high in toxicity and not suitable in their native form for vaccination studies and vaccine development. A less toxic adjuvant that still elicits a good antibody response is the non-toxic recombinant B-subunit of the *E. coli* enterotoxin (EtxB), which is immunogenic by both parental and mucosal routes and elicits a strong antibody response when administered mucosally (Millar, Hirst & Snider, 2001).

Another type of adjuvant is the ISCOM (immune stimulating complex), which is a particle with a cage-like structure, typically 40-100 nm in diameter, comprised of cholesterol, phospholipid and *Quillaia* saponin (Morein *et al.*, 1987). The ISCOM-matrix is formed when the antigen is mixed with the ISCOM, sometimes leaving the antigen inside the ISCOM-particle. When the ISCOM is administrated mucosally a strong IgA response is induced, both locally and in remote mucosal areas (Morein, Hu & Abusugra, 2004). Several ISCOM-based vaccines are under development and one vaccine against equine influenza (EQUIP F) is currently on the market (Crouch *et al.*, 2005).

Immunisations and challenge procedure in a mouse model

A mouse model has previously been used in testing whether other recombinant proteins (SFS, EAG and FNZ of *S. equi*) could be used as antigens in a vaccine against subsp. *equi* infections (Flock *et al.*, 2004). Combinations of these proteins were also used in horses to investigate whether they could give antibodies

recognising these antigens. The results showed that vaccination in mice had a protective effect and that horses responded to the introduced antigens. It was therefore thought to be interesting to investigate whether vaccination in the mouse model using SclC also resulted in a protective effect.

The mice were immunised intranasally (i.n.) with SclC, followed by booster vaccinations on days 7, 14 and 21. The challenge, when bacteria of subsp. *equi* were introduced into the nose of the animals, took place 28 days after the first vaccination. The animals were then monitored for weight change and growth of subsp. *equi* in the nose in order to determine the level of protection.

The mice immunised were found to have significantly less weight change than the non-immunised animals (Fig. 7), as well as having less colonisation of bacteria in the nose tract (Fig. 6 in **III**). In addition, the antibody levels were inversely correlated to the weight change (r-values = 0.62) *i.e.* the weight loss was less in animals with higher antibody levels, indicating that it was the antibodies against SclC that were responsible for the protection.

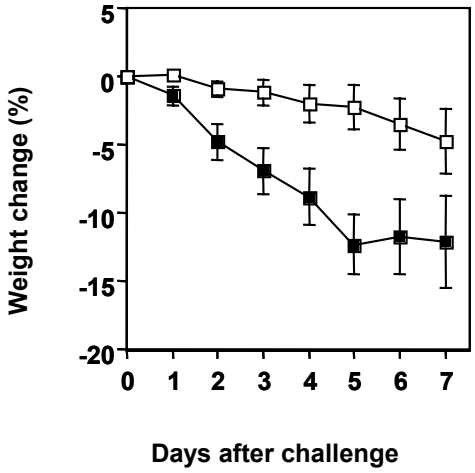

Figure 7. Weight change (%) in mice immunized with SclC. Mice (n=15) (open squares) were immunized with SclC and control mice (n=15) (closed squares) with adjuvant only, followed by infection with subsp. equi. *Mean values of weight change ± SE are shown.*

In another experiment mice were vaccinated with CNE and EAG, one at the time and and in combination. The results of these immunisations turned out to be positive in aspect of degree of protection. The combination of CNE and EAG was more efficient than CNE alone. Interestingly, vaccination with both the collagen-binding protein CNE and the collagen-like protein SclC gave protection. However, the fibronectin-binding protein FNEB that also was tested as an antigen did not result in any protection. The conclusion from these experiments was that when continuing with the immunisation studies in horses, a vaccine that combines the proteins SclC, CNE and EAG would be interesting for further studies.

The effect of recombinant protein from *S. equi* on collagen lattice contraction (IV)

Studies of the recombinant protein FNZN

Mesenchymal cells, *e.g.* fibroblasts or smooth muscle cells, that are cultured in a three-dimensional collagen lattice contract the lattice typically within 24 hours and this process is referred to as cell-mediated collagen gel contraction. Cells from the murine myoblast cell line C2C12 mediate collagen lattice contraction after stimulation with platelet-derived growth factor (PDGF). These cells lack expression of collagen-binding β_1 integrins (*e.g.* $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$ and $\alpha 1_1\beta_1$) and use the $\alpha_V\beta_3$ integrin for collagen gel contraction (Lidén *et al.* Manuscript in preparation). Several different extracellular proteins from *S. equi* were tested as to their ability to modulate collagen gel contraction mediated by C2C12 cells and by a cell line, (C2C12-α2) in which the collagen-binding β_1 integrin $\alpha_2\beta_1$ has been reintroduced by transfecting the cells Since the C2C12 does not express collagen-binding β_1 integrins, contraction of these cells could be due to activation of the $\alpha_V\beta_3$ integrin and the investigation of this can further studied when by adding a specific inhibitor of $\alpha_V\beta_3$.

