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Identification and characterisation of a novel adhesin of *Streptococcus suis* and its use as a target of adhesion inhibition and bacterial detection

by

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ABSTRACT

Streptococcus suis is an important pig pathogen but it is also zoonotic, i.e. capable of causing diseases in humans. Human *S. suis* infections are quite uncommon but potentially life-threatening and the pathogen is an emerging public health concern. This Gram-positive bacterium possesses a galabiose-specific (Galα1–4Gal) adhesion activity, which has been studied for over 20 years. P-fimbriated *Escherichia coli*–bacteria also possess a similar adhesin activity targeting the same disaccharide.

The galabiose-specific adhesin of *S. suis* was identified by an affinity proteomics method. No function of the protein identified was formerly known and it was designated streptococcal adhesin P (SadP). The peptide sequence of SadP contains an LPXTG-motif and the protein was proven to be cell wall–anchored. SadP may be multimeric since in SDS-PAGE gel it formed a protein ladder starting from about 200 kDa. The identification was confirmed by producing knockout strains lacking functional adhesin, which had lost their ability to bind to galabiose. The adhesin gene was cloned in a bacterial expression host and properties of the recombinant adhesin were studied. The galabiose-binding properties of the recombinant protein were found to be consistent with previous results obtained studying whole bacterial cells. A live-bacteria application of surface plasmon resonance was set up, and various carbohydrate inhibitors of the galabiose-specific adhesins were studied with this assay. The potencies of the inhibitors were highly dependent on multivalency. Compared with P-fimbriated *E. coli*, lower concentrations of galabiose derivatives were needed to inhibit the adhesion of *S. suis*. Multivalent inhibitors of *S. suis* adhesion were found to be effective at low nanomolar concentrations. To specifically detect galabiose adhesin–expressing *S. suis* bacteria, a technique utilising magnetic glycoparticles and an ATP bioluminescence bacterial detection system was also developed.

The identification and characterisation of the SadP adhesin give valuable information on the adhesion mechanisms of *S. suis*, and the results of this study may be helpful for the development of novel inhibitors and specific detection methods of this pathogen.

Key words: anti-adhesion therapy, bacterial adhesion, galabiose, glycoparticle, *Streptococcus suis,* surface plasmon resonance

Annika Kouki

***Streptococcus suis* -bakteerin uuden adhesiinin tunnistaminen ja karakterisointi sekä käyttö adheesioinhibition ja bakteeridetektion kohteena**

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TIIVISTELMÄ

Streptococcus suis on tärkeä sikojen taudinaiheuttaja, mutta sillä on myös kyky aiheuttaa zoonooseja eli ihmisiin tarttuvia eläintauteja. Ihmisillä tämän Gram-positiivisen bakteerin aiheuttamat taudit ovat kuitenkin harvinaisia, joskin ne voivat olla henkeä uhkaavia. *S. suis* -bakteerin on havaittu tunnistavan spesifisesti galabioosi-sokerirakennetta (Galα1–4Gal), johon myös P-fimbriallisen *Escherichia coli* -bakteerin tiedetään sitoutuvan adheesiossa.

Väitöskirjatyössä tämä galabioosi-spesifinen adhesiini, jonka ominaisuuksia on tutkittu jo yli 20 vuotta, tunnistettiin affiniteettiproteomiikan avulla. Tunnistetulle proteiinille ei ennen tätä tutkimusta ollut tiedossa yhtään tehtävää, ja se nimettiin streptokokin adhesiini P:ksi (SadP). Adhesiinin aminohapposekvenssissä on LPXTG-motiivi (motif) ja sen osoitettiinkin olevan soluseinäproteiini. SadP saattaa olla multimeerinen proteiini, koska SDS-PAGE -analyysissä se muodosti tikapuu-kuvion, joka alkoi noin 200 kDa:n kohdalta. Adhesiinin tunnistaminen todennettiin valmistamalla poistogeenisiä bakteerikantoja, jotka eivät tuottaneet toimivaa adhesiinia eivätkä sitoutuneet galabioosiin. Adhesiinigeeni kloonattiin tuottokantaan ja rekombinanttiproteiinin sitoutumisominaisuuksien havaittiin olevan samankaltaisia kokonaisten bakteereiden sitoutumisominaisuuksien kanssa. Galabioosia tunnistavien adhesiinien inhibiittoreiden tutkimiseksi kehitettiin pintaplasmoniresonanssia hyödyntävä sovellutus, joka mittasi elävien bakteereiden adheesiota. Inhibiittoreiden multivalenttisuus lisäsi huomattavasti inhibitiotehokkuutta. *E. coli* -bakteereiden adheesion inhibitioon verrattuna pienempiä pitoisuuksia galabioosiyhdisteitä tarvittiin inhiboimaan *S. suis* -bakteereiden sitoutumista. Multivalenttiset inbibiittorit estivät *S. suis* -bakteereiden adheesiota jo käytettäessä nanomolaarisia konsentraatioita. Galabioosiin perustuvan adheesion mittaamista varten kehitettiin menetelmä, jonka avulla voitiin spesifisesti tunnistaa adhesiinia ilmentävät bakteerit. Menetelmä hyödynsi magneettisia glykopartikkeleita, joiden sitomien bakteereiden määrä selvitettiin ATP-bioluminesenssi -menetelmällä.

SadP-proteiinin tunnistaminen ja sen ominaisuuksien tutkiminen tuottivat lisätietoa *S. suis* -bakteerin adheesiomekanismeista. Väitöstutkimuksen tulokset saattavat olla hyödyllisiä myös uudenlaisten antibakteeristen lääkeaineiden sekä taudinaiheuttajien spesifisten detektiomenetelmien kehitystyössä.

Avainsanat: antiadheesioteriapia, bakteeriadheesio, galabioosi, glykopartikkeli, pintaplasmoni-resonanssi, *Streptococcus suis*

CONTENTS

ABBREVIATIONS

3-pgk	3-phosphoglycerate kinase
BSA	bovine serum albumin
Cer	ceramide
Cfu	colony forming units
CSF	cerebrospinal fluid
Dpr	Dps-like peroxide resistance protein
Ef-Tu	elongation factor Tu
ELISA	enzyme-linked immunosorbent assay
Gal	galactose
Galα1–4Gal	galabiose, galactosyl-α1–4-galactose
GalNAc	*N*-acetylgalactosamine
GBL	glucan-binding lectin
GbO_3	globotriaosylceramide (also known as CD77 antigen) (Galα1–4Galβ1–4Glcβ1–1'Cer)
GbO_4	globoside (GalNAcβ1–3Galα1–4Galβ1–4Glcβ1–1'Cer)
GbO_5	Forssman antigen (GalNAcα1–3GalNAcβ1–3Galα1–4Galβ1–4Glcβ1–1'Cer)
GBP	glucan-binding protein
Glc	glucose
GlcNAc	*N*-acetylglucosamine
HA	haemagglutination
Hsa	haemagglutinating streptococcal antigen
IC_{50}	the concentration which causes half-maximal inhibition
IF-2	translation initiation factor 2
Kan^r	contains kanamycin resistance gene
MIC	minimal inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
MurNAc	*N*-acetylmuramic acid
Neu5Ac	*N*-acetylneuraminic acid (a sialic acid)
P_1	Galα1–4Galβ1–4GlcNAc
PAGE	polyacrylamide gel electrophoresis
Pap	pyelonephritis-associated pili
PAMAM	polyamidoamine
PCR	polymerase chain reaction
P^k	Galα1–4Galβ1–4Glc
PVDF	polyvinylidene difluoride
Rha	rhamnose
SadP	streptococcal adhesin P
SDS	sodium dodecyl sulphate
SLT	Shiga-like toxin
Spc^r	contains spectinomycin resistance gene
SPR	surface plasmon resonance
SrtA	sortase A
SS2	*Streptococcus suis*, serotype 2
UTI	urinary tract infection
WBC	white blood cell

LIST OF ORIGINAL PUBLICATIONS

This thesis by Annika Kouki (née Salminen) is based on the following original publications and some unpublished results. The publications are reproduced with the permissions of the American Society for Biochemistry and Molecular Biology (I), Oxford University Press (II) and The Royal Society of Chemistry (III and IV). In the text, the original publications will be referred to by the roman numerals (I–IV).

I Kouki, A.*, Haataja, S.*, Loimaranta, V., Pulliainen, A.T., Nilsson, U.J. and Finne, J. (2011) Identification of a novel streptococcal adhesin P (SadP) recognizing galactosyl-α1–4-galactose–containing glycoconjugates: convergent evolution of bacterial pathogens to binding of the same host receptor. *Journal of Biological Chemistry* **286**:38854-38864

II Salminen, A., Loimaranta, V., Joosten, J.A.F., Khan, A.S., Hacker, J., Pieters, R.J. and Finne, J. (2007) Inhibition of P-fimbriated *Escherichia coli* adhesion by multivalent galabiose derivatives studied by a live-bacteria application of surface plasmon resonance. *Journal of Antimicrobial Chemotherapy* **60**: 495-501

III Branderhorst, H.M., Kooij, R., Salminen, A., Jongeneel, L.H., Arnusch, C.J., Liskamp, R.M.J., Finne, J. and Pieters, R.J. (2008) Synthesis of multivalent *Streptococcus suis* adhesion inhibitors by enzymatic cleavage of polygalacturonic acid and 'click' conjugation. *Organic & Biomolecular Chemistry* **6**: 1425-1434

IV Parera, N.*, Kouki, A.*, Haataja, S., Branderhorst, H.M., Liskamp, R.M.J., Visser, G.M., Finne, J. and Pieters, R.J. (2010) Detection of pathogenic *Streptococcus suis* bacteria using magnetic glycoparticles. *Organic & Biomolecular Chemistry* **10**: 2425-2429

*Equal contribution of the authors.

1. INTRODUCTION

Microbial infection is a complex process that involves a series of events that may lead to host tissue malfunction or destruction (Kahn et al. 2002). The genus *Streptococcus* consists of a wide variety of species, some of which are important human pathogens and some are zoonotic pathogens of domestic and wild animals (Moschioni et al. 2010). According to definition, zoonoses are infectious diseases that are transmitted from animals to man under natural conditions (Schaapveld and Treurniet 1995). Streptococci have developed sophisticated assets that enable response to environmental changes. The fact that streptococci are able to colonise almost any human tissue (Mitchell 2003) demonstrates how successful these strategies have been and still are. However, the properties that have been the strengths of streptococci, such as the ability to adhere to tissues, may also be targets that can be exploited when developing new ways to prevent and control infections. The identification and characterisation of the molecules involved in the interaction of the bacteria with the host cells could give valuable information for the understanding of the pathogenesis of the disease and is crucial for development of novel antibacterial agents or vaccines against major Gram-positive pathogens.

Sequences of complete genomes of bacteria have become available, and a next step is to find out the functions of the encoded proteins and how all these proteins come together to create a living cell. The functions of many proteins can be inferred from the known functions of homologous proteins, but there are a large number of putative proteins that do not even have a hypothetical function. The genome of a zoonotic swine pathogen *Streptococcus suis* has been sequenced (Holden et al. 2009). Cell surface proteins of *S. suis* are expected to have important roles during different stages of infection. These proteins are also expected to be potential vaccine components like in other streptococci (Larsson et al. 1997, Areschoug et al. 2004). Some proteomics studies have already shed light on the surface proteins of *S. suis* (Zhang and Lu 2007, Geng et al. 2008, Jing et al. 2008, Wu et al. 2008, Zhang et al. 2008, Chen et al. 2011, Wu et al. 2011, Zhang et al. 2011). However, the list of *S. suis* surface proteins is far from complete and the functions of many of the proteins are waiting to be solved. On the other hand, *S. suis* has been known to possess a galabiose (galactosyl-α1–4-galactose, Galα1–4Gal) binding activity but the molecule responsible for the binding has remained unknown.

The cell surface proteins may be utilised for the detection of the pathogen. Traditionally bacteria have been detected mainly by culturing or by methods based on immunoassays or PCR (polymerase chain reaction) (Leung et al. 2006). Bacterial culturing is time-consuming, antibodies used in immunoassays need to be refrigerated, which is problematic in field applications, and PCR is sensitive to contaminants in samples, just to mention some of the disadvantages of these traditional methods (Hatch et al. 2008). Therefore, there is a call for new and inventive detection techniques.

The present study focuses on the galabiose-specific adhesin of *S. suis*. The identification of the adhesin and the inhibition of whole bacterial cells or recombinant adhesin by synthetic derivatives of the disaccharide will be covered. The *S. suis* adhesin will be compared with a galabiose binding adhesin of *Escherichia coli* and a glyconanoparticle technique for the detection of adhesin expressing bacteria will be presented.

2. REVIEW OF THE LITERATURE

2.1. Streptococcus suis

Streptococcus suis is a Gram-positive facultative anaerobe that is coccoid or ovoid in shape. It occurs as single cells, in pairs, or in short chains (Figure 1). When grown on a plate, the colonies of *S. suis* are small (1–2 mm in diameter) and grey or transparent in colour. The bacteria may produce narrow zones of α- or β-haemolysis on blood agar plates (Staats et al. 1997).

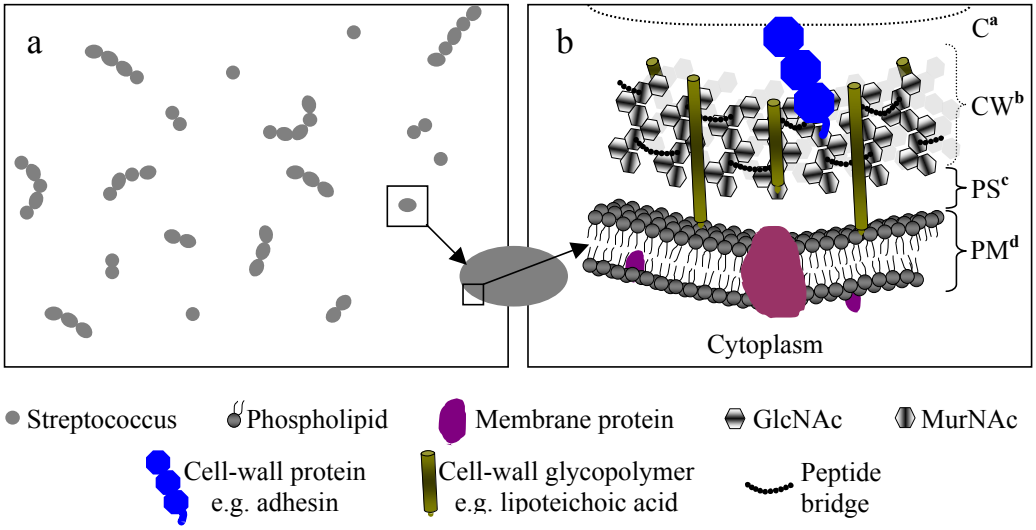

Figure 1. Chains of streptococci (a) and a simplified schematic cell wall of Gram-positive bacteria (b).

[a] C, capsule, which some strains have over the peptidoglycan layer (Charland et al. 1998).
[b] CW, cell wall, which consists of monosaccharides and amino acids (peptidoglycan) that form a three-dimensional mesh with cell wall glycopolymers (Dmitriev et al. 1999, Weidenmaier et al. 2008).
[c] PS, periplasmic space
[d] PM, plasma membrane, which consists of lipid bilayer with embedded proteins.

The classification of *S. suis* is rather complex due to historical reasons. Originally *S. suis* strains were classified as Lancefield groups R, S, RS, and T (Demoor 1963), but later strains of *S. suis* were shown to share antigens with group D streptococci (Elliott 1966) and *S. suis* can be regarded as a member of that group (Reuter 1992). Currently there are 33 serotypes of *S. suis* based on the capsular polysaccharide antigens. Two serotypes previously classified as *S. suis* have been proven to be serotypes of *Streptococcus orisratti*. Previously serotyping was done by methods based on capsular reaction, capillary precipitation or coagglutination. (Higgins and Gottschalk 1990). PCR is today a useful technique not only for serotyping (Rasmussen and Andresen 1998) but also detecting *S. suis* infections in humans for clinical diagnosis (Matsuo et al. 2003). To better distinguish isolates potential to cause disease and to find out the genetic relatedness between *S. suis* strains,

multilocus sequence typing has been used to classify *S. suis* isolates. In this scheme, the sequence types are classified based on various alleles found in certain housekeeping gene loci. *S. suis* sequence type 1 (not to be confused with serotype) has been found to be the most virulent one. (King et al. 2002)

S. suis is phylogenetically distant to other streptococcal species as about 40 % of its 2 Mb genome is unique. However, within *S. suis* species the genome is highly conserved and virtually all of the genome is common to the *S. suis* strains but there is small scale sequence variation from strain to strain. Larger varying regions carry coding sequence associated with drug resistance, and small-scale sequence variation has been found in regions coding for putative virulence and colonisation factors. (Holden et al. 2009)

2.2. Porcine S. suis *infections*

S. suis is one of the most significant swine pathogens and it causes substantial economical losses worldwide. In addition to swine, *S. suis* has been isolated also from humans (see section 2.3.) and other mammalian (wild boars, horses, dogs, cats) as well as avian species (Gottschalk et al. 2007, Lun et al. 2007). Serotypes 1, 2, 1/2, 7 and 9 are most common in strains isolated from swine (Haesebrouck et al. 2004) and *S. suis* serotype 2 (SS2) is considered to be the most virulent type to swine and humans (Gottschalk et al. 2007). The natural habitat of *S. suis* is the porcine upper respiratory tract, particularly the tonsils and nasal cavities, and the genital and alimentary tracts (Haesebrouck et al. 2004). Hence, it is usually transmitted nasally or orally, and subsequently colonises these niches. In some swineherds, the carrier rate can reach almost 100 %. (Staats et al. 1997) Young piglets at the age of 3 to 12 weeks are most susceptible to *S. suis* infection, especially after weaning (Lamont et al. 1980). Clinical presentation of infected pigs varies from asymptomatic bacteraemia to fulminant systemic disease. Pigs may even die without any apparent clinical signs but generally fever, depression and anorexia are common signs of the acute disease. These first signs may be followed by dyspnoea, tremors, nystagmus, erythema, ataxia, convulsions, blindness, deafness and paralysis. *S. suis* causes a wide range of clinical disease syndromes in pigs: septicaemia, meningitis, endocarditis, pneumonia, arthritis, polyserositis, abortions and abscesses. The necropsy findings depend on the duration of the disease and the tissues affected but congestion of the meninges, lymph nodes and lungs, purulent meningitis, cardiac lesions such as vegetative valvular endocarditis and pulmonary lesions such as interstitial or bronchopneumonia are common findings. (Staats et al. 1997)

Various types of vaccines have been developed for pigs but there is no truly effective vaccine available for pigs yet, even though *S. suis* infections cause great economical losses in the farming industry (Haesebrouck et al. 2004). The vaccines developed and tested in swine so far have been based on capsular polysaccharides (Elliott et al. 1980), whole bacterial cells (Fittipaldi et al. 2007) or proteins such as suilysin (Jacobs et al. 1996), muramidase-released protein and extracellular factor (Wisselink et al. 2001). The protective effect of these vaccines has been either strain or serotype dependent and the results of vaccination studies have been mostly indefinite (Li et al. 2006). However, proteomics studies performed in recent years have identified several promising

immunogenic proteins such as HP0245 (Li et al. 2011c) and SsPepO (Li et al. 2011b). A recombinant peptide containing an extracellular region of HP0245 elicited protection against *S. suis* challenge in a murine model (Li et al. 2011c). SsPepO was found to be conserved in all of the sequenced strains of *S. suis* serotypes 2, 7 and 9. Furthermore, it conferred a partial protection against a lethal dose challenge with *S. suis* serotype 2 in a swine model. (Li et al. 2011b). Further studies of these proteins are needed, and meanwhile inactivated autogenous vaccines are still used. Autogenous vaccines are prepared from the virulent strain isolated from infected pigs on the farm. They are not available immediately after the infection outbreak and they may not always be very efficient or safe (Haesebrouck et al. 2004).

2.3. Human S. suis *infections*

In addition to being an important pathogen of pigs, *S. suis* is also an emerging zoonotic agent (Lun et al. 2007). Zoonoses in general are mainly foodborne infections (for example Salmonella or Campylobacter infections) or acquired by professional exposure to animals or animal products (Schaapveld and Treurniet 1995). This holds true also for human *S. suis* infections which have been almost exclusively related to occupational exposure to pigs or pork products in the more developed countries (Staats et al. 1997, Lun et al. 2007). *S. suis* infections have also been reported among housewives in Thailand, presumably because they have been handling contaminated meat (Vilaichone et al. 2002). *S. suis* has been isolated from the pharynxes of healthy abattoir workers in Germany. The carriage rate of the employees was 5.3 % while those without contact to pigs were consistently tested negative (Strangmann et al. 2002). Therefore, zoonotic pathogens may seem to influence only a marginal group. However, zoonoses may have unexpected consequences. An example of such a consequence is the recent observation that there is a connection between zoonotic bacteria and antibiotic resistance development (Prescott 2008). Considerable effort has been made to reduce antimicrobial drug usage in humans in order to reduce resistance and its spread. However, in food animal farming, for instance in piggeries, antibiotics are still used or even abused for growth promotion and disease prevention (Prescott 2008). The inappropriate use of antibiotics in animal production may select for resistant strains. Therefore livestock, such as pigs, may constitute a growing reservoir of highly resistant zoonotic bacteria, as has been the case with methicillin-resistant *Staphylococcus aureus* (MRSA) (Springer et al. 2009).

The first human *S. suis* infection case was reported in Denmark in 1968 (Perch et al. 1968). Since the 1960's, human *S. suis* infections have been reported worldwide (Table 1). However, the number of cases is probably in reality much greater owing to unreported and undiagnosed cases. For example in Thailand, five of eight *S. suis* infection cases had been initially erroneously diagnosed as *Streptococcus viridans* infections, and identified as *S. suis* infections when these cases were studied retrospectively (Donsakul et al. 2003). *S. suis* is thought to infect humans through skin wounds or the oral route (Gottschalk et al. 2010). In situations of immunodeficiency, *S. suis* may become an opportunistic pathogen. Splenectomy, alcoholism, diabetes mellitus and malignancy have been suggested as predisposing factors for *S. suis* infection (Gallagher 2001, Watkins et al. 2001, Huang et al. 2005). There is no *S. suis* vaccine for humans yet. Diseased pigs are the main source of human

infections, and therefore vaccination of pigs would indirectly protect also humans by decreasing *S. suis* carriage in pigs.

Table 1. Cases of human patients infected with *S. suis* worldwide summarised from published papers since 1968 until 2012. The table has been updated (Kennedy et al. 2008, Nghia et al. 2008, Poggenborg et al. 2008, Tramontana et al. 2008, Aspiroz et al. 2009, Galbarro et al. 2009, Ishigaki et al. 2009, Kerdsin et al. 2009, Laohapensang et al. 2010, Kerdsin et al. 2011, Kim et al. 2011), originally from Lun et al. 2007.

Country	Cases	Deaths
China	283	54
Thailand	62	12
Netherlands	34	1
Spain	8	0
Germany	6	1
UK	6	1
France	5	1
Croatia	4	2
Denmark	4	0
Australia	3	0
Japan	3	0
Belgium	2	1
Canada	2	0
Italy	2	0
Vietnam	1	1
Argentina	1	0
Austria	1	0
Greece	1	0
Hungary	1	0
Korea	1	0
New Zealand	1	0
Singapore	1	0
Sweden	1	0
USA	1	0
Total	429	73 (17 %)

Meningitis is the most common presentation of human diseases caused by *S. suis* (Table 2) and may result in death or have serious permanent sequelae such as hearing loss. *S. suis* is actually the most common bacterial pathogen causing meningitis in Vietnam (Mai et al. 2008, Ho Dang Trung et al. 2012), which may perhaps be surprising. In fact, *S. suis* is one of the main meningitis-causing pathogens also elsewhere in Southeast and East Asia (Suankratay et al. 2004, Hui et al. 2005). In addition to meningitis, *S. suis* infection may cause either separate or simultaneous arthritis,

pneumonia, peritonitis and endocarditis, as well as septicaemia and streptococcal toxic shock like–syndrome (Table 2), which are consequences of excessive or poorly regulated immune response to a pathogen (Tsiotou et al. 2005).

Table 2. Types of disease manifestations reported worldwide in human patients infected with *S. suis*, adapted from Lun et al. 2007.

	Number of cases
Meningitis	264 (73 %)
Septicaemia and septic shock	88 (24 %)
Arthritis	4 (1.1 %)
Endocarditis	4 (1.1 %)
Pneumonia	3 (0.8 %)
Peritonitis	1 (0.3 %)
Total	364 (100 %)

The clinical signs of *S. suis* infection are manifold. In an acute form of meningitis patients have high fever, headache, chills, nausea, vomiting and vertigo. At later stage of infection patients may suffer from petechia, ecchymosis, rashes, articular pain, neck stiffness, severe myalgia, rhabdomyolysis, walking ataxia, peripheral and facial paralysis and coma. Again, in an acute form of toxic septic shock, there may be hypotension, tachycardia, subcutaneous haemorrhage, liver dysfunction, disseminated intravascular coagulation, acute renal failure and acute respiratory distress syndrome, in addition to the same symptoms as in acute meningitis. Hence, death often follows septic shock. Care in an intensive care unit may be required to maintain blood glucose levels, to prevent iatrogenic infections and to prevent damage to the liver, kidneys and the circulatory system. (Lun et al. 2007)

The diagnosis of human *S. suis* infection is based on the clinical signs, laboratory examinations (Table 3) and possibly anamnestic information about direct contact with sick pigs. Patients are often treated with penicillin G accompanied by one or more other antibiotics including cefriaxone, gentamicin, chloramphenicol and ampicillin. (Lun et al. 2007) Nevertheless, penicillin-resistant strains as well as strains resistant to other commonly used antibiotics have been observed (Gottschalk et al. 1991, Prieto et al. 1994, Aarestrup et al. 1998). The most common pathological findings in humans (and pigs) are congestion of the meninges, lymph nodes and lungs. There may also be oedema of the brain, increased amount of cerebrospinal fluid (CSF), hyperaemia of myocardium, widespread haemorrhage, disseminated intravascular coagulation as well as necrosis of hepatocytes and kidney cells. (Lun et al. 2007)

Table 3. Laboratory findings in *S. suis* infection (Lun et al. 2007)

	***S. suis* infected patients**	**Reference range[a]**
Blood white blood cell (WBC) count	$13.8–26.6\times10^9$ [b]	$3.4–8.2\times10^9$
Plasma C-Reactive Protein (CRP)	130–236 mg/L	< 10 mg/L
Plasma Alanine Aminotransferase (ALAT)	increased	10–70 U/L
Plasma Aspartate Aminotransferase (ASAT)	increased	15–45 U/L
CSF WBC	$1.25–3.24\times10^3$ /µL	0–3 /µL
CSF protein	very low	150–450 mg/L
CSF glucose	very low	2.2–3.9 mmol/L
Gram-stain (CSF, blood, joint fluid)	short chains of coccoid rods	no bacteria

[a] for males (http://ohjekirja.tykslab.fi/)
[b] 81–95 % were polymorphonuclear neutrophils

Outbreaks of human *S. suis* infection are uncommon. However, there have been three large outbreaks in China: two in Jiangsu province during the summers 1998 and 1999 and the largest outbreak so far in Sichuan province in the summer of 2005 with 215 human cases out of which 39 died. These outbreaks happened simultaneously with large disease outbreaks in pigs. Virtually all patients had a history of direct contact with diseased pigs, no person-to-person transmission was reported during these outbreaks. (Yu et al. 2006) However, in Southeast and East Asia *S. suis* seems to be an emerging pathogen also in general population (Gottschalk et al. 2010). When the pathogen causing these outbreaks was analysed by sequencing certain housekeeping gene loci, an emerging and highly virulent sequence type 7 was identified. This sequence type is a single-locus variant of the sequence type 1 previously reported to be the most virulent one (Ye et al. 2006).

2.4. Virulence factors of S. suis

According to definition, virulence factors operate in establishing the disease state (Jenkinson and Lamont 1997). Pathogenesis of *S. suis* infections is poorly understood as it has been unclear how *S. suis* initially adheres to host cells, how it is able to translocate through the mucosal epithelia of the upper respiratory tract, how it travels in the bloodstream and how it invades the subarachnoid space (Haesebrouck et al. 2004).

S. suis adheres to the epithelial cells in pig tonsils and in human respiratory tract (Lalonde et al. 2000, Fittipaldi et al. 2012). A galabiose-specific adhesin, which may enable the first critical step of infection, was identified in the present study. This protein, as well as the adhesion process, will be discussed in detail later. *S. suis* has also been reported to interact with components of the extracellular matrix such as fibronectin, collagen and plasminogen (Esgleas et al. 2005). Some proteins taking part in these interactions have been identified (Table 4), namely autolysin, di-peptidyl peptidase IV, enolase as well as fibrinogen- and fibronectin-binding protein. Interestingly, *S. suis* has been reported to possess an IgA1 protease, which is able to cleave human mucosal

antibodies, and therefore help *S. suis* to resist host immune defence (Zhang et al. 2010). Mere adhesion of a pathogen to a tissue does not cause disease. Specific responses leading to infection are needed as well. It is known that *S. suis* induces the release of several proinflammatory cytokines and chemokines in the host, which have been detected in acutely infected pigs and humans (Vanier et al. 2009, Ye et al. 2009). It was recently suggested that *S. suis* activates the complement system, an important part of innate immunity, mainly via the alternative pathway (Pian et al. 2012). In the bacteria, the interaction with the host leads to changes in global regulators (Table 4) that allow responses to the changing environment as has been shown with *E. coli* (Abraham et al. 1998, Schilling et al. 2001).

S. suis also produces a haemolysin, named suilysin (Gottschalk et al. 1995), which is able to destroy epithelial cells, monocytes and neutrophils by forming transmembrane pores, and could play a role in the dissemination of the bacteria in the bloodstream. Possibly, suilysin might also be involved in the disruption of the blood–brain barrier by affecting the endothelial cells, and have a role in the escape from opsonophagocytosis (Haesebrouck et al. 2004, Gottschalk et al. 2007, Lecours et al. 2011). The cell wall lipoteichoic acid, too, seems to have a role in the escape from host immune clearance and in moving across host barriers (Fittipaldi et al. 2008b).

Other virulence factors have been suggested as well (Fittipaldi et al. 2012). Some virulence-associated factors have been categorised according to their properties or functions in Table 4. As an example, the availability of trace metals and other nutritients may be scarce in the infected host and therefore several proteins involved in metal homeostasis have evolved. As another example, bacterial cell wall properties are important for the survival in host tissues. The cell wall capsular polysaccharide (Charland et al. 1998) is one of the well-established virulence factors since knockout mutants lacking the capsular polysaccharide are almost avirulent. The capsule is useful for the pathogen after invasion to the deeper tissues or circulation. It has been reported to help the bacterium to escape phagocyte killing (Smith et al. 1999). The SS2 capsule consist of the repeating unit $[(\text{Neu5Ac}\alpha2\text{–}6\text{Gal}\beta1\text{–}4\text{GlcNAc}\beta1\text{–}3)\text{Gal}\beta1\text{–}4(\text{Gal}\alpha1\text{–}3)\text{Rha}\beta1\text{–}4\text{Glc}\beta1\text{-}]_n$ (Van Calsteren et al. 2010). Terminal sialic acid residues prevent the deposition of complement protein C3 on the surface of group B streptococci (Marques et al. 1992). These residues might similarly prevent the activation of the alternative pathway that would lead to opsonin-dependent intracellular killing of *S. suis*. During adhesion and invasion however, the capsular polysaccharide seems to block the adhesins at the cell surface, since unencapsulated strains haemagglutinate and adhere more strongly to HEp-2 cells than encapsulated strains (Tikkanen et al. 1996, Benga et al. 2004). It has been hypothesised that prior to adhesion the expression of the capsular polysaccharide is downregulated in response to signals from the environment (Fittipaldi et al. 2012).

After adhesion, invasion and surviving in circulation, *S. suis* needs to cross the blood–brain barrier or the blood–cerebrospinal fluid barrier in order to cause infections in the central nervous system. Adhesins responsible for attachment to the brain microvascular endothelial cells are not known but adhesion to and invasion of these cells have been described *in vitro* (Vanier et al. 2004). A great number of virulence-associated factors, also some genes with unidentified functions, have been identified in recent years (Table 4).

Table 4. Proposed virulence-associated factors of *S. suis*

Virulence factor	Characteristics/Evidence	Reference
LPXTG-motif proteins		
Amylopullulanase (ApuA)	Promotes adhesion to porcine epithelium and mucus *in vitro*.	Ferrando et al. 2010
IgA1 protease	Cleaves human mucosal IgA1. Immunogenic, convalescent sera. KO[a]	Zhang et al. 2010, Zhang et al. 2011
Di-peptidyl peptidase IV	Interacts with human fibronectin. KO	Ge et al. 2009
Muramidase-released protein	NE[b], P[c]	Vecht et al. 1991, Wisselink et al. 2001
Surface antigen one (Sao)	P	Li et al. 2006, Li et al. 2007
Serum opacity–like factor	Confers serum opacification activity but may contain function in adhesion, too. Does not affect colonisation. KO	Baums et al. 2006
Subtilisin-like protease (SspA)	Induces the secretion of proinflammatory cytokines and chemokines by macrophages. KO	Bonifait et al. 2011, Hu et al. 2010
Cell wall synthesis		
Capsular polysaccharide	Constitutes a physical barrier that protects from phagocytosis and killing in blood and solid tissues. KO, P	Elliott et al. 1980, Charland et al. 1998
CpsE/F, Cps2C, NeuB	Roles in capsular polysaccharide biosynthesis. KO	Smith et al. 1999, Smith et al. 2000, Wilson et al. 2007
D-alanylation of cell wall lipoteichoic acid (DltA)	Mutant strain more susceptible to the action of cationic antimicrobial peptides and killing by porcine neutrophils. KO	Fittipaldi et al. 2008b
N-deacetylation of peptidoglycan (PgdA)	A deacetylase may bring about resistance to neutrophil lysozyme-mediated killing. KO	Fittipaldi et al. 2008a
Sortase A (SrtA)	Anchors proteins to the cell surface. KO	Vanier et al. 2008

Table 4. Proposed virulence-associated factors of *S. suis* (continued)

Virulence factor	Characteristics/Evidence	Reference
Toxins		
Suilysin	Destroys epithelial cells, monocytes and neutrophils. P	Gottschalk et al. 1995, Jacobs et al. 1996
Autolysin	Binds to fibronectin. Participates in cell autolysis, separation of daughter cells and biofilm formation. KO	Ju et al. 2012
Metal homeostasis		
AdcR	Zinc uptake regulator, KO	Aranda et al. 2010
Dpr	Resistance to iron-mediated toxicity. Counteracts oxidative stress.	Pulliainen et al. 2003
FeoB	Iron transporter, KO	Aranda et al. 2009
Fur	Iron uptake regulator, KO	Aranda et al. 2010
SSU0308	A lipoprotein involved in zinc uptake. KO	Aranda et al. 2012
TroA	Manganese uptake. Counteracts oxidative stress. KO	Wichgers Schreur et al. 2011
Zur	Resistance to zinc-mediated toxicity.	Feng et al. 2008
Moonlighting proteins		
6-phosphogluconate-dehydrogenase	Recombinant protein inhibits adhesion to HEp-2 and HeLa cells. P	Tan et al. 2008
Amylopullulanase (ApuA)	Promotes adhesion to porcine epithelium and mucus *in vitro*. LPKTGE cell wall-anchoring motif.	Ferrando et al. 2010
Glyceraldehyde-3-phosphate dehydrogenase	Recombinant protein inhibits adhesion to porcine tracheal rings and HEp-2 cells. Immunogenic in pigs.	Brassard et al. 2004, Wang et al. 2007, Zhang et al. 2008
Enolase	Binds to fibronectin and plasminogen, export mechanism unknown, recombinant protein inhibits adhesion to HEp-2 cells. NE, P	Esgleas et al. 2008, Feng et al. 2009
Glutamate dehydrogenase	NE	Okwumabua et al. 2001
Glutamine synthetase	A role in colonisation of organs. KO	Si et al. 2009