The recombinant proteins tested were the fibronectin-binding proteins FNZ, SFS and FNEB, the collagen-binding protein CNE and collagen-like protein SclC (Fig. 1 in **IV**). FNZ is called FNZN in this study since only the N-terminal part of the protein is present in the recombinant protein, thus lacks the C-terminal fibronectin-binding repeats. The only protein that had an effect on contraction was the protein FNZN. None of the other recombinant proteins had any clear effect on collagen gel contraction mediated by the two different cell lines. In the cell line C2C12, FNZN was found to enhance the contraction, which would not take place unless PDFG is added. In the transfected cell line C2C12-α2, where the cells contract by themselves, this effect could not be observed. Furthermore, the activation of the contraction could be blocked by adding specific $\alpha_V\beta_3$ inhibitor, indicating that FNZN somehow works by stimulating the $\alpha_V\beta_3$ integrin, which leads to contraction in the gel.

To investigate whether the FNZN protein could bind directly to cells, *i.e.* through binding to $\alpha_V\beta_3$, and increase the binding of cells to different ligands, an interaction study of the two types of cell lines with three extracellular matrix (ECM) ligands were performed. These ECM ligands were; fibronectin, collagen type I and denatured collagen type I. The ECM proteins were immobilised in plastic dishes, the myoblast cells pre-incubated with FNZN and then added into the plastic dishes. The result showed that FNZN did inhibit the adhesion of the C2C12 cells to denatured collagen I but not to the other ligands tested.

In order to establish weather FNZN could bind directly to the cells, FNZN was labelled with ^{125}Iodine and incubated with suspended C2C12 cells to see if any such binding could be detected. As shown in Fig. 6 in **IV** FNZN binds to the cells. Since the myoblast cells bind denatured collagen, FNZN was further studied in terms of binding to fibronectin, native collagen and denatured collagen.

Microwells were coated with the ligands and ^{125}I-labelled FNZ was added. The results shows that FNZN binds to both fibronectin and native collagen I (Fig. 4 in **IV**). Since FNZN binds to the 40k region of the fibronectin molecule (Lannergard *et al.*, 2005), a region also responsible for the binding to collagen, it would be interesting to further investigate whether FNZN can inhibit binding of fibronectin to collagen.

In conclusion the effect of FNZN to the collagen lattice contraction suggest that the FNZN protein stimulate contraction of C2C12 cells and do this via the $\alpha_V\beta_3$ integrin. This intergrin is involved in the reactions against infection and inflammation and counteracts edema formation in tissue (Lidén *et al.*, submitted). Gram-negative bacteria can cause edema due to release of lipopolysaccharides (LPS) and their stimulation of tissue macropahges to produce interleukin-1 in the connective tissue structures surrounding the capillary beds (Nedrebo & Reed, 2002, Nedrebo *et al.*, 2004). The vascular support system breaks down and fluid leaks into the tissue, thus creating edema in which the tissue can actually be said to collapse. In that the $\alpha_V\beta_3$ integrin can counteract the effect. The recombinant protein FNZN from Gram-positive *S. equi* seems to have the opposite effect in the cell system used.

Contraction studies of SclC

Humotse *et al.* (Humtsoe *et al.*, 2005) have reported that certain motifs of the bacterial collagen-like proteins are recognised by, and react with, mammalian collagen-binding integrins. These motifs are party described as GLOGER, but there also seem to be some unknown motifs that bind as well. To establish whether SclC has an unknown motif reacting with mammalian collagen, it was tested in the collagen lattice contraction with C2C12 and C2C12-α2 cells.

The results indicated that there was no reaction when SclC was added, which is consistent with previously reported data, since this protein does not have any of the reported binding sites to the collagen-binding integrins. This also indicates that SclC does not have any of the unrecognised motifs previously reported. The remaining six collagen-like proteins from subsp. *equi* should also be tested to further investigate their role in the bacterial-cell interaction.

Concluding remarks

This thesis describes seven novel collagen-like proteins found in a pathogenic streptococcal strain and investigates the possible biological role of one of them in particular. When studying extracellular proteins in the obligate pathogen *S. equi* subsp. *equi*, signal phage display was shown to be a powerful tool in identifying potential proteins harbouring a signal sequence. Furthermore, by combining this technique with bioinformatic tools, a novel family of cell surface-anchored proteins was identified. This family of collagen-like proteins consists of seven proteins, all sharing the same features. These proteins differ from collagen structures found in mammals since they do not contain hydroxyproline. The possibility that these proteins can form stable triple helices has not been tested. Although, earlier reports on similar proteins in *S. pyogenes* have shown that these proteins can form triple helices, which indicate that also the collagen-like proteins of subsp. *equi* can form this type of structures.

The biological role of these proteins remains to be clarified. SclC was tested in a collagen lattice contraction study using mouse cells to see whether the protein had any effect on the contraction of the gels, which was found not to be the case.