Table 4. Proposed virulence-associated factors of *S. suis* (continued)

Virulence factor	Characteristics/Evidence	Reference
Global regulators		
Sugar metabolism regulator catabolite control protein A (CcpA)	Sugar regulator Deletion leads to markedly reduced thickness of capsule and reduced resistance to killing by neutrophils.	Willenborg et al. 2011
CiaRH	Two-component signal transduction system deletion mutant showed decreased adherence to HEp-2 and PIEC cells and increased clearance from blood. KO	Li et al. 2011a
CovR	Orphan transcriptional regulator. Negative regulator of virulence. Deletion of covR gene leads to formation of longer bacterial chains, thicker capsules, increased haemolytic activity and adhesion.	Pan et al. 2009
LuxS	Regulates transcription levels of many virulence genes that affect biofilm formation, cell adhesion and haemolytic activity. KO	Wang et al. 2011
RevS	Orphan response regulator, KO	de Greeff et al. 2002a
RevSC21	An orphan transcriptional regulator, deletion mutant showed decreased adherence to HEp-2 cells and had lost haemolytic activity. KO	Wu et al. 2009
Rgg-like regulator	Global transcriptional regulator, deletion mutant showed increased adherence to HEp-2 cells and haemolytic activity, and reduced utilisation of nonglucose carbohydrates. KO	Zheng et al. 2011
SalK/SalR signal transduction system	Two-component signal transduction system, KO	Li et al. 2008

Table 4. Proposed virulence-associated factors of *S. suis* (continued)

Virulence factor	Characteristics/Evidence	Reference
Function in pathogenesis unknown		
Endo-β-*N*-acetylglucosaminidase D	Putatively degrades host surface carbohydrates. KO	Wilson et al. 2007
Extracellular factor	NE, P	Vecht et al. 1991, Wisselink et al. 2001
Fibrinogen- and fibronectin -binding protein	Binds to human fibronectin and fibrinogen *in vitro*. NE	de Greeff et al. 2002b
Genes involved in metabolism: *cdd*, *gtfA*, *manN*, *purA*, *purD*, *scrB*, *scrR*	Putative functions respectively: Cytidine deaminase, sucrose phosphorylase, mannose-specific phosphotransferase system component IID, adenylosuccinate synthetase, phosphoribosylamine-glysine ligase, sucrose operon repressor, sucrose-6-phosphate hydrolase KO	Wilson et al. 2007
Genes involved in transcription regulation: *nadR*, SMU61, *treR*	Putative transcriptional regulators KO	Wilson et al. 2007
GnlH	KO	Wilson et al. 2007
Lpp	Lipoprotein, KO	Wilson et al. 2007
Permease	ABC-type amino acid transporter, KO	Wilson et al. 2007
Spr1018	Conserved hypothetical protein, KO	Wilson et al. 2007
Trag	KO	Zhang et al. 2010
VirA	KO	Li et al. 2010

[a] KO, knockout mutant lacking this factor is almost avirulent/attenuated in an animal model.
[b] NE, not essential for the virulence.
[c] P, recombinant protein elicits protective immune reaction (in an animal model).

2.5. Fimbriae of Gram-positive bacteria

Fimbriae or pili are long filamentous structures (Figure 2) extending from the surface of most bacterial pathogens (Telford et al. 2006). These two terms are interchangeable, although the term pilus may sometimes be reserved solely to the structures required in bacterial conjugation (Llosa et al. 2002), and therefore surface appendages not involved in DNA transfer will be referred to as fimbriae in the present study. Fimbriae often participate in the initial adhesion of bacteria to host tissues during colonisation and biofilm formation (Yamaguchi and Matsunoshita 2004). In addition to adhesion, fimbriae mediate the aggregation of bacteria, which may promote the colonisation and aid bacteria to resist host defences. The aggregation also allows different bacterial species to interact. (Kirn et al. 2000) Fimbriae participate in the infection process also after the colonisation of tissues since they assist the invasion of host cells (Maisey et al. 2007) and possess immunomodulatory capabilities (Barocchi et al. 2006, Maisey et al. 2008).

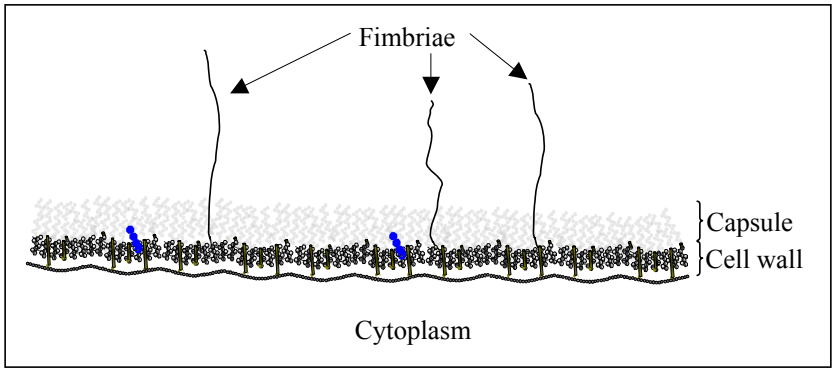

Figure 2. Fimbriae are hairlike structures that in Gram-positive bacteria are covalently linked to the cell wall.

Corynebacterium renale was the first Gram-positive bacterium that was observed to possess fimbria-like structures on its surface (Yanagawa et al. 1968, Yanagawa and Otsuki 1970). Since then, these surface appendages have been found in many other Gram-positive species, including streptococcal pathogens that cause invasive disease in humans: group A streptococci (*S. pyogenes*) (Mora et al. 2005), group B streptococci (*S. agalactiae*) (Lauer et al. 2005), *S. pneumoniae* (Barocchi et al. 2006) and, in addition to the several other virulence factors already discussed, also *S. suis* is known to possess fimbriae (Jacques et al. 1990, Fittipaldi et al. 2010). The fimbrial backbones are made up of major protein subunits called pilins. In contrast to Gram-negative fimbriae that are usually formed by non-covalent homopolymerisation, the pilins in Gram-positive fimbriae are covalently linked. (Proft et al. 2009) The origin of fimbriae in Gram-positive bacteria is unclear but, based on the genomic organisation of fimbria-operons, they may be acquired by horizontal transfer of a pathogenicity island (Telford et al. 2006).

Two types of fimbriae have been found in streptococci: short, thin fibrils that extend 70 to 500 nm from the bacterial surface (McNab et al. 1999) and flexible rods that are much longer, up to 3 µm long (Mora et al. 2005). The longer rod-like fimbriae are comprised of polymerised, covalently

linked pilins each containing a Gram-positive cell wall anchor motif (often an LPXTG sequence). This motif is the target of sortase enzymes that are transpeptidases linking the threonine residue in the motif to the bacterial cell wall. When catalysing the fimbria formation, sortases assist the joining of the pilin subunits to each other and to the peptidoglycan cell wall. It is predicted that the pilin subunits are added to the base of the growing fimbria until the last subunit is covalently joined to the cell wall peptidoglycan. (Telford et al. 2006) The pilins are polymerised by fimbria-specific sortases encoded in the fimbria gene clusters (Nobbs et al. 2008). In *S. suis*, the four putative fimbria gene clusters found so far, *srtBCD*, *srtE*, *srtF* and *srtG* (Osaki et al. 2002), have been named after the sortase gene. In addition, there is a housekeeping sortase, sortase A (SrtA), which along with other roles seems to be involved in anchoring the fimbria to the cell wall peptidoglycan, but it is not needed for the polymerisation of the fimbria (Fittipaldi et al. 2010). SrtA is found in virtually all Gram-positive genomes sequenced hitherto (Nobbs et al. 2009). A *S. suis* SrtA knockout mutant strain has been reported to show less adherence to endothelial cells, which suggests that peptidoglycan-anchored, LPXTG motif-containing adhesins are important for interactions of the pathogen and host cells (Vanier et al. 2008).

Gram-positive fimbriae are usually made up of three protein subunits, and each subunit has a distinct function. One of the subunits, pilin, forms the backbone of the fimbrial structure as already discussed. In addition, there are usually two ancillary proteins (Telford et al. 2006). It has been speculated that the ancillary proteins may have a role in adhesion, like many minor fimbrial proteins of Gram-negative *E. coli* have (Westerlund-Wikström and Korhonen 2005). The backbone component can produce homopolymers, but the ancillary proteins cannot polymerise without the backbone component. During fimbrial assembly, certain ancillary proteins are added occasionally to the backbone structure while some proteins are added only to the tip of the fimbria. Therefore, the initial contact with the target cell may be achieved by the tip of the fimbria and then more intimate interaction could be accomplished by interactions between the target cell and the whole length of the fimbrial shaft. (Telford et al. 2006) For instance, P-fimbriae of *E. coli* are are composed of a major subunit and three minor proteins, one of which facilitates adherence to galabiose and the two other minor proteins bind to fibronectin (Westerlund et al. 1991).

The model of Gram-positive fimbria assembly is not all-encompassing as it was recently noticed in *S. suis* that also the ancillary protein in the *srtG* cluster is needed for the fimbria backbone formation, and furthermore that *S. suis* has only one ancillary protein that seems to be tip-associated (Okura et al. 2011). PAPI-2b, an ancillary protein of SS2, has recently been reported to be immunoprotective (Garibaldi et al. 2010) and hence fimbrial subunits may prove useful in the development of vaccines against *S. suis*. Interestingly, more fimbriae are formed if bacteria are grown in temperatures below 30 °C than when the growth temperature is above 30 °C. It may seem odd that less fimbriae are formed under physiological conditions but it should be remembered the surface temperatures of the external parts of pigs, such as snout, ears and back, range from 20 to 30 °C at room temperature, and therefore it may beneficial for the bacteria to express more fimbriae if the temperature of the environment is below the body temperature. (Okura et al. 2011). However, all *S. suis* strains may not express fimbriae on their surface, as several mutations have been found in fimbria gene clusters of some strains (Takamatsu et al. 2009). For instance, the fimbriae encoded by the *srtF* fimbria cluster are formed exclusively by the major pilin subunit in many SS2 strains, as

there are nonsense mutations in the ancillary protein gene (a putative adhesin). Therefore, it seems that many *S. suis* strains produce fimbriae, but these structures may not always contain adhesins. (Fittipaldi et al. 2010)

2.6. Bacterial adhesins

The initial event in most infectious diseases is the adherence of pathogens to the host organism (Beachey 1981). Bacterial engagement of host receptors can be expedient to target a pathogen to a particular niche, capturing underlying signaling pathways, establishing persistent infections and inducing invasion (Finlay and Cossart 1997). That said, it ought to be remembered that adhesion is a dynamic process: It may be beneficial for bacteria to detach from the surface if the conditions on the surface are not favourable (Nobbs et al. 2009).

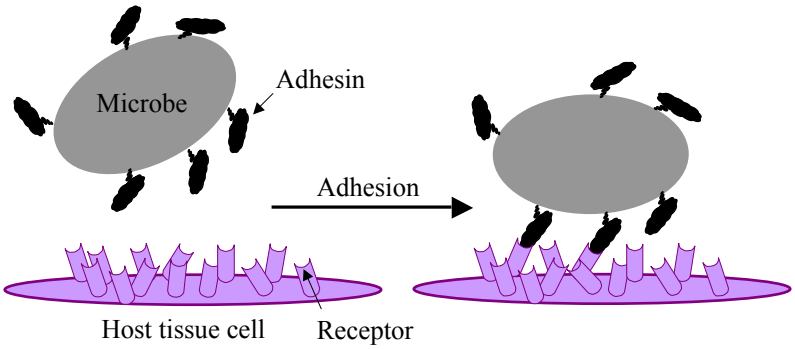

Figure 3. A simple model of microbial adhesion to receptors of host cells

Adhesion is in many cases mediated by the interaction between an adhesin on the surface of the infectious organism and specific receptor on the host cells (Figure 3). Microbial adherence is often based on lectin-like protein–carbohydrate recognition but adherence may be based on protein–protein or carbohydrate–carbohydrate interactions as well (Nobbs et al. 2009). The lectin-like adhesins often recognise the carbohydrate moieties of glycoproteins or glycolipids (Strömberg et al. 1990, Backhed et al. 2002). Components of the extracellular matrix, such as fibronectin, collagen, fibrinogen, fibrin, elastin, vitronectin, laminin, decorin and heparin sulphate-containing proteoglycans (Westerlund and Korhonen 1993, Moschioni et al. 2010) as well as serum components, such as albumin, plasmin and immunoglogulins (Jenkinson and Lamont 1997), are also very common host receptors. Response to receptor binding and response to other information from the bacterial surroundings is mediated by transcription regulators that control gene expression. In streptococci, there are two main transcription regulator types, the two-component signal transduction systems and the stand-alone regulators that repress or activate also the expression of surface adhesins. (Nobbs et al. 2009)

In addition to host tissues, bacteria adhere to other microbial cells and to various surfaces (Jenkinson and Lamont 1997). This kind of adhesion is a prerequisite for the formation of bacterial communities or biofilms (Nobbs et al. 2009). An interesting example of such bacterial coadhesion

and subsequent communication between bacteria is the binding of *Porphyromonas gingivalis* short fimbriae to *Streptococcus gordonii* adhesins, an event that is the beginning of a signalling pathway finally leading to mixed oral biofilm formation (Jenkinson and Lamont 2005). Inter-bacterial binding assists colonisation and may also promote nutritional relationships between organisms (Jenkinson and Lamont 1997).

Several adhesins of *E. coli* have been studied in detail (Wright and Hultgren 2006). Common features of these adhesins related to urinary tract infections (UTI), are that they are fimbrial, for instance part of F1C, P, S or type 1 fimbriae, and that they recognise carbohydrate structures such as GalNAcβ1–4Gal, galabiose, sialylated ligands or α-linked mannose derivatives, respectively (Ott et al. 1987, Khan et al. 2000). A single *E. coli* strain can express several adhesins as part of different fimbriae, which enables adhesion to a range of receptors (Westerlund-Wikström and Korhonen 2005). The P-fimbriae of *E. coli* studied in the present work contain a fimbrial tip-associated PapG adhesin that recognises galabiose (Strömberg et al. 1990). There are four classes of PapG adhesins, PapGI–IV, each binding to distinct globo series isoreceptors. These isoreceptors differ in having a variable number of *N*-acetylgalactosamine moieties added to galabiose and possibly having additional sialic acid residues. Host tissues possess particular isoreceptors and PapG classes prefer certain isoreceptors, which leads to host tropism of P-fimbriated *E. coli.* The binding preference of PapGI adhesin is globotriaosylceramide (GbO_3, abundant on human uroepithelial cells), PapGII prefers globoside (GbO_4, abundant on human uroepithelial cells) and PapGIII Forssman antigen (GbO_5, predominant on canine, but not human uroepithelial cells). (Westerlund-Wikström and Korhonen 2005, Lane and Mobley 2007). The binding specificity of PapGIV is still unknown.

A predominant feature of streptococcal adhesins, even if they differ in size and conformation, is that they often contain amino acid repeat motifs (Hollingshead et al. 1986, Talay et al. 1994, Handley et al. 2005). Different strains have variable numbers of repeats in these motifs, which may hamper vaccine development as the antibodies produced may not recognise all the repeat-number variants. Another typical feature of streptococcal adhesins and other surface proteins is that they break down into discrete fragments. The proteolytic degradation of the proteins also hinders adhesion research and vaccine development. Furthermore, the simultaneous effects of multiple adhesins may be complicated and, for instance, deletion of a single adhesin gene may only have a minor effect on bacterial adhesion. In general, Gram-positive bacteria including streptococci have a more extensive and complex surface proteome as compared to Gram-negative bacteria. (Nobbs et al. 2009) A versatile surface protein collection enables bacteria to have multiple interactions with various host molecules depending upon the receptors available.

2.7. Carbohydrate-specific adhesins and related proteins in streptococci

Carbohydrates have been identified to be the host receptors for bacterial binding in many instances (Table 5). The oligosaccharide chains of glycoproteins and -lipids in different cell types of the body are ideal receptors for bacterial attachment, not only because of the great variety of oligosaccharide structures but also because of the specificity of bacterial lectin-like adhesins (Sharon 1987, Karlsson 1989). A striking example of a minor difference that has a major consequence is the *E. coli* strain K99 which binds to *N*-glycolylneuraminic acid but not to *N*-acetylneuraminic acid containing glycolipids. A single hydroxyl group is the only difference between these two sugars, and this difference defines the host specificity of the pathogen. *N*-glycolylneuraminic acid residues are found in porcine, bovine, and ovine (sheeps) but not in the human glycoproteins or glycolipids. (Kyogashima et al. 1989) Carbohydrate recognition is also dependent on the orientation and conformation of the saccharide. Factors affecting the recognition can be identified by studying synthetic saccharide derivatives or isomers. (Haataja et al. 1994) Common monosaccharides recognised in host oligosaccharide chains of glycolipids and -proteins are galactose, mannose, glucose, fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine and sialic acids (Nobbs et al. 2009). A general feature in the structures of carbohydrate-binding proteins is pairs or triplets of aromatic amino acid residues that may lead to the formation of hydrophobic pockets that can enclose non-polar parts of sugars. Another common feature is a secondary structure where β-pleated regions are flanked by helices. (Talay et al. 1994)

It was estimated by atomic force microscopy that the force required to unbind lectin–carbohydrate bonds, formed by multiple lectin molecules on the surface of a single cell, was 120 pN. By comparison, the force required to unbind a biotin–avidin bond is about 160 pN. (Touhami et al. 2003) Adhesion mediated by protein–carbohydrate pairs is strengthened by the usually large number of adhesin–receptor pairs involved (Zopf and Roth 1996).

Table 5. Carbohydrate receptor specificities of streptococcal adhesins

Specificity	Adhesin	Species	Reference
Glucans	Glucan-binding lectin (GBL)	*S. cricetus*	Drake et al. 1988
	Glucan-binding protein 2 (GBP-2)	*S. sobrinus*	Smith et al. 1998
	Glucan-binding protein A (GbpA)	*S. mutans*	Russell et al. 1985, Matsumura et al. 2003
	Glucan-binding protein C (GbpC)	*S. mutans*	Sato et al. 1997, Lynch et al. 2007
Sialic acid	Choline-binding protein A (CbpA)	*S. pneumoniae*	Rosenow et al. 1997
	Platelet-binding protein (GspB)	*S. gordonii*	Bensing et al. 2004
	Haemagglutinating streptococcal antigen (Hsa)	*S. gordonii*	Bensing et al. 2004, Loimaranta et al. 2005
	M type 6 protein	*S. pyogenes*	Ryan et al. 2001
	Sialic acid–binding protein (SABP)	*S. mitis*	Murray et al. 1986
	Serine-rich protein A (SrpA)	*S. sanguinis*	Plummer et al. 2005
Glycosaminoglycans	Alpha C protein	*S. agalactiae*	Baron et al. 2004
	Glycosaminoglycan-binding protein (GAG-BP)	*S. pyogenes*	Winters et al. 1993
	M proteins	*S. pyogenes*	Frick et al. 2003
Galabiose	Streptococcal adhesin P (SadP)	*S. suis*	Haataja et al. 1993, the present study (I)

Adhesins of oral streptococci

In oral streptococci, the ability to synthesise extracellular glucans contributes to plaque formation and to the subsequent development of dental caries. This ability is dependent on the function of glycosyltransferases and dextranase that are capable of converting dietary sucrose to water soluble (α-(1,6)-linked) and insoluble (α-(1,3)-linked) glucan (Sun et al. 1995). Glucan-binding lectins (GBL) of *S. cricetus* (Drake et al. 1988, Singh et al. 1996) as well as glucan-binding proteins of *S. sobrinus* (GBP1–5) (Landale and McCabe 1987, Ma et al. 1996, Smith et al. 1998) and *S. mutans* (GbpA–D) (Russell et al. 1985, Smith et al. 1994, Sato et al. 1997, Matsumura et al. 2003, Lynch et al. 2007) have a high affinity for glucans rich in α-(1,6) linkages. Although all these proteins bind to glucans, only GBL, GBP-2, GbpA and GbpC have been shown to function as adhesins (Drake et al. 1988, Smith et al. 1998, Matsumura et al. 2003, Lynch et al. 2007). α-(1,6) linkages are not the

only prerequisite of binding since for instance the GBL of *S. cricetus* recognises glucans that are at least nine hexose residues long and linear (Drake et al. 1988). Since only the longer α-(1,6)-linked glucans are recognised, dietary carbohydrates such as glucose, maltose or starch do not inhibit the adhesion of these lectins. The specificity of glucan-binding proteins and lectins contributes to the colonisation of oral pathogens in glucan-containing plaque in oral cavity (Drake et al. 1988).

In addition to the glucan-binding proteins, the AgI/II family adhesins also participate in dental plaque formation. AgI/II family adhesins are produced by most oral streptococci, as well as *S. pyogenes*, *S. agalactiae* and *S. suis*. (Brady et al. 2010) These proteins adhere to salivary constituents, host cell matrix proteins and other oral bacteria (Jenkinson and Demuth 1997). Adhesins characterised to bind to surface-bound salivary glycoprotein gp340 include SpaP (or P1) of *S. mutans* (Kelly et al. 1989), SspA/SspB of *S. gordonii* (Jenkinson and Demuth 1997), Pas of *S. intermedius* (Jakubovics et al. 2005), AspA (or Spy1325) and Spy0843 of *S. pyogenes* (Zhang et al. 2006, Loimaranta et al. 2009). The interactions of *S. mutans* and *S. gordonii* have been reported to be sialic acid–specific (Loimaranta et al. 2005). However, the AgI/II family adhesins are not exclusively carbohydrate-specific as peptide–peptide interactions with the backbone sequences of gp340 are also involved in the adhesion (Jakubovics et al. 2005) and the recognition may involve leucine-rich repeats in the proteins (Loimaranta et al. 2009).

S. sanguinis is the most common oral bacterium that causes infective endocarditis (Plummer et al. 2005) and *S. gordonii* is also an oral bacterium that can be further classified as a commensal viridans group streptococcus (Loimaranta et al. 2005). Haemagglutinating streptococcal antigen (Hsa) and platelet-binding protein of *S. gordonii* as well as serine-rich protein A of *S. sanguinis* belong to the serine-rich repeat polypeptides, which mediate binding to sialylated carbohydrates (Bensing et al. 2004, Plummer et al. 2005). However, there may be some diversity in the preferred epitopes to which these adhesins bind, for instance, the platelet-binding protein binds to either α-(2,3) or α-(2,6)-linked sialic acid, with a slight preference for α-(2,3) linkages (Bensing et al. 2004), whereas Hsa prefers SLe^x/3'sialyllactose–structures (Loimaranta et al. 2005). Hsa has been reported to aggregate platelets (Takahashi et al. 2004), which probably facilitates bacterial colonisation of platelet–fibrin vegetations in endocarditis. In addition to platelets, Hsa binds to erythrocyte surface sialoglycoproteins and causes haemagglutination, which may increase the severity of the disease (Yajima et al. 2008).

Sialic acid-recognising adhesins

Sialic acids are recognised also by other than oral streptococci since a sialic acid–binding protein has been identified also in *S. mitis* (Murray et al. 1986) and sialic acid recognition seems to be involved in the colonisation of pharyngeal epithelial cells by *S. pyogenes*. Namely, M type 6 protein binds *in vitro* to human pharyngeal cells, which contain α-(2,6)-linked sialic acid residues on their surface. The M proteins are major surface-expressed virulence factors of *S. pyogenes* and in addition to the sialic acid–containing cells, the M protein binds to sialic acid moieties on mucins, the main glycoproteins of mucus. (Ryan et al. 2001)

Most of the carbohydrate-specific adhesins listed in Table 5 contain an LPXTG motif and are therefore anchored to the cell wall peptidoglycan. Choline-binding protein A of *S. pneumoniae* is an exception since it does not contain the motif. This protein and other choline-binding proteins are non-covalently bound to the phosphoryl-choline of the bacterial cell wall teichoic acid. The choline-binding protein A binds to sialic acid and lacto-*N*-neotetraose. This binding seems contribute to pneumococcal adherence to cytokine-activated human cells and colonisation of the nasopharynx. (Rosenow et al. 1997)

Glycosaminoglycan-recognising adhesins

S. agalactiae colonises mucosal surfaces of the human gastrointestinal and female reproductive tracts. The bacterial surface alpha C protein interacts with host cell glycosaminoglycans. This interaction may be inhibited by sodium chlorate pre-treatment of the cells, which inhibits sulphate incorporation into glycosaminoglycans, or by degrading the heparan sulphate chains with heparitinases. Addition of soluble heparin, heparan sulphate or host cell-derived glycosaminoglycans also inhibit the bacterial binding. (Baron et al. 2004) The glycosaminoglycan-binding region has already been identified (Baron et al. 2007) and recent results from insect and mammalian infection models indicate that the interaction between alpha C protein and glycosaminoglycans is important for the establishment of central nervous system infections (Chang et al. 2011). A glycosaminoglycan-binding protein has also been found in *S. pyogenes* and it binds selectively to the basal laminae of human cardiac muscle (Winters et al. 1993). M proteins of *S. pyogenes*, one of which has already been discussed to bind to sialic acids, mediate binding to various forms of glycosaminoglycans, namely dermatan sulphate, highly sulphated fractions of heparan sulphate and heparin (Frick et al. 2003). Thus, the M proteins may present an example of bacterial surface proteins that possess multiple carbohydrate-binding specificities.

Carbohydrate-recognising enzymes

The biological role of cell-surface attached carbohydrate-degrading enzymes, such as fructosidase (Burne and Penders 1992), dextranase (Wanda and Curtiss 1994), hyaluronidase (Berry et al. 1994), *N*-acetylhexoseaminidase (Clarke et al. 1995) or neuraminidases (sialidases) (Manco et al. 2006), in streptococcal adhesion is uncertain. The main function of the carbohydrate-degrading enzymes seems to be to provide nutrients for the bacterium, but they may degrade the extracellular matrix to reveal receptors for bacterial adhesion or, as discussed below in the cases of β-galactosidase (Limoli et al. 2011) and pullulanase (Hytönen et al. 2003), these enzymes may provide protective and adhesive effects (Marion et al. 2011). Interestingly, a common feature in the structure of many glycosidases is carbohydrate-binding modules that target the enzymes to a particular substrate (Lammerts van Bueren et al. 2007). For instance, sialidases have sialic acid–specific carbohydrate-binding modules. Currently the carbohydrate-binding modules are classified into 64 families on the basis of amino acid sequence (see http://www.cazy.org/Carbohydrate-Binding-Modules.html).

It was recently suggested that the exoglycosidase β-galactosidase (BgaA) might contribute to the *S. pneumoniae* adherence to human epithelial cells. β-galactosidase could possibly enhance adhesion by cleaving sugars and revealing receptors on host cells but this does not seem to be the case since

pretreatment of host cells with β-galactosidase did not restore the adherence of a *bgaA* knockout mutant. The receptor of this adhesin has not been characterised in detail yet but it may be the glycan part of a glycosphingolipid since trypsin or proteinase K treatments of the epithelial cells did not abolish the bacterial adherence. (Limoli et al. 2011)

Pullulanases are another group of carbohydrate-degrading enzymes, whose role in virulence is not clear. Human oropharynx and lungs, the main sites of *S. pyogenes* and *S. pneumoniae* infection, contain abundantly intra- and extracellular α-glucans such as glycogen (Lammerts van Bueren et al. 2007, Shelburne et al. 2009). In the lungs, pneumococci adhere preferentially to surfactant-producing alveolar type II cells, and host glycoproteins are probable adhesion receptors of both pathogens (Cundell et al. 1994, Hytönen et al. 2003). At least *S. pneumoniae* is known to be able to invade host cells without killing them (Kadioglu et al. 2001). Once the bacteria are inside alveolar cells, surface-anchored pullulanases from *S. pyogenes* (PulA) and *S. pneumoniae* (SpuA) adhere multivalently to intracellular lung glycogen and degrade it (Lammerts van Bueren et al. 2007). The *pulA* gene encoding pullulanase was found in all *S. pyogenes* strains tested, also in strains that were unable to degrade starch, whereas a starch-degrading phenotype correlated with the presence of the *amyA* gene. Therefore, degrading starch may not be the main function of *S. pyogenes* cell surface pullulanase. The *amyA* gene associated with the starch-degrading phenotype encodes cyclomaltodextrin α-glucanotransferase that degrades α-glucans into cyclic chains composed of glucose. Interestingly, it was suggested that the cyclic maltodextrins produced by AmyA decrease transepithelial resistance and therefore facilitate translocation and invasion of the host. As an energy source, the pathogen may utilise linear maltodextrins degraded by human α-amylase. (Shelburne et al. 2009) These findings undermine the view that providing a carbon source is the main function of surface-anchored pullulanases. Conversely, a homologous protein to PulA and SpuA has been found in *S. agalactiae*, and this pullulanase (SAP) has an important role in α-glucan metabolism (Santi et al. 2008).

Another interesting cell-surface attached group are the glycolytic enzymes such as glyceraldehyde-3-phosphate-dehydrogenase (Pancholi and Fischetti 1992), enolase, phosphoglycerate kinase, phosphoglycerate mutase, triosephosphate isomerase (Kinnby et al. 2008), 6-phosphogluconate dehydrogenase (Daniely et al. 2006) and fructose bisphosphate aldolase (Blau et al. 2007). Some of them have been found also on the surface of *S. suis* (Table 4). These proteins may be called moonlighting proteins since they seem to have another function in addition to their role in glycolysis (Jeffery 1999). Glycolytic enzymes have been reported to bind to fibronectin, lysozyme, myosin and/or actin (Pancholi and Fischetti 1992) as well plasminogen (Kinnby et al. 2008). Even if 3-phosphate-dehydrogenase has not been reported to recognise carbohydrates, it appears to be an adhesin of *S. suis*, since adhesion to porcine tracheal rings was reported to be inhibited by pre-incubation with recombinant 3-phosphate-dehydrogenase (Brassard et al. 2004).

Unidentified adhesins

In addition to the bacteria in Table 5, also other streptococci have been reported to recognise carbohydrates, for instance, *S. pneumoniae* specifically binds to GalNAcβ1–4Gal (Krivan et al. 1988) and *S. suis* strains have an adhesion activity recognising sialylated poly-*N*-acetyllactosamine

glycans (Liukkonen et al. 1992), but the adhesins responsible for the binding to these carbohydrates are not known. Carbohydrate microarrays have become available for bacterial surface lectin specificity studies (Disney and Seeberger 2004). New techniques now available hold out hope of faster discovery of carbohydrate-recognising adhesins.

2.8. Galabiose-specific adhesion of S. suis

Bacteria that have sugar-specific lectins on their surface can attach to red blood cells and cause agglutination of the cells *in vitro*. This phenomenon is called haemagglutination and it is based on multivalent recognition of the cell surface carbohydrates by the bacterial protein molecules. (Hansen et al. 1997). Haemagglutination has been used for the characterisation of bacterial carbohydrate recognition. A galabiose-specific adhesion activity of *S. suis* has been discovered by haemagglutination (Haataja et al. 1993). Galabiose-binding adhesins can be studied with haemagglutination since galabiose is present in the glycolipids representing the P blood group antigens P^k, P and P_1 (Table 6). For example, GbO_3 (CD77) is a neutral glycolipid that represents the P^k antigen (Haataja et al. 1993). Human and animal erythrocytes have different glycolipid compositions, and the predominant isoreceptors in leporine (rabbit), human and ovine (sheep) erythrocytes are GbO_3, GbO_4 or GbO_5, respectively (Haataja et al. 1994). Native as well as glycosidase-modified erythrocytes have been used in haemagglutination studies (Kurl et al. 1989). Depending on the binding specificity, glycosidase treatment may enhance or inhibit haemagglutination. The effect of glycosidase treatment can also be indirect, for instance, sialidase treatment of human erythrocytes exposes GbO_3 and reduces the negative charge of the erythrocyte surface (Haataja et al. 1993). Interestingly, tumor cell lines derived from cancer tissues such as astrocytoma, renal cell carcinoma, colon cancer and breast cancer have been also reported to express GbO_3 (CD77) (Devenica et al. 2011).

Table 6. Galabiose-containing glycolipids and corresponding blood group antigens

Glycolipid	Structure	Blood group antigen
GbO_3, globotriaosylceramide	Galα1–4Galβ1–4Glcβ1–1'Cer	P^k
GbO_4, globoside	GalNAcβ1–3Galα1–4Galβ1–4Glcβ1–1'Cer	P
GbO_5, Forssman antigen	GalNAcα1–3GalNAcβ1–3Galα1–4Galβ1–4Glcβ1–1'Cer	Forssman
	Galα1–4Galβ1–4GlcNAcβ1–3–Galβ1–4Glcβ1–1'Cer	P_1

Galabiose is also recognised by other bacterial proteins (Table 7). GbO_3 has already been discussed to be the binding preference of *E. coli* PapGI adhesin, and *Pseudomonas aeruginosa* lectin I is another bacterial adhesin that preferentially binds to this glycolipid (Blanchard et al. 2008). The galabiose-unit is also recognised by some bacterial toxins, which according to definition made by Sir E. R. Lankester in 1886, are "poisons for animals produced by pathogenic bacteria" (Popoff and Alouf 2004). Shiga toxin, as well as verotoxin 1 (Shiga-like toxin, SLT-I), verotoxin 2 (SLT-II) and

verotoxin 2c (SLT-IIvh [human variant]), recognise GbO_3, whereas verotoxin 2e (Pig edema disease toxin, SLT-IIvp [pig variant]) binds to GbO_4 and to a lesser extent to GbO_3 (Lindberg et al. 1987, Lingwood et al. 1987, Waddell et al. 1988, DeGrandis et al. 1989, Samuel et al. 1990). Unlike the galabiose-specific adhesin of *S. suis*, the interactions of these toxins cannot be inhibited by monosaccharides (Haataja et al. 1994). As galabiose is present in many naturally occurring glycoconjugates and is recognised by several lectins, adhesins and toxins, it is a promising target for anti-adhesion therapy (discussed in section 2.10.).