However, using sera from horses diagnosed with strangles it could be concluded that these proteins are most likely expressed during an infection of subsp. *equi*. Antibodies raised in a rabbit against one of the proteins, *i.e.* SclC, are cross-reactive with the collagen-like region of the remaining six proteins, indicating that the antibodies formed after immunisations could be reactive against all collagen-like proteins found in subsp. *equi*. This finding will be important if immunisation with SclC is protective in horses, since only one protein is needed in order to get antibodies reacting with all the collagen-like proteins from this subspecies.

Immunisations with SclC in a strangles model in the mouse gave protection as measured by less weight loss and lower bacteria counts in vaccinated animals. Combining this protein with the other recombinant proteins (EAG and CNE) found to have a positive effect in the vaccination study would be interesting. This since these two recombinant proteins were found to enhance the rate of protection in combination with each other.

In fact, the SclC protein has been introduced as a vaccine component in a horse vaccination trial including challenge. The results from this vaccination have not yet been fully evaluated, but the overall outcome looks promising. Consequently, further studies of the collagen-like proteins expressed by subsp. *equi* are needed in order to clarify their biological role and importance in virulence of this pathogenic bacterium.

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Acknowledgement

The work in this thesis was supported by FORMAS, Carl Trygger's foundation and the Swedish Horse Board.

I would like to thank the people who have contributed to this thesis in different ways.

My supervisor Professor **Bengt Guss** for accepting me as an undergraduate student and introducing me to this fascinating bacterial species and later for believing in the project of collagen-like proteins. I really will remember those times in the lab talking about 'bugs' and I will always try to 'simma lugnt', even during more hectic times.

My assistant supervisor Associate Professor **Karin Jacobsson** for giving excellent comments on research plans and writing as well as being so organised and efficient in every aspect. I'll think about 'as well as'… and the summer BBQs!

My former assistant supervisor Associate Professor **Lars Frykberg** for having a vast knowledge in research from the smallest virus particles to seeing the larger scale of things, and of course for teaching the craft of 'boule'.

My co-authors **Margareta Flock** (all those mice…) and Professor **Jan-Ingmar Flock** for the interesting discussions on immunity and vaccine development.

My co-authors **Åsa Lidén** (the patience and efforts you made!)**, Lena Persson, Cecilia Rydén** and Professor **Kristoffer Rubin**, for patiently explaining the world of integrins & cell systems and for a collaboration that gave a new angle of looking at bacterial proteins.

NordVacc läkemedel for horse sera and plasma samples. Especially **Rune Bergman** and **Kenneth Janzon** for bringing me to England with you. I will never look at a head of a horse again without interesting pictures on my mind ;)

Present and former members of the adhesion group; my room-mate and co-author **Jonas Lannergård** for giving me insights in the world of fibronectin-binding proteins. **Christer, Nora, Martin, Anna, Joakim** and **Azar** for welcoming me into the group early on and making me feel like home. Not to mention all the talks about staphylococci and their proteins. It has not been the same since you left!

Also I would like to express gratitude to late Professor emeritus **Martin Lindberg** who was the first professor to welcome and introduce a lost and confused undergraduate student to university and research.

Some members of the Department have had more impact on me than others,
Klara for being a dear friend and always laughing with me even when things aren't looking good. I'll always remember Arvika, where coffee never smelled better…
Torun, we really have spent lots of nights together at the Department writing, talking and thinking. Now we are free to spend quality time outside of here!

The girls **Ingela, Karin E, Karin N, Klara, Lotta J, Mia and Torun** for the wonderful dinners and drink nights where we discussed everything…

All members, past and present, of the Department of Microbiology that I have met and gotten to know, who helped making this place what it is, especially some of the fikapeople, **Anders**, **Cecilia, Helena, Jennifer, Jesper, Katrin, Kristin** (die kleine Zeitungsente) **Lisa, Lotta L, Magnus**, **Petter, Ullis** for the chats about lots of stuff... Also thank you **Ingemar, Anki** and **Barbro** for technical support and assistance.

My PUMA-mentor **Harriet Hedin** for encouragement, sharing of life wisdom and just being an interesting person to know.

My dear friends **Johanna Paléus, Åsa Nilsson**, **Susanne Paul**, **Agneta "Nene" Ormes** & **Emma Eriksson** for all your support and the never ending fun times we have spent together; from Midsummer's Eve parties to Canada, weddings and life 'planning'. I am grateful to have you as friends!

Also **Joakim Blomkvist** and the rest of 'syntgänget' for some of the best times of my life, including dinners, playing games and "clubbing".

All my other friends who I enjoy spending time with are greatly thanked for letting me know there is a life outside the lab. Including you I've gotten to know during those dark Sunday evenings - it (I) would not have been the same without those times. A special notice goes to **Gabrielle, Thea** and **Vilhelm** for showing me the new world.

My family, my brother **Per** & **Maria** and **William** for Sunday dinners, crayfish parties and being "the Big Brother" at all times.

My mother and father, **Lena & Christer**, for always believing in my ideas and me as a person. Also the fact that you have supported me all times, through the phone and through trips to London, Florence and Canada. "Har du bara pass & pengar så ordnar det sig alltid!"

And **Dan,** because you exist in my life and want to share it with me. You have really put up with lots of things these last months. Jag älskar dig!