Table 7. Galabiose-binding proteins

Galabiose-binding protein	Organism	Preferred glycolipid	Reference
Adhesins			
Lectin I	*Pseudomonas aeruginosa*	GbO_3	Blanchard et al. 2008
PapGI adhesin	*Escherichia coli*	GbO_3	Strömberg et al. 1990
SadP	*Streptococcus suis*	GbO_3	Haataja et al. 1993, the present study (I)
Toxins			
Shiga toxin	*Shigella dysenteriae*	GbO_3	Lindberg et al. 1987
Verotoxin 1	*Escherichia coli*	GbO_3	Lingwood et al. 1987
Verotoxin 2	*Escherichia coli*	GbO_3	Waddell et al. 1988
Verotoxin 2c	*Escherichia coli*	GbO_3	Samuel et al. 1990
Verotoxin 2e	*Escherichia coli*	GbO_4	DeGrandis et al. 1989

The galabiose-specific adhesion of *S. suis* has been studied for quite some time. Already in 1989, it was recognised that the *S. suis* haemagglutination is heat, trypsin and pronase labile, which suggests that the adhesin involved is a protein (Kurl et al. 1989). The adhesin was thought to have been identified in 1995 (Tikkanen et al. 1995), but the protein was later identified as Dps-like peroxide resistance protein (Dpr) (Pulliainen et al. 2003) and it is not the galabiose-specific adhesin (I) even though Dpr binds to carbohydrates and is located on the cell surface (Tikkanen et al. 1996). For the present, the carbohydrate-binding property of Dpr remains unknown.

Two *S. suis* variants of the galabiose-specific adhesin have been identified and they both prefer the disaccharide in terminal position. One of the variants, referred to as type P_N, is inhibitable by galactose and *N*-acetylgalactosamine and the other one (type P_O) is inhibitable by galactose only. Another difference between the types is that the P_O type seems to be more dependent on the terminal position of galactose than the P_N strains. In the galabiose structure, the essential key hydroxyls for *S. suis* type P_N adhesin binding are HO-4', HO-6', HO-2 and HO-3 (Figure 4) and in addition to these four essential hydroxyls, P_O strains are in weak interactions also with HO-3' and HO-6 in the disaccharide (Figure 4). (Haataja et al. 1994)

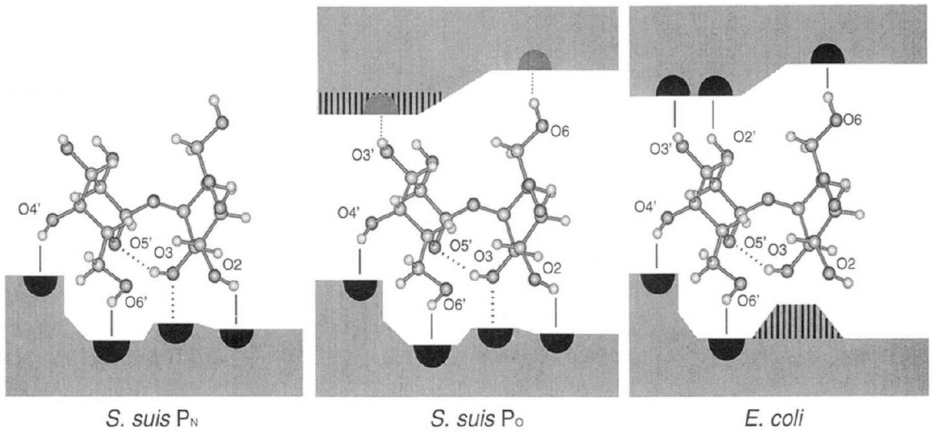

Figure 4. Binding pattern of the galabiose-recognising adhesins in *S. suis* types P_N and P_0, and in *E. coli*. Figure kindly provided by S. Haataja (Haataja et al. 1994).

S. suis binds to the glycolipid GbO_3 (Table 6), but not GbO_4 or GbO_5 (Haataja et al. 1993), which is different from P-fimbriated *E. coli* discussed earlier as the PapG adhesins bind all the three glycolipids. There is also difference in the hydrogen bonding pattern of galabiose-binding between these bacteria. As for the adhesin in P-fimbriated *E. coli* strain, it recognises HO-2', HO-3', HO-4', HO-6' and HO-6 hydroxyls in the disaccharide (Figure 4). Therefore, these two bacteria seem to recognise the disaccharide from different sides. Because the adhesins do not recognise just a simple monosaccharide but an extended sugar sequence, bacteria are able to bind receptor structures at the cell surface more specifically. Thus, bacteria may be better targeted to certain tissues (Haataja et al. 1994).

The inhibition efficiency of various natural and synthetic carbohydrate derivatives of *S. suis* has been studied in haemagglutination and solid-surface binding assays (Haataja et al. 1993, Haataja et al. 1994, Hansen et al. 1997). These inhibition studies have indicated the essential role of the terminal α-galactose. Galabiose was found to be a 2000-fold better inhibitor that the other galactose disaccharides containing α-(1,3) or α-(1,6) linkages. The trisaccharide Galα1–4Galβ1–4Glc was an even better inhibitor than the disaccharides but further extensions to the reducing end did not increase the inhibitory potential. The terminal α-galactose moiety is probably essential for the binding as lactose (Galβ1–4Glc) is inactive as adhesion inhibitor. (Haataja et al. 1993) In the trisaccharide the same hydroxyls as in the disaccharide, namely the 4'', 6'', 2' and 3'-hydroxyls, are critical for the inhibitory activity. In contrast, the hydroxyls of the glucose moiety are not essential for binding. (Haataja et al. 1999) Therefore, many aspects of the interaction of the galabiose-specific adhesin of *S. suis* had already been characterised before the present study but the identity of the adhesin molecule had remained obscure. In the present study this adhesin was finally identified.

2.9. Adhesion inhibition by multivalent carbohydrates

Carbohydrates are not likely to be immunogenic or toxic, quite the contrary: many of them are natural components of body fluids and cell surfaces (Sharon and Ofek 2000, Ofek et al. 2003). For instance, human breast milk contains many saccharides that inhibit adhesion (Kunz et al. 2000). However, the interactions between adhesin proteins and carbohydrates are often weak and therefore high concentrations of simple sugars are needed to inhibit adhesion (Pieters 2011). Inhibitor concentrations needed for complete adhesion inhibition are typically in the micro- to millimolar region, which probably is too high for practical applications (Hansen et al. 1997). Nature overcomes the weakness of a single interaction by multiple binding, and hence bacteria have developed an ability to bind to more than one host receptor simultaneously (Jenkinson and Lamont 1997). Thus multivalency is evidently very important in the interactions of carbohydrates and proteins. Synthetic polyvalent macromolecules can be designed to simultaneously engage many adhesin molecules at the bacterial surface to form a stable complex (Zopf and Roth 1996). Multivalent systems can be created by covalently linking carbohydrates together utilising polymeric carriers (Pieters 2011). These polyvalent oligosaccharides are orders of magnitude better adhesion inhibitors than their monovalent forms (Hansen et al. 1997, Joosten et al. 2004). However, multivalent drug design is likely to produce molecules of high molecular weight that may not easily cross biological membranes and therefore their use in therapy applications may be limited (Hansen et al. 1997).

In mycobacteria, adhesins are not randomly distributed over cell surface, but concentrated in areas called adherosomes (Dupres et al. 2005). Adhesins could be unevenly presented also in other bacteria, and therefore it may be advantageous to design multivalent inhibitors corresponding to the topology of adherosomes. The distance between the carbohydrate binding sites probably varies in different adhesins. Individual binding sites are likely to be well separated if adhesins are located at the tip of separate fimbriae. Long polymers may be needed to inhibit this kind of adhesion. The distance between binding sites may be shorter if adhesin proteins are associated on the cell surface, and the binding sites could be tightly spaced if there are recognition subsites within a single protein. Multivalent systems have the advantage of a chelate effect and have favourable entropy as carbohydrate ligands bridged by suitable spacers simultaneously bind to neighbouring binding sites in the adhesin. Entropic barriers are thought to be reduced after binding of the first ligand. (Pieters 2007)

Some advancement has been made in the design of adhesion inhibitors as, for example, a tetravalent galabioside inhibitor of *S. suis* adhesion with rather long and flexible aromatic linkers to allow effective adhesion to the bacterial surface proteins was 600-fold more potent inhibitor than its monovalent counterpart (Hansen et al. 1997). Recently, flexible and dynamic multivalent structures have been developed. As an example of many possibilities, glyco-pseudopolyrotaxanes are structures where carbohydrates lie like beads on a string. In these structures, carbohydrates are able to freely rotate about and move along the polymer backbone to find optimal positions and orientations for optimal interactions with adhesins. The pseudopolyrotaxanes had about 300 times higher inhibitory potency than the monomeric carbohydrates. (Kim et al. 2010) Inhibitors can be designed very precisely if the three-dimensional structure of ligand–receptor pair is known. The

crystal structure of Shiga-like toxin, which enters mammalian cells by simultaneously binding to at least five oligosaccharide residues on host cell surface, has been determined. The structure was used to design an inhibitor with correct dimensions for binding to the toxin. The design of the Shiga-like toxin inhibitor was very successful as the *in vitro* inhibitory activity of the oligovalent carbohydrate ligand was million-fold higher than that of univalent ligands (Kitov et al. 2000).

Adhesion inhibitors are searched for also among berries and other plants (Toivanen et al. 2010, Bensch et al. 2011). Cranberry and lingonberry extracts possess anti-adhesion potential against P-fimbriated *E. coli* and *S. suis*. Carbohydrates may contribute to the adhesion inhibition but anthocyanidin/proanthocyanidin compounds found in cranberry are thought to be the main reason for the adhesion inhibition. (Toivanen et al. 2010) In an extensive review over cranberry and UTI, it was concluded that there is no evidence that cranberry can be used to treat UTIs. Furthermore, the preventive efficacy of cranberry has been modest at best in clinical trials, and there have been adverse events such as gastrointestinal intolerance, weight gain and drug–cranberry interactions (inhibition of the cytochrome P450-mediated drug metabolism by flavonoids). (Guay 2009)

2.10. Adhesin-based therapy and vaccination

The adhesion of bacteria to their host receptors can be inhibited by soluble ligands that mimic the receptors. This tactics to prevent or treat infections, called anti-adhesion therapy, holds promise for battle against pathogens in the era of increasing antibiotic resistance (Ofek et al. 2003). Bacteria may be eradicated by the administration of antibiotics. However, killing of bacteria brings on selection pressure which promotes the development of resistance (Figure 5). In anti-adhesion therapy, bacteria remain alive but they are rendered non-infective and removed by natural cleansing mechanisms such as mucus and ciliary movement in the respiratory tract or urinary flow in the urinary tract (Sharon 2006, Pieters 2007). Thus, there is no selection of resistant bacteria. Since the chemical structures of the adhesion inhibitors are similar to the natural attachment ligands used by bacteria, it is unlikely that resistance would give the bacteria the capability to defeat the inhibitory effect of the anti-adhesive drug without deteriorating their ability to adhere to the host cell (Hansen et al. 1997).

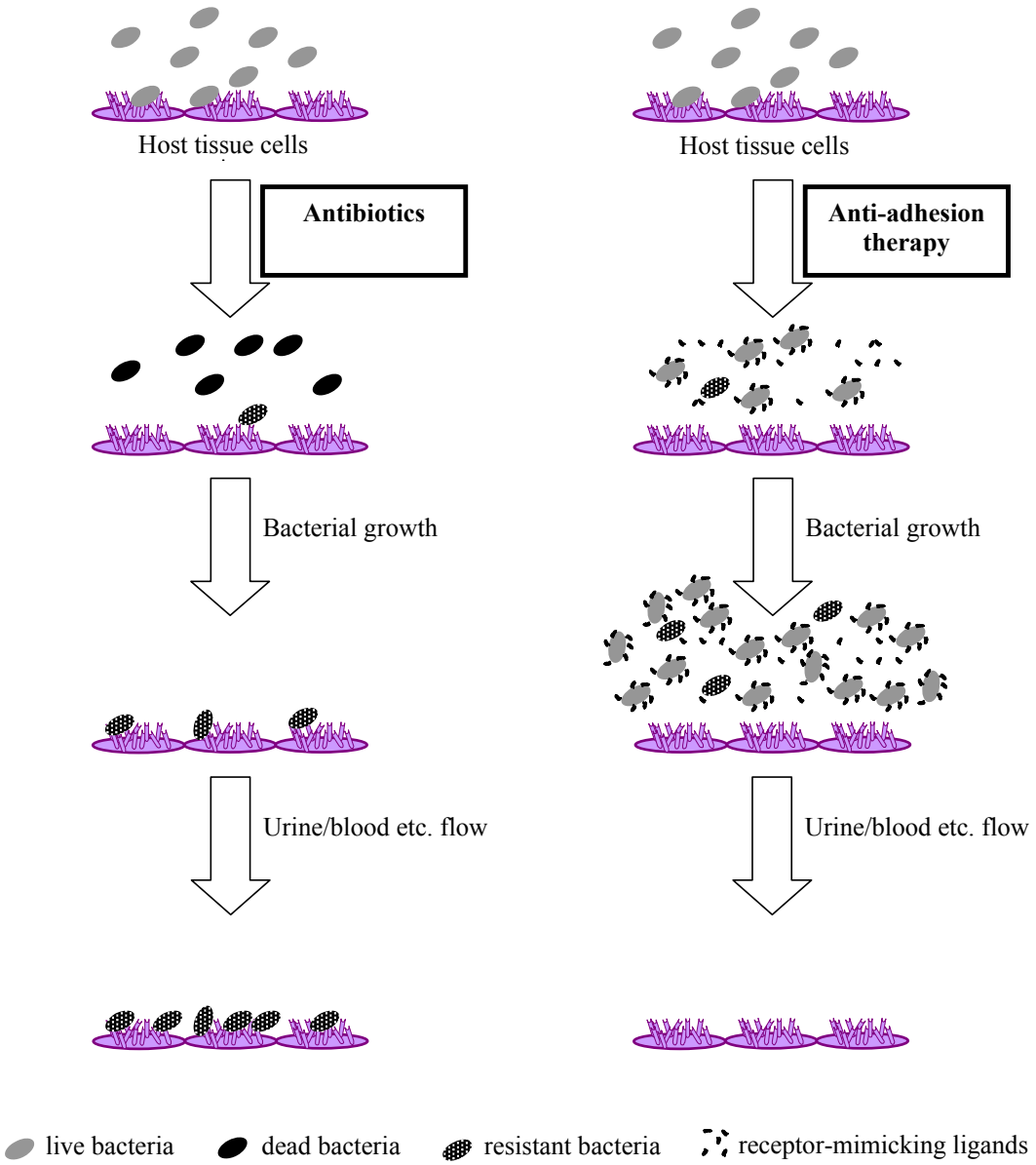

Figure 5. Comparison of the effects of antibiotics and anti-adhesion therapy on bacterial infection. Antibiotics kill most bacteria, which promotes the development of resistance. Anti-adhesion therapy prevents live bacteria from adhering to the carbohydrate receptors on host cells. As bacteria are not killed, there is no selection of resistant bacteria. When antibiotics are used to treat infection, mutant bacteria develop resistance against antibiotics but still have the capacity to adhere to the host cells. In the case of anti-adhesion therapy, bacteria may develop resistance against carbohydrates used in the therapy if the carbohydrate-recognising adhesin is mutated, but at the same time mutant bacteria lose their capacity to bind to the host cells.

Only a few human clinical trials on anti-adhesion therapy have been reported so far, and these studies have not given the results desired (Sharon 2006). In relation to streptococci, a phase II clinical trial has been conducted where 500 infants aged 10–24 months were given prophylactically the pentasaccharide 3′-sialyllacto-*N*-neotetraose that was administered by a nasal spray for 3 months. However, the colonisation of the nasopharynx by *S. pneumoniae* or *Hemophilus influenzae* was not reduced, nor was the frequency of acute otitis media infections. The absence of efficacy could be attributed to insufficient doses of the inhibitor as the 50 % inhibitory concentration is as high as 1–2 mmol/L, or to the presence of multiple lectins, that would require a cocktail of saccharides in order that anti-adhesion therapy could be effective. (Ukkonen et al. 2000)

In addition to carbohydrates, the adhesion of the bacteria may be inhibited by an adhesin analogue or more practically by a peptide fragment of the adhesin that binds to the host receptor and blocks the adhesion of the bacteria. For instance, the binding of a *S. mutans* adhesin SpaP to glycoprotein gp340 has been inhibited by a 20-amino-acid peptide fragment corresponding to twenty consecutive amino acid residues of the adhesin (Kelly et al. 1999). Peptides have also been used to inhibit the assembly of fimbriae, which leads to a situation where bacteria no longer are able to adhere and cause disease (Svensson et al. 2001). In addition to peptides, also other molecules have been designed to interfere with the interaction of fimbrial proteins and chaperones (Chorell et al. 2012).

Immunisation with bacterial adhesins has been used in order to provoke lasting immune responses against pathogens and to inhibit adhesion (Wizemann et al. 1999). Antibodies against P-fimbriae (Roberts et al. 1984), type 1 fimbrial adhesin (Abraham et al. 1985), FimH adhesin (Langermann et al. 1997) and Dr fimbrial adhesin (Goluszko et al. 2005) have been protective against *E. coli* urinary tract infections in experimental animal models. Intranasal immunization with a fragment of fimbrial-tip adhesin MrpH conferred effective protection against the UTI-causing pathogen *Proteus mirabilis*, which is also associated with urolithiasis (Li et al. 2004). In Gram-positive bacteria, vaccination of mice with a recombinant fragment of a collagen adhesin has been found to provide protection against otherwise lethal *Staphylococcus aureus* infections (Nilsson et al. 1998), intranasal vaccination with the fibronectin-binding protein SfbI provided protection against *S. pyogenes* (Guzman et al. 1999) and mucosal immunisation with a fragment of antigen I/II adhesin inducted protective immunity against *S. mutans* (Huang et al. 2001). Recently, mice immunised with a laminin-binding adhesin of *Salmonella enterica* serovar Typhi were protected against subsequent bacterial challenge. Antibodies against this adhesin have also been found in the sera of naturally infected humans. (Ghosh et al. 2011) However, it is not known if the antibodies produced are binding to the receptor binding domains in the adhesins or to the vicinity of the domains so that the adhesion is inhibited by steric hindrance, or if the protection is exclusively caused by the immune response elicited by antibodies on the bacterial surface.

Adhesins are attractive targets for vaccine development since adhesin-based vaccines may induce production of antibodies that prevent the primary stage of infection before the invasion of the host has occurred (Wizemann et al. 1999). Adhesins are thought to reside outside the bacterial cell wall and the capsule, available to interact with protective antibodies, which would be an advantage of adhesin-based vaccines. However, a major problem with adhesin vaccines is that adhesins, like other cell surface structures, are variable and therefore antibodies induced by vaccination may be

directed against a subclass of bacteria. Polyvalent vaccines in which carefully chosen regions of multiple proteins representing multiple strains are linked together may be a solution to this problem (Bronze and Dale 2010). It is a rather difficult task to produce protective immune responses against mucosal pathogens. The delivery route and adjuvants are especially relevant in the mucosal immunisation (Pavot et al. 2012). However, antibodies produced through other than mucosal route may also be released into external secretions since for instance in the case of *E. coli* FimH adhesin vaccination considerable amounts of IgG antibodies specific to FimH were found in the urine of mice previously immunised subcutanously with the vaccine (Langermann et al. 1997). Another obstacle of vaccine development against streptococci is that many pathogenic strains are antigenically related to commensal bacteria and therefore antibodies induced by vaccination may upset the balance between the commensals and the host (Jenkinson and Lamont 1997). In addition, streptococcal antigens have induced antibodies that cross-react with host proteins, particularly in heart tissue, or generate immune complexes that may cause acute glomerulonephritis (Lymbury et al. 2003, Batsford et al. 2005). These issues may be addressed by carefully choosing the antigens, delivery method, adjuvant and immunisation schedule. (Jenkinson and Lamont 1997)

In addition to vaccinations and antimicrobial agents including anti-adhesion therapy, replacement therapy is another approach suggested to fight infectious diseases. The aim of replacement therapy is to create commensal strains that colonise the host organism and replace potentially pathogenic strains in the host flora. Such commensal strains, improved by genetic engineering, should have enhanced colonisation potency (including appropriate adhesion activity) and possibly also an ability to produce bacteriocins, which are ribosomally synthesised small antimicrobial peptides produced by bacteria (Kjos et al. 2011). Such a strain is expected to have a survival benefit at the expense of pathogenic streptococci. (Jenkinson and Lamont 1997) However, it seems that the real life−applications of replacement therapy are still under development (Allaker and Douglas 2009).

Irrespective whether carbohydrates, adhesin analogues or antibodies are used, anti-adhesion therapy is complicated by the fact that most pathogens are likely to possess multiple adhesins (Hasty et al. 1992). To overcome this, it will probably be necessary to simultaneously use more than one agent to specifically inhibit each type of adhesin. (Ofek et al. 2003) In order to develop novel adhesion inhibitors and in order to understand how adhesion contributes to the emergence of bacterial disease, there must be knowledge of the molecular interactions between the bacterial adhesins and host cells. Identification and cloning of adhesins are the basis for the development of novel drugs and vaccines inhibiting adhesion.

3. AIMS OF THE PRESENT STUDY

The purpose of the research was to study the galabiose-specific adhesion of *Streptococcus suis,* and to use this adhesion activity as a target for inhibition of bacterial binding and for specific detection.

The detailed aims were:

• To identify the molecule responsible for the galabiose-specific adhesion of *Streptococcus suis*.

• To study the specificity of the interactions between the bacterial adhesin and the glycoconjugate receptor.

• To investigate multivalent galabiose derivatives as inhibitors of adhesion.

• To develop an approach for the specific detection of bacteria which carry an adhesin for galabiose.

4. MATERIALS AND METHODS

The detailed description of the materials and methods used in the present study is given in the original publications I–IV.

4.1. Bacterial strains, plasmids and primers

The bacterial strains, plasmids and primers are listed in Tables 8, 9 and 10 respectively.

Table 8. Bacterial strains

Bacterial strain	Characteristics	Source/Reference
Escherichia coli		
BL21(DE3)pLysS	Expression strain, genotype: F^-, ompT, $hsdS_B(r_B^- m_B^-)$, gal, dcm, (DE3), pLysS (Cam^R)	Novagen, WI, USA
DH5α	General purpose host strain for cloning, genotype: F^-, Φ80*lacZ*ΔM15, Δ(*lacZYA-argF*), U169, *rec*A1, *end*A1, *hsd*R17, (rK^-, mK^+), *pho*A, *sup*E44, λ^-, *thi*-1, *gyr*A96, *rel*A1	Meselson and Yuan 1968
HB101	Host strain for cloning, genotype: F^-, *hsd*S20(r_B^- m_B^-), *xyl*5, l^-, *rec*A13, *gal*K2, *ara*14, *sup*E44, *lac*Y1, *rps*L20(str^r), *leu*B6, *mtl*-1, *thi*-1	AllianceBio, CA, USA
HB101 ($PapG_{J96}$)	Expresses the $PapG_{J96}$ adhesin	Lindberg et al. 1984
HB101 ($PapG_{AD110}$)	Expresses the $PapG_{AD110}$ adhesin	van Die et al. 1983
HB101 ($PrsG_{J96}$)	Expresses the $PrsG_{J96}$ adhesin	Lund et al. 1988
Lactococcus lactis		
MG1363	Culture originally from the National Collection of Dairy Organisms, England	Gasson 1983
Streptococcus gordonii		
DL1 (Challis)	Origin not recorded, possibly human blood	M. Kilian (Århus University, Denmark)

Table 8. Bacterial strains (continued)

Bacterial strain	Characteristics	Source/Reference
Streptococcus suis		
D282	Wild-type strain, serotype 2, HA type P_N, isolated from a pig with clinical signs of meningitis	Vecht et al. 1989
D282-*mits*	*mits*::pSF151, Kan^r, *mits*-gene inactivated by insertional mutation	I
D282-*sadP*	*sadP*::pSF151, Kan^r, *sadP*-gene inactivated by insertional mutation	I
D282-*srtA*	*srtA*::pSF151, Kan^r, *srtA*-gene inactivated by insertional mutation	I
D282-Δ*dpr*	Spcr, *dpr*-gene inactivated by allelic replacement mutantion	Pulliainen et al. 2003
D282-33	*dpr*::pKUN19, Spc^r, *dpr*-gene inactivated by insertional mutation	Pulliainen et al. 2005
D282-981782	981782::pSF151, Kan^r, *ssu981782*-gene inactivated by insertional mutation	I
P1/7	Wild-type strain, serotype 2, sequence type 1, HA type P_N, isolated from ante-mortem blood culture from a pig dying with meningitis. Genomic sequence of the strain is available in Sanger Institute database.	Clifton-Hadley 1981
P1/7-*sadP*	*sadP*::pSF151, Kan^r, *sadP*-gene inactivated by insertional mutation	I
166’	Wild-type strain, serotype 2, non-haemagglutinating, isolated from a pig with meningitis	Berthelot-Herault et al. 2001
628	Wild-type strain, serotype 2, HA type P_N, isolated from human brain	Kurl et al. 1989

Table 9. Plasmids

Plasmid	Characteristics	Source/Reference
N(31–295)SadP	N-terminal 1 kb PCR product of *sadP* cloned in pET-28a	I
pBR322	4.4 kb cloning vector, used as control	Lindberg et al. 1984
pDL278	6.6 kb *E. coli*–Enterococcus shuttle vector	LeBlanc et al. 1992
pDL278-SadP	2.7 kb PCR product of *sadP* cloned in pLZ12Spc for complementation	Present study
pET-28a	5.4 kb expression plasmid with polyhistidine and S-Tag	Novagen
pLZ12Spc	3.9 kb *E. coli–S. pyogenes* shuttle vector, Spc^r	Husmann et al. 1995
pLZ12Spc-SadP	2.7 kb PCR product of *sadP* cloned in pLZ12Spc for complementation	Present study
pSadP	2.1 kb PCR product of *sadP* cloned in pET-28a	I
pSadP-KO	*sadP*::pSF151, internal 422 bp PCR product of gene *sadP* cloned in vector pSF151 for insertional inactivation	I
pSF151	3.5 kb plasmid capable of replicating in *E. coli* but not in streptococci because of the absence of a streptococcal origin of replication, Kan^r, 10 unique restriction sites	Tao et al. 1992
p-SrtA-KO	*srtA*::pSF151, internal 573 bp PCR product of gene *srtA* cloned in vector pSF151 for insertional inactivation	I
pUB1000	5.6 kb expression plasmid with lactococcal P1 promoter and the signal peptide coding sequence of *L. lactis usp45* followed immediately by a *Sal*I site	Heddle et al. 2003

Table 10. Primers

Primer	UniProtKB Gene name	Sequence	Comments and usage
3-pgk a	SSU0154	gaggagaagcccggtctatttttcagtcaaagc	Cloning (phosphoglycerate kinase to expression host *E. coli*)
3-pgk s	SSU0154	gacgacgacaagatggcaaaattgactgtt	Cloning (phosphoglycerate kinase to expression host *E. coli*)
Suispgkantisense	SSU0154	ttttttgaattctaccaccaccgataagaact (*Eco*RI underlined)	Mutagenesis (phosphoglycerate kinase gene knockout)
Suispgksense	SSU0154	tttttctgcaggttcttgttcgtgtggactt (*Pst*I underlined)	Mutagenesis (phosphoglycerate kinase gene knockout)
Eft a	SSU0482	gaggagaagcccggtttaagcttcgatttc	Cloning (elongation factor Tu to expression host *E. coli*)
Eft s	SSU0482	gacgacgacaagatggcaaaagaaaaatac	Cloning (elongation factor Tu to expression host *E. coli*)
Eft KO a	SSU0482	tttttgaattctaccacgaccagtgattgag (*Eco*RI underlined)	Mutagenesis (elongation factor Tu gene knockout)
Eft KO s	SSU0482	tttttctgcagggtacaattggacacgttga (*Pst*I underlined)	Mutagenesis (elongation factor Tu gene knockout)
Eftseq1	SSU0482	cttgtagtagcttcaactga	Sequencing (elongation factor Tu gene)
Eftseq2	SSU0482	ctgaaatggtaatgcctggtg	Sequencing (elongation factor Tu gene)
Mits a	SSU05_2069	gaggagaagcccggtttaccaaactggctcatc	Cloning (ABC-type amino acid transport/signal transduction system to expression host *E. coli*)
Mits s	SSU05_2069	gacgacgacaagatgaaattaaaaaaattgttt	Cloning (ABC-type amino acid transport/signal transduction system to expression host *E. coli*)
Mits KO a	SSU05_2069	tttttgaattccaaccttgatcaaaccagc (*Eco*RI underlined)	Mutagenesis (Mits gene knockout)
Mits KO s	SSU05_2069	tttttctgcaggtaggtgtacttgctgcatgt (*Pst*I underlined)	Mutagenesis (Mits gene knockout)
pUB-Eft s	SSU0482	acgcgtcgacatggcaaaagaaaaatacgat (*Sal*I underlined)	Cloning (elongation factor Tu to *S. gordonii* and *L. lactis*)
pUB-Eft a	SSU0482	acgcggatccagcttcgatttctgtaaccat (*Bam*HI underlined)	Cloning (elongation factor Tu to *S. gordonii* and *L. lactis*)

Table 10. Primers (continued)

Primer	UniProtKB Gene name	Sequence	Comments and usage
SadP a	SSU0253	tatctcgagttaacttgcttcgcctgtat (*Xho*I underlined)	Cloning (streptococcal adhesin P to expression host *E. coli*)
SadP s	SSU0253	gttggatccgaatcgctagaac (*Bam*HI underlined)	Cloning (streptococcal adhesin P to expression host *E. coli*)
SadP KO a	SSU0253	ttttttgaattcactttccactgaatctga (*Eco*RI underlined)	Mutagenesis (streptococcal adhesin P gene knockout)
SadP KO s	SSU0253	tttttttctgcagattcaacgacaacggtta *(Pst*I underlined)	Mutagenesis (streptococcal adhesin P gene knockout)
SadPLIC s	SSU0253	gacgacgacaagatgagcaagcagaaagtt	Sequencing (streptococcal adhesin P gene knockout strain)
N(31–328) a	SSU0253	tatctcgagttattattctttctcaagggtaatctc (*Xho*I underlined)	Cloning (N-terminal fragment of streptococcal adhesin P to expression host *E. coli*)
SAPSeq1	SSU0253	aaggtcattcaggagttg	Sequencing (streptococcal adhesin P gene)
SAPSeq2	SSU0253	ctgggaaggaaacttctc.	Sequencing (streptococcal adhesin P gene)
SAPSeq3	SSU0253	tattaacaactcagccagtc	Sequencing (streptococcal adhesin P gene)
SAPSeq4	SSU0253	ttatgagcaagcagaaag	Sequencing (streptococcal adhesin P gene)
SAPSeq5	SSU0253	gaaagtaacttttgatacaagc	Sequencing (streptococcal adhesin P gene)
SAPSeq6	SSU0253	gaatccaaaaccagagc	Sequencing (streptococcal adhesin P gene)
SRTA-3'-KO	SSU0925	ttttttgaattcacgtaggtgtaaacattggtc (*Eco*RI underlined)	Mutagenesis (sortase A gene knockout)
SRTA-5'-KO	SSU0925	tttttttctgcagcaagaagaagcgtaaaggttc (*Pst*I underlined)	Mutagenesis (sortase A gene knockout)
SRTA-PROM	SSU0925	tgccttggtagatgctgccg	Sequencing (sortase A gene knockout strain)
pSF151MCS a		tctgtgactggtgagtactt	Sequencing (inserts cloned to pSF151 plasmid)
pSF151 MCS		ttagctcactcattaggcac	Sequencing (inserts cloned to pSF151 plasmid)
pUB1000 s		gtcatcttacctcttttattag	Sequencing (genes cloned to pUB1000 plasmid)
T7 promoter		taatacgactcactataggg	Sequencing (genes cloned to pET-28a plasmid)
T7 terminator		gctagttattgctcagcgg	Sequencing (genes cloned to pET-28a plasmid)

4.2. Laboratory methods

The laboratory methods are summarised in Table 11.

Table 11. Laboratory methods

Method	Described in
Affinity purification of the galabiose-binding proteins[a]	I
LC-MS/MS analysis[b]	I
Inactivation by insertional mutation[c]	I
Cloning of *sadP*	I
Expression and purification of recombinant proteins[d]	I
DNA sequence analysis[e]	I
Detection of galabiose-binding proteins (Eastern blot)	I
Solid-phase glycoprotein binding assays[f]	I
Nanoparticle oligosaccharide inhibition assay[f]	I
Surface plasmon resonance analysis[g]	I, II
Haemagglutination assay	I, II, III
Synthesis of galabiose derivatives[h]	I, II, III, IV
Cell adhesion assay[i]	II
Preparation of glycoparticles	IV
Detection of bacteria by glycoparticles	IV

[a] The affinity purification matrix was produced by Docent Sauli Haataja at the University of Turku (I).

[b] The LC-MS/MS analyses were performed by Dr Anne Rokka at the Turku Centre for Biotechnology.

[c] *dpr* and *srtA* inactivation mutant strains were produced by Dr Arto T. Pulliainen at the University of Turku.

[d] The purification of recombinant SadP was optimised and performed by Docent Sauli Haataja at the University of Turku.

[e] The DNA sequence analyses were performed by Oso Rissanen at the Turku Centre for Biotechnology.

[f] The solid-phase glycoprotein binding assay and the nanoparticle oligosaccharide inhibition assay were performed by Docent Sauli Haataja at the University of Turku.

[g] The SPR assay was performed in I and guided in II by Dr Vuokko Loimaranta at the University of Turku.

[h] The galabiose derivatives were synthesised by the group of Professor Roland J. Pieters at the University of Utrecht (II, III, IV) and by the group of Professor Ulf J. Nilsson at the University of Lund (I).

[i] The cell adhesion assay was developed and performed by the late Dr A. Salam Khan at the University of Würzburg.

5. RESULTS

5.1. Initial attempts to identify the galabiose-specific adhesin

Heat, trypsin and pronase treatments abolish *S. suis* haemagglutination, and therefore it has been deduced that the galabiose-specific adhesin is a protein (Kurl et al. 1989). In order to identify the adhesin protein, various affinity proteomics methods described below were utilised. The methods described did not lead to the identification of the galabiose-specific adhesin but they may well be practicable to use in the identification of adhesins in general. Due to the sometimes very low abundance of adhesins (Ilver et al. 1998), an affinity enrichment step is usually required in the identification procedure. Furthermore, adhesins are often fimbrial or membrane bound, and do not resolve routinely in polyacrylamide gels. Therefore they may not be directly identifiable by electrophoresis and a subsequent fingerprinting by mass spectrometry and database search.

In the present study, a technique called receptor activity–directed affinity tagging was initially used. The strategy behind this technique is to add a biotin-tag to the adhesin molecule. The biotin-tag could subsequently be used for the purification and identification of the adhesin. (Ilver et al. 2003) The technique was based on a Sulfo-SBED probe that consisted of three parts: (i) a galabiose unit (ii) a photo reactive aryl azide group, which forms covalent bonds between the probe and neighbouring molecules such as the adhesin, and (iii) a cross-linker with a biotin group. The probe was bound by living bacteria and was therefore, in theory, in close proximity to the adhesin. The first probe was based on a 2-aminoethyl-galabiose molecule which was synthetised by the group of Professor Ulf J. Nilsson at Lund University. In addition to 2-aminoethyl-galabiose, pigeon ovomucoid (I), which is a glycoprotein that contains a terminal galabiose sequence in its glycans (Suzuki et al. 2006), was attached to the Sulfo-SBED. The third probe was based on a galabiose-bovine serum albumin (BSA) conjugate containing about twenty galabiose residues. This probe was synthetised by the group of Professor Roland J. Pieters at Utrecht University.

The adhesin candidates biotinylated utilising the pigeon ovomucoid-Sulfo-SBED probe were enriched from a mixture of *S. suis* proteins utilising magnetic streptavidin particles. After enrichment, the proteins were eluted from the particles and separated by polyacrylamide gel electrophoresis (PAGE). A single major band was observed and excised from the silver-stained gel and was subjected to mass spectrometry. Based on the peptide sequence, 3-phosphoglycerate kinase (3-pgk, SSU05_0157) was identified as a candidate adhesin. Interestingly, 3-pgk has previously been reported to be a plasminogen-binding adhesin of oral streptococci (Kinnby et al. 2008). The 3-pgk gene was cloned and the recombinant protein was expressed and purified. Recombinant 3-pgk inhibited haemagglutination weakly at high concentrations, but attempts to produce a knockout mutant of the *3-pgk* gene were not successful. This gene may be essential for the bacterium and it may not therefore be possible to produce a knockout mutant of the gene. Recombinant 3-pgk did not bind to pigeon ovomucoid in an Eastern blot assay (which differs from a Western blot assay in that galabiose-binding proteins were detected with pigeon ovomucoid instead of antibodies), and therefore it was deduced that 3-pgk was not the galabiose-binding adhesin.

As another approach, 2-aminoethyl-galabiose, galabiose-BSA and pigeon ovomucoid were used to generate affinity columns and beads. These molecules were coupled to prepacked affinity columns and the galabiose-BSA–conjugate was also coated to latex microbeads. It has been reported previously that the adhesin may be detached from *S. suis* cells by sonication and concentrated by fractional ammonium sulphate precipitation (Tikkanen et al. 1995). Therefore, a sonicated and ammonium sulphate precipitated cell fraction was incubated with the columns and beads. This fraction was further fractionated by ion-exchange chromatography. Various solutions were used to elute the putative adhesin from the columns, and the adhesin-content of the eluates was determined by haemagglutination assay. Unfortunately, the adhesin could not be purified with these columns or the microbeads so that it could have been identified as a band in gel electrophoresis. Interestingly, it was found that the adhesin activity was bound by a weak anion-exchanger but not by a weak cation-exchanger at pH 6 and 8. Based on these studies, it was deduced that the adhesin has a pI value lower than 6. This proved later to be correct, as the pI of the adhesin finally identified is 4.6.

A nanoprobe-based affinity mass spectrometry technique (Chen et al. 2005) that was based on magnetic nanoparticles was also utilised. The P^k-nanoparticles used in this technique were synthesised by the group of Professor Chun-Cheng Lin at the National Chiao Tung University, Taiwan. These magnetic nanoparticles were used in order to separate and enrich the adhesin from a mixture of *S. suis* cell wall proteins. Washing steps were relatively simple since a magnet could be used to separate the particles from the liquid phase. After subsequent molecular mass fingerprinting and a search against the *S. suis* genome sequence, another candidate adhesin, elongation factor Tu (Ef-Tu, SSU0482), was identified. Even though the known function of Ef-Tu is in protein synthesis on the ribosomes, this protein has recently been identified as a cell surface protein in *S. suis* proteome analyses (Jing et al. 2008, Chen et al. 2011, Wu et al. 2011). Furthermore, Ef-Tu of *S. gordonii* has been identified to bind to salivary mucin (Kesimer et al. 2009). The *ef-Tu* gene was cloned and the recombinant protein was expressed and purified. Concentrated recombinant Ef-Tu inhibited haemagglutination moderately. An *ef-Tu* gene knockout mutant could not be produced, and therefore Ef-Tu was cloned in *Streptococcus gordonii* and *Lactococcus lactis* in order to produce the candidate adhesin on the surface of these bacteria. However, a haemagglutination-positive phenotype could not be achieved. The binding of the recombinant Ef-Tu to the P^k-nanoparticles could not be inhibited by pigeon ovomucoid, 1 mM octavalent galabiose inhitor or 5 mM galabiose. In addition, it was found that Ef-Tu bound not only to P^k-nanoparticles but also to nanoparticles functionalised with sialic acid. Ef-Tu seems therefore to bind to the nanoparticles themselves, not to the carbohydrates attached. It was concluded that Ef-Tu was not the galabiose-specific adhesin.

5.2. Identification and molecular properties of the galabiose-specific adhesin (I)

A cell wall protein fraction extracted from live *S. suis* strain D282 by the lysozyme treatment strongly inhibits haemagglutination. To simplify the assay setting, the mere cell wall fraction was purified with an affinity matrix instead of all bacterial proteins. The proteins released by the lysozyme treatment were incubated with the pigeon ovomucoid glycoprotein that had been coupled

to an affinity matrix. In accordance with containing galabiose-terminals in its glycans, pigeon ovomucoid efficiently inhibits *S. suis* haemagglutination (Haataja et al. 1993). After washing of the pigeon ovomucoid–affinity matrix, the cell wall proteins bound were detached by SDS treatment, separated by gel electrophoresis and subjected to mass spectrometric identification (Figure 1 in I).

The protein identified by the affinity matrix technique in *S. suis* (SSU0253) had no previously known function, but it had been annotated as a putative surface-anchored protein due to the LPXTG-sequence. This protein did not travel as a single band but formed a ladder-like pattern in a SDS-PAGE gel starting from approximately 200 kDa. It was designated **s**treptococcal **ad**hesin **P** (SadP) since it recognises the P blood group antigen P^k. SadP protein ladder could be seen in the SDS-PAGE gels of all the three wild-type strains analysed (Figure 4 in I). The *sadP* gene was cloned in an *E. coli* expression host to produce a recombinant protein (I). The predicted sizes of SadP and the truncated fusion protein are 84,300 Da and 79,600 Da, respectively. The native and recombinant proteins had the same mobility in native PAGE. For unknown reason, the native and recombinant SadP proteins had much higher molecular weights (approximately 200 kDa) than predicted in native and SDS-PAGE (Supplemental Figure 4 in I). No traces of other proteins co-purifying with the recombinant SadP were found in mass spectrometric analysis.

To test the specificity of SadP, the glycoproteins fetal calf serum fetuin (contains terminal NeuNAcα2,3/6–residues) and porcine thyroglobulin (contains terminal Galα1–3Gal–residues) were coupled to the affinity matrix as controls. After the cell wall proteins had been detached from these control affinity matrixes and separated by gel electrophoresis, it could be seen that numerous proteins had bound to these control glycoproteins, but SadP was not among them. SadP did not bind to BSA-affinity matrix either. Furthermore, no protein binding at all was observed when plain affinity matrix was incubated with the cell wall proteins. In an ELISA-based assay (I), SadP did not bind other glycoproteins either: hen egg ovalbumin or invertase (both contain high mannose glycans), human milk lactoferrin (contains Fucα1–3–residues) or bovine submandibular asialomucin (contains GalNAcα1–residues).

The binding specificity of SadP was verified and studied further with Eastern blot analysis as well as with ELISA and surface plasmon resonance (SPR) (I). SadP bound specifically to pigeon ovomucoid in Eastern blot assay. In ELISA, SadP was found to bind to pigeon ovomucoid and P^k-BSA in a concentration dependent manner. SadP protein was also found to strongly bind to the glycolipid GbO_3 and weakly to GbO_4 but it did not bind to the control glycolipids sialosylparagloboside or lactosylceramide. Binding to pigeon ovomucoid was also verified in an SPR assay. Consistently with ELISA, the SPR signal was found to be increased in a dose-dependent manner. Therefore, it can be concluded that SadP bound galabiose-containing glycoproteins and glycolipids in a manner that is consistent with the previous results obtained studying whole *S. suis* bacteria.

The necessary hydroxyls in galabiose for *S. suis* adhesin binding are HO-4', HO-6', HO-2 and HO-3 (Figure 4). Consistently with the previous results from whole-bacteria studies, 4'-, 6'-, 2- or 3-deoxy derivatives of galabiose did not inhibit the binding of SadP to pigeon ovomucoid (I). SadP was found to have a binding specificity of the P_N subtype, since galabioses derivatised at the O2'

and C3', positions not tolerated for substitution in the case of P_O subtype, were strong inhibitors of SadP binding to pigeon ovomucoid.

Two *sadP*-gene knockout mutant strains were constructed to determine the haemagglutination phenotype of the mutants (I). As discussed earlier, agglutination of human red blood cells is dependent on the multiple binding of *S. suis* bacteria to galabiose-units on the red blood cell surface, and it was expected that bacteria unable to produce the galabiose-specific adhesin would show a haemagglutination-negative phenotype. The *S. suis* strains used for the knockout constructs were D282, which was used also in the identification of the adhesin, and P1/7 (Table 8). The knockout mutant strains D282-*sadP* and P1/7-*sadP* (Table 8) had haemagglutination-negative phenotypes, as expected. No SadP protein ladder could be seen from D282-*sadP* extracts in the PAGE gel (Figure 4 in I). The complementation of the *sadP* gene in the *S. suis* D282-*sadP* knockout mutant strain with the pLZ12Spc-SadP or pDL278-SadP plasmids (Table 9) was not successful. This may be due to the fact that these shuttle vectors are designed to be used in *E. coli* and *S. pyogenes* or enterococci, not in *S. suis*.

The peptide sequence of SadP contains some structural features common to many streptococcal cell surface proteins (Figure 6). One of the common features in the Gram-positive surface proteins is an LPXTG cell wall sorting signal recognised by sortase. A *srtA* knockout mutant strain was constructed and this mutant strain had a haemagglutination-negative phenotype. Therefore, the galabiose-specific adhesin is probably anchored to the cell wall by sortase, and without this anchorage the bacteria are unable to adhere to galabiose. Another common feature in the streptococcal cell surface proteins is a signal sequence, which is usually 30 to 40 amino acids long. It directs the proteins to the secretion apparatus for export to the cell surface and is cut away from the precursor structure by a signal peptidase (Rigel and Braunstein 2008). This sequence was also found in SadP (Figure 6).

SadP was found to contain a C-terminal repeat region. To study its significance for galabiose recognition, a truncated N-terminal fragment (amino acids 31–328), which did not contain this repeat region, was produced. The fragment was found to still retain the pigeon ovomucoid-binding activity, which was as strong as in full-length SadP (I). However, the minimal region required for the binding has not been determined yet. In SPR assays the truncated and full-length proteins had different binding kinetics in binding to surface-bound pigeon ovomucoid. The truncated molecule had faster association and dissociation rates than the whole-length SadP. The binding of both molecules could be specifically inhibited by a synthetic galabiose inhibitor (Figure 8 in I).

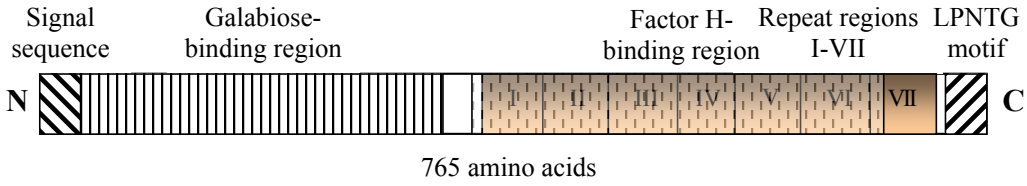

Figure 6. Structural features of the SadP protein (modified from I).

5.3. A live-bacteria application of surface plasmon resonance (II)

The purpose of adhesins is to retain bacteria attached to their target surfaces. Hence, the interaction between an adhesin and the target surface must stand the shear stress caused by the flowing body fluids such as urine or blood (Thomas et al. 2002). It may be informative to measure the interactions of adhesins under flow conditions, like in an SPR assay, since interactions formed under static conditions may not be physiologically relevant (Beauharnois et al. 2006). Therefore, an SPR assay to measure the adhesion of live bacteria to a galabiose-surface, was set up to characterise adhesion and inhibitory potencies of adhesion inhibitors (II). The effectiveness of galabiose derivatives to inhibit bacterial adhesion was recorded as changes in the refractive index. This live-bacteria application of SPR established was found to be practicable for measurement of bacterial adhesion. The inhibitory concentrations measured under flow conditions were consistently somewhat lower than those measured under static conditions. Bacteria mostly dissociated from the galabiose-surface after the injection of bacteria was finished but the binding was not fully reversible and therefore the galabiose-surface needed to be regenerated between the runs. The chips could be used for repeated analysis for several days.

5.4. Inhibition of S. suis and E. coli adhesion by galabiose derivatives (II, III)

The $PapG_{J96}$ adhesin of the uropathogenic *E. coli* belongs to the PapGI class and recognises terminal galabiose (Strömberg et al. 1991), which is also recognised by SadP. In order to search agents that inhibit the binding of *S. suis* and *E. coli* to cells displaying galabiose, the relative inhibition potencies of various synthetic mono- and multivalent derivatives were determined (Table 12) by the SPR assay developed (II) as well as by ELISA-based cell adhesion (II) and haemagglutination assays (II, III). Inhibitors used in the studies had been synthesised either by a fully chemical synthesis approach (II) or semi-synthetically utilising enzymatic degradation of polygalacturonic acid (III), which is more cost-effective than the fully chemical synthesis of carbohydrates. The dendrimer moieties, composed mainly of hydrocarbon, were identical in II and III, and the spacer between the dendrimer and galabiose molecules were almost the same, too. However, the slight difference in the linking moiety did not seem to have an effect since tetravalent inhibitors with different spacers had similar minimal inhibitory concentration (MIC) values (III).

All the divalent inhibitors were more potent than the monovalent inhibitors (Table 12). With *S. suis*, the inhibitory potency per sugar was not further enhanced when the valency was increased from tetravalent to octavalent (Table 12). The tetravalent inhibitors had 40 to 60 times higher inhibitory potencies per sugar than the monovalent inhibitors (III). However, additional galactose residues in a tetravalent inhibitor did not increase the inhibitory potency since the tetravalent inhibitor containing terminal trisaccharides (Galα1–4Galα1–4Gal-) had significantly reduced inhibitory potency in comparison to the tetravalent disaccharide derivative. The MICs of the tetravalent inhibitors were at the low nanomolar level (III).

Table 12. Inhibitory potencies of galabiose derivatives against *S. suis* and *E. coli* adhesion

Bacterium and inhibitor	Valency	Relative potency	Potency per sugar
***S. suis* 628**[a]			
Monovalent	1	1.0	1.0
Divalent, short spacer arms	2	13	6.7
Divalent, long spacer arms	2	12	5.8
Tetravalent	4	250	63
Octavalent	8	310	39
Octavalent PAMAM	8	260	32
***S. suis* D282**[b]			
Monovalent	1	1.0	1.0
Divalent	2	50	25
Tetravalent	4	170	42
Tetravalent galatriose[c]	4	8.3	2.1
Octavalent	8	100	13
***E. coli* $PapG_{J96}$**[a]			
Monovalent	1	1.0	1.0
Divalent, short spacer arms	2	2.6	1.3
Divalent, long spacer arms	2	3.3	1.6
Tetravalent	4	7.7	1.9
Octavalent	8	43	5.3
Octavalent PAMAM	8	6.1	0.8

[a] The IC_{50} values, which are the concentrations of the inhibitor that cause a half-maximal inhibition, were determined in the SPR assay (II).
Relative potency = IC_{50} or MIC of the monovalent inhibitor divided by that of inhibitor in question.
Relative potency per sugar = Relative potency / Valency

[b] MIC values were determined in the haemagglutination assay (III).

[c] Other inhibitors in the table were galabiose derivatives.

The inhibitory potencies against P-fimbriated *E. coli* increased when the valency increased from monovalent to octavalent (II) but the effect of multivalency was not as strong as with *S. suis*. An octavalent galabiose derivative was found to be the most effective inhibitor of *E. coli* adhesion with an IC_{50} value of 2 μM. For comparison, the IC_{50} value of the tetravalent inhibitor was 11 μM. Overall in studies with *E. coli*, the inhibitory concentrations were in the micromolar range and in studies with *S. suis* in the nanomolar range. The inhibition of *S. suis* and *E. coli* adhesion were both dependent on the concentration of the galabiose derivatives. Control mannose ligands did not cause any inhibition even at high concentrations (II).

5.5. Detection of S. suis with magnetic glycoparticles (IV)

In the adhesion-based *S. suis* detection technique developed (IV), biotinylated mono- or tetravalent galabiose molecules were coupled to streptavidin particles (∅ 250 nm). These glycoparticles were utilised to capture galabiose-binding bacteria from solution, and the captured bacteria were detected with a standard luminescence-based ATP detection system. The presence of ATP indicates that bacteria are metabolically active, in other words, the detection system does not measure dead bacteria. Bacteria were bound to the galabiose-modified surface of the particles. The binding to the particles was galabiose-specific since only low signals were obtained from control particles containing a nonbinding ligand (GlcNAc). The results could be obtained in less than two hours and the detection limit of this technique was in the range of 10^4–10^5 cfu/mL.

Factors affecting the performance of the technique, such as the particle size and the valency of the derivatives, were also studied. In the *S. suis* adhesion inhibition studies, tetravalent galabiose derivatives were superior to the monovalent ones (see section 5.4.). However, in the glycoparticle detection application, a similar difference was not seen. In fact, the number of bacteria detected with monovalent particles was somewhat higher than with tetravalent particles (IV). Contrary to the inhibition studies, the use of multivalent derivatives coupled to particles did not bring any additional value. A possible explanation could be that monovalent galabioses are multivalently presented at the particle surface due to the high surface density of the molecules coupled.

For comparison, galabiose compounds were also coupled to conventional microparticles (∅ 50 μm). However, the signals detected from these were down to 50 times lower than the signals measured from the submicron particles, and consequently the detection limits of the bacteria were much higher than the ones achieved with the smaller particles.

6. DISCUSSION

6.1. Streptococcal adhesin P of S. suis

Our group has a long history studying the galabiose-specific adhesion as *S. suis* was reported to have a galactose-specific adhesion activity already in 1989 (Kurl et al. 1989). The galabiose-specific adhesin was identified in the present study by a method based on affinity purification, mass spectrometry and database search, and was named SadP. The adhesin protein contained an LPXTG-motif and had a molecular mass of about 200 kDa in SDS-PAGE gels. Sequences of proteins similar to SadP were searched and compared utilising bioinformatics. SadP did not show any sequence similarity with the other carbohydrate-specific adhesins (Table 5). However, highly similar proteins were found in various *S. suis* strains with slight differences in amino acid sequences and protein sizes. SadP has an almost identical amino acid sequence with two identical proteins from different *S. suis* strains, IF-2 (NCBI accession YP 001199825) or HP0272 (HP, hypothetical protein, NCBI accession YP 001197640). IF-2 has been automatically annotated as translation initiation factor 2 but this annotation seems to be misleading. The IF-2/HP0272 protein is slightly shorter than SadP (698 versus 765 amino acids) but all the 698 amino acids in IF-2/HP0272 are identical with the SadP sequence. IF-2/HP0272 contains 14 additional amino acids at the N-terminus but it lacks amino acids 360–440 (81 amino acids) in the SadP sequence.

Although the function of the slightly shorter protein (IF-2/HP0272) has not been known, it has been the subject of several studies. Interestingly, based on results of the proteomics studies, it has been deduced that it is one of the most abundant cell wall proteins on the surface of *S. suis* strain P1/7 (Mandanici et al. 2010). The gene was found in the genomes of many (29 of 47) serotype 2 field strains and it was present in 17 of 33 *S. suis* serotypes tested (Chen et al. 2010). The genes were not totally identical in all the serotypes tested since the size of the PCR products varied. The *in vivo* expression of IF-2/HP0272 studied by real time PCR was reported to be upregulated during infection (Chen et al. 2010). Inactivation of the *IF-2*/*hp0272* gene was found to significantly reduce bacterial virulence since the knockout mutant was not lethal in a pig infection model. In addition, resistance to phagocytosis in human blood was reported to be diminished as a consequence of the deletion of the gene (Pian et al. 2012). Hence, this protein seems to contribute to bacterial virulence and bacterial survival in blood.

After the completion of the present work, another activity of the SadP protein has been discovered. The shorter but otherwise identical IF-2/HP0272 protein has been reported to bind to human factor H, which is a glycoprotein that regulates the complement system (Pian et al. 2012). More specifically, factor H and its variant factor H–like protein 1 are the main serum proteins that regulate the alternative pathway of the complement system. Factor H is a cofactor of factor I which cleaves complement protein C3b into inactive product, iC3b. In addition to functioning as a cofactor, factor H moderates complement activation by interfering with the association of factor B with C3b and by promoting the dissociation of preformed C3 convertase (C3bBb). Several pathogens, including group A and B streptococci as well as *Yersinia enterocolitica*, are known to

bind factor H and thus protect themselves from complement attack. (Biedzka-Sarek et al. 2008) In the very recent *S. suis* study, IF-2/HP0272 was reported to interact with factor H and this interaction was found to inhibit complement C3b deposition on *S. suis* cells, and thus helped bacteria to evade innate immune defences. The region interacting with factor H has so far not been determined conclusively but a repeat sequence, which has a high degree of similarity to the factor H–binding protein of *S. agalactiae*, may be responsible for this interaction. (Pian et al. 2012) In the SadP amino acid sequence this repeat spans from amino acids 353 to 682 (Figure 6). The galabiose-binding activity is in the N-terminal fragment of SadP, in the region of amino acids 31 to 328. Therefore, the galabiose and factor H–binding motifs are likely to be separate in SadP and the protein seems to have at least two binding targets, which is comprehensible considering the limited resources and genome sizes of microbes. Similar multifunctionality has also been found, for instance, among moonlighting proteins (Table 4) as well as in eukaryotes, among ectoenzymes that regulate leukocyte trafficking (Salmi and Jalkanen 2012).

IF-2/HP0272 has been reported to be a highly immunogenic protein (Geng et al. 2008, Zhang et al. 2008, Chen et al. 2010, Mandanici et al. 2010, Chen et al. 2011). In an immunoproteomics study, IF-2/HP0272 was recognised by both hyperimmune and convalescent sera of pigs, and antibodies against this protein were found in a large number of pigs (Zhang et al. 2008). Antibody levels against IF-2/HP0272 were observed to be higher in human patient sera than in control sera but the difference was not significant (Geng et al. 2008). These observations indicate that this protein is expressed *in vivo*, and that it elicits an immune response. In mice immunised with a recombinant fragment of IF-2/HP0272, increased survival (80 %) and decreased bacterial burden were observed after a challenge with a lethal dose of SS2 (Mandanici et al. 2010). In another study, recombinant IF-2/HP0272 elicited a protective antibody response against a lethal dose of SS2 infection in a mouse model (Chen et al. 2010). By all appearances, SadP is a promising vaccine candidate.

The apparent masses of native and recombinant SadP in SDS-PAGE did not match with the calculated masses. The experimental molecular weight of IF-2/HP0272 was also found to be much higher than the theoretical molecular weight (Geng et al. 2008). Similar discrepancies have previously been reported for other *S. suis* surface proteins (Li et al. 2006). An unusual amino acid composition could be one explanation for the molecular mass discrepancy. The peptide sequence of full-length SadP contains a high proportion of proline residues (15 %) and other proline-rich proteins have been reported to migrate more slowly in SDS-PAGE gels (Podbielski et al. 1996). However, the percentage of proline residues in the truncated recombinant protein (4.7 %) can be considered normal, and as the truncated protein also had a higher molecular weight when ran in the gel, proline residues in the peptide sequence are not likely to be the explanation for the molecular mass discrepancy. Post-translational modifications (Chen et al. 2010) might also play a role in the molecular mass discrepancy and they could be different in *S. suis* and the expression host *E. coli*. For that reason post-translational modifications are not a probable explanation for the discrepancy, either. Another explanation could be that the adhesin may be a part of fimbrial structures that various *S. suis* strains produce (Fittipaldi et al. 2010). The fimbriae of Gram-positive bacteria consist of polymerised pilin subunits that are covalently linked together and cannot therefore be dissociated by boiling in SDS-PAGE sample buffer. Such proteins form high-molecular-weight ladders when run in SDS-PAGE. (Okura et al. 2011) SadP forms a ladder in electrophoresis gels

and may therefore be speculated to be a fimbria-associated adhesin. The ladder was not found to contain any other proteins than SadP when the excised gels were subjected to mass spectrometry. In spite of the apparent absence of other proteins, SadP could be fimbrial protein since mass analysis does not always reveal all the present proteins, especially if they are not very abundant, if the proteins are modified, if the proteins are small and have such an amino acid composition that proteases do not not produce any analysable peptides or if the proteins are resistant to proteases. For instance, when pneumococcal fimbriae were studied with mass spectrometry, only peptides from the protein forming the backbone of the fimbriae were detected, even though the fimbriae also possess two ancillary proteins, which were shown to be present by immunoelectron-microscopy. (Hilleringmann et al. 2008)

If SadP is not associated with fimbriae, it could be present on the cell surface as an oligomer in a structure resistant even to SDS-treatment. Most Gram-negative bacteria do not have sortase genes encoded by their genome (Pallen et al. 2001), and therefore the supposed multimerisation of SadP cannot be sortase-dependent since the recombinant protein produced in *E. coli* was not a monomer (I). The attachment of SadP to the cell wall peptidoglycan is most likely mediated by the housekeeping sortase A as the SrtA knockout mutant had a haemagglutination-negative phenotype (I). Disruption of *srtA* gene has been reported to markedly diminish the ability of the *S. suis* bacteria to adhere to human epithelial (Wang et al. 2009) and porcine microvascular endothelial cells (Vanier et al. 2008). In addition, the virulence of these *srtA* knockout mutants was reported to be somewhat attenuated in piglets (Wang et al. 2009). The diminished adherence and the attenuated virulence could be due to the lack of SadP adhesin on the bacterial surface.

The pathogenesis of bacterial diseases involves complex bacteria–host interactions (Rhen et al. 2003). The possible pathogenic role of the SadP adhesin in such a series of interactions remains unknown for the time being. The interaction between the bacterium and the host mediated by SadP probably facilitates a bidirectional biological crosstalk, which has an effect on both the host and the bacterium, and eventually leads to the development of disease. The pathogenic role of SadP could be clarified by studying the virulence of deletion mutant strains in animal models.

The three-dimensional structure of SadP has not been resolved so far, neither are the amino acid residues that are crucial for carbohydrate-binding. The crystal structure of SadP is under investigation, and the residues participating in carbohydrate interactions are now being studied by site-directed mutagenesis. As more information about the adhesin–receptor interaction becomes available, highly specific inhibitors that block the interaction could be designed. The three-dimensional structure of *E. coli* PapG adhesin, including the globoside-binding site, has been determined (Dodson et al. 2001, Westerlund-Wikström and Korhonen 2005). Thus, the structural comparison of these two adhesins will be possible when the crystal structure of SadP becomes available. In addition to SadP and PapG adhesins, galabiose is also recognised by several bacterial toxins and plant lectins, which are structurally unrelated. These galabiose-binding proteins are a fascinating example of convergent evolution involving carbohydrate–protein interactions. The evolutionary conservation of the galabiose-binding properties in both Gram-positive and -negative pathogens is remarkable and suggests that the galabiose-specific adhesion of the unrelated proteins confer a significant advantage for the bacteria.

6.2. Inhibition of streptococcal adhesin P

The SPR assay measuring galabiose-specific adhesion of whole bacteria and adhesion inhibition with soluble carbohydrate derivatives is based on detecting changes in refractive index upon binding of bacteria to the receptor-containing chip surface. The SPR measurements require significantly less inhibitors and adhesion receptor molecules as compared with more conventional assays such as a haemagglutination or ELISA. An SPR assay could be considered to simulate the conditions of natural infections since it records whole bacterial cells under flow conditions. The difference between results under static and flow conditions might be even bigger in the case of some other adhesins. However, the flow conditions may be totally different in the SPR flow cells than *in vivo*. Either way, the SPR assay has some advantages as compared to other assays: SPR can be used in inhibition studies even if the target adhesin does not cause haemagglutination or if there is no cell line available for ELISA, and with the SPR assay quantitative inhibition results of intact bacteria are detected in real time.

Polyvalent inhibitors have the potential to simultaneously occupy several adhesin molecules on the bacterial surface. For comparison, the inhibition of *E. coli* adhesion was only somewhat influenced by the valency of inhibitors but the importance of multivalency was more pronounced in the adhesion inhibition of *S. suis*. One possible explanation for this difference in the effect of multivalency might be that the PapGI adhesins of *E. coli* are located on the tip of the P-fimbriae and therefore the distance between the adhesin molecules is likely to be large so that multivalent inhibitors cannot reach multiple adhesins simultaneously. Indeed, the distance between the galabiose molecules in the multivalent inhibitors seems to affect the inhibitory potency: galabiose spacing was important also with divalent inhibitors differing in length of the spacer arms between galabioses were studied. The inhibitor with longer spacer arms was a more potent adhesion inhibitor of P-fimbriated *E. coli* than the inhibitor with short spacer arms. Another difference between the adhesion inhibition of these two bacteria was that considerably higher concentrations of inhibitors were needed to inhibit the adhesion of P-fimbriated *E. coli* than that of *S. suis* (micromolar vs. low nanomolar).

6.3. Specific detection of adhesin-expressing S. suis

Discrimination between virulent and avirulent bacteria would be of importance when detecting bacteria from clinical and other samples such as food or water. Pathogenic bacteria are heterogenous: there are strains that are highly virulent but not all strains of a potentially pathogenic bacterium constantly express virulence factors due to phase variation (Quessy et al. 1995). Such variation may have a great effect on the virulence of a pathogen. For instance, the P-fimbriae of *E. coli*, which are known to be expressed *in vivo* during acute UTI, are under the phase variation (Pere et al. 1987). The expression of streptococcal adhesins is known to be regulated in response to environmental and growth conditions, but factors affecting the regulation of adhesin genes in Gram-positive bacteria are not known in detail (Moschioni et al. 2010).

The virulence of many streptococcal species correlates with the expression of adhesins (see section 2.7.), and some streptococcal adhesins have been found to affect the pathogenesis of infection. For example in *S. pyogenes*, there is a connection between plasminogen-binding activity and skin infections (Wistedt et al. 1995), and binding to the complement-regulating plasma protein clusterin is associated with glomerulonephritis (Åkesson et al. 1996). As a general rule, carbohydrate-recognising adhesins are essential virulence factors since lectin-deficient mutants often do not have the ability to cause infection (Sharon 2006). Therefore, the ability to distinguish whether bacteria recognise certain carbohydrate receptors or not, may be of clinical significance. Pathogens may be detected based on their carbohydrate interactions, and in a carbohydrate array format, even the identification of bacterial species may be possible (Disney and Seeberger 2004).

Glycoparticles are a novel and promising approach for the detection of bacteria that contain carbohydrate-recognising adhesins on their surface. The particles used previously have mainly been conventional microparticles. The presentation of carbohydrate ligands on the surface of particles may allow interactions that are similar to interactions of bacteria with host cells (de la Fuente and Penadés 2006). In addition to carbohydrate ligands, also lectins themselves have been coupled to the surface of particles (Gao et al. 2010). Glycoparticles have been previously utilised for detection only in a few studies (El-Boubbou et al. 2007, Hatch et al. 2008, Huang et al. 2009, Liu et al. 2009) and submicrometer glycoparticles seem not to have been used to detect Gram-positive bacteria.

The bacterial detection limit of the glycoparticles utilised in the present work was 10^4–10^5 cfu/mL, which could be considered to be in a clinically relevant range. The detection limits of assays measuring different pathogens cannot be directly compared. However, as an example of a clinical assay, patients whose urine contain over 10^5 cfu/mL, have been reported to likely have a clinically significant UTI (Kwon et al. 2012). Like most bacterial detection methods, the method used to determine the number of bacteria in urine measures any coliform bacteria in the sample, whether they are virulent or not, whereas the adhesion-based technique developed measures specifically live bacteria that express the galabiose-recognising adhesin, potentially differentiating between virulent and avirulent bacteria.

The technique to detect adhesin-expressing bacteria developed in the present work could be considered as a prototype of tests to be developed in the future. The speed of detection could be improved by using shorter incubation times. The detection limit on the other hand could be improved by optimising several factors. To detect bacteria more sensitively, more carbohydrates could be coupled to the particle surface, or alternatively the derivatives could be attached to the surface of a chip to create an array. In addition, bacteria could be detected with a more sensitive method than the luminescence-system used. For instance, a system based on nuclear magnetic resonance (Shao et al. 2010) would probably be quite sensitive, especially when used with magnetic particles. Submicron-particles are smaller than a bacterium and this is why more than one particle may attach to a single bacterial cell (El-Boubbou et al. 2007). As the diameter of particles is reduced, the particle surface area in relation to the volume increases. The large surface area seems to improve the capture efficiency, which is essential for the sensitive detection of bacteria in this technique. The submicron particles (∅ 250 nm) used were found to be superior to the microparticles (∅

50 µm) and the detection limit could possibly be further improved by the use of even smaller particles.

The adhesin-capturing glycoparticle technique could have further potential applications that should be tested. The antibiotic susceptibility of the pathogens bound by glycoligands during detection could probably be tested by eluting the bound bacteria from the surface of the particles. The determination of the MIC values of antibiotics seems feasible since bacteria stay alive on the surface of the glyconanoparticles unlike, for instance, in PCR and other methods, which require the destruction of bacteria. A subsequent antibiotic testing step has been successfully used in a microarray application (Disney and Seeberger 2004). The stability of detection assay components is another important factor especially if the assay needs to be performed under field conditions like when measuring environmental samples on site. Carbohydrates are ideal ligands since they are stable at elevated temperatures and during prolonged storage (Hatch et al. 2008). To better assess if samples contain pathogens with the capacity to cause disease, especially the essential virulence factors are excellent targets for detection, since they are related to the pathogenic potential of the pathogen. Therefore fast and sensitive techniques detecting bacteria that express virulence factors are highly desirable.

6.4. Summary and conclusions

The present study deals with galabiose-specific adhesion of the zoonotic Gram-positive pathogen *S. suis* and is based on four original research publications. A galabiose-specific adhesin of *S. suis* was identified and its molecular properties were studied. In addition, an application of surface plasmon resonance that measures live bacteria was set up. Inhibition of *S. suis* and *E. coli* adhesion with several galabiose derivatives was investigated with the surface plasmon resonance application. The galabiose-recognising adhesion activity was further utilised for the detection of *S. suis* bacteria with magnetic glycoparticles. The identification and characterisation of the molecules involved in the interaction of the bacteria with the host cells is likely to provide valuable information for the understanding of the pathogenesis of the infection. The results of the present study may also be useful in the development of novel drugs and vaccines for humans as well as pigs. On the basis of the results obtained, the following conclusions can be made:

1. SadP is the galabiose-specific adhesin of *S. sui*s.

2. The adhesion inhibition of live bacteria can be measured by the SPR assay set up, and with this assay quantitative results are obtained in real time.

3. The inhibition potency of *S. suis* adhesion inhibitors is highly dependent on the multivalency of the inhibitor.

4. Glycoparticles hold promise for future bacterial detection that distinguishes between adhesin-expressing and non-expressing strains.

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