Bacterial modulation of host glycosylation - in infection, biotechnology, and therapy

Sjögren, Jonathan

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ACADEMIC DISSERTATION

Bacterial modulation of host glycosylation

- in infection, biotechnology, and therapy

Jonathan Sjögren

2015

DOCTORAL DISSERTATION

By due permission of the Faculty of Medicine, Lund University, Sweden,
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Abstract
A majority of the proteins of the immune system are glycosylated and the glycans of IgG are essential for its functionality. Bacteria display enzymes that modulate the glycans of the immune system to weaken the host defense and favor bacterial survival. In this thesis we aimed at exploring bacterial modulation of host glycosylation in infection and to evaluate the usefulness of bacterial enzymes in biotechnology and for therapeutic use.
The role of IgG endoglycosidase EndoS in streptococcal virulence was evaluated in a murine model of invasive infection and we found significant contribution when heterologously expressed. We also discovered and characterized EndoS2, a novel enzyme specific and conserved in serotype M49 of streptococci, with enzymatic activity on the glycans of IgG and α-acid glycoprotein. Enterococcal pathogenesis was studied, and we found that the endoglycosidase EndoE cleaved glycans of lactoferrin to reduce the antibacterial functions and to support bacterial growth. A glycoform specificity difference between EndoS and EndoS2 was observed, and we suggested a method for quantification of high-mannose glycans on therapeutic antibodies, a key quality attribute. Finally, we explored the importance of Fc glycosylation of IgE and showed that EndoS cleaved glycans of this immunoglobulin causing a reduction of the immune cell activation *in vivo,* a potential new therapeutic strategy for severe IgE mediated allergies.
In this thesis we demonstrate that glycans are an integral part of the immune system, and that the study of bacterial effectors of glycosylation paves the way for a deeper understanding of infections, for novel tools supporting the biotech arena, and for new therapeutic strategies.

Key words: *Streptococcus pyogenes,* endoglycosidase, IgG, glycosylation, EndoS

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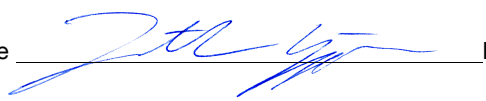
Signature  Date February 8th 2015

Bacterial modulation of host glycosylation

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The cover features an oil painting on canvas by the Swedish artist Barbro Öhrling, interpreting an antibody and the Fc-glycans.

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PREFACE

My hope with this thesis is that you will learn something new. There could have been long traditional chapters on the immune system and microbiology. Instead, I focused on what I find particularly interesting and it is my hope that everyone who reads this thesis will learn something they did not know before.

As scientists, we thrive at the border of the unknown. Through scientific methodology and empirical evidence, our task is to shed light on what is not yet known. To ask questions, to search for answers, to do re-search, is to discover and describe the nature that already exists. This task has for the past five years been both challenging and rewarding. It is indeed a small enzyme, only barely secreted, and carries a number two after its name; still, EndoS2 was not described before the work of this thesis. To be creative in science is to ask new questions, to elegantly describe, and to find new ways of revealing the complexity of nature. Still we only re-search what is already there. The true geniuses are those who create what did not exist before; the pianissimo beginning of "Aftonen", or an oil-painting of an antibody with its Fc-glycans. In our creative work we sense the source of all creativity. For now we see only a reflection as in a mirror.

The pursuit to describe the world around us is accompanied by a great humbleness for the beauty and complexity of nature. To track the footsteps of the source of creativity has for me been a true privilege. God knows I am thankful.

Jonathan Sjögren
Lund, 2015

"For now we see only a reflection as in a mirror; then we shall see face to face. Now I know in part; then I shall know fully, even as I am fully known."

1 Corinthians 13:12

CONTENTS

ABSTRACT

A majority of the proteins of the immune system are glycosylated and the glycans of IgG are essential for its functionality. Bacteria display enzymes that modulate the glycans of the immune system to weaken the host defense and favor bacterial survival. In this thesis we aimed at exploring bacterial modulation of host glycosylation in infection and to evaluate the usefulness of bacterial enzymes in biotechnology and for therapeutic use.

The role of IgG endoglycosidase EndoS in streptococcal virulence was evaluated in a murine model of invasive infection and we found significant contribution when heterologously expressed. We also discovered and characterized EndoS2, a novel enzyme specific and conserved in serotype M49 of streptococci, with enzymatic activity on the glycans of IgG and α_1-acid glycoprotein. Enterococcal pathogenesis was studied, and we found that the endoglycosidase EndoE cleaved glycans of lactoferrin to reduce the antibacterial functions and to support bacterial growth. A glycoform specificity difference between EndoS and EndoS2 was observed, and we suggested a method for quantification of high-mannose glycans on therapeutic antibodies, a key quality attribute. Finally, we explored the importance of Fc glycosylation of IgE and showed that EndoS cleaved glycans of this immunoglobulin causing a reduction of the immune cell activation *in vivo*, a potential new therapeutic strategy for severe IgE mediated allergies.

In this thesis we demonstrate that glycans are an integral part of the immune system, and that the study of bacterial effectors of glycosylation paves the way for a deeper understanding of infections, for novel tools supporting the biotech arena, and for new therapeutic strategies.

ORIGINAL PAPERS

This thesis is based on the studies reported in the following original papers.

Paper I **Jonathan Sjögren**, Cheryl Okumura, Mattias Collin, Victor Nizet, Andrew Hollands.
Study of the IgG endoglycosidase EndoS in group A streptococcal phagocyte resistance and virulence
BMC Microbiolology 11, 120 (2011)

Paper II **Jonathan Sjögren**, Weston Struwe, Eoin Cosgrave, Pauline Rudd, Martin Stervander, Maria Allhorn, Andrew Hollands, Victor Nizet, Mattias Collin.
EndoS2 is a unique and conserved enzyme of serotype M49 group A *Streptococcus* that hydrolyses N-linked glycans on IgG and α_1-acid glycoprotein
Biochemical Journal 455, 107–118 (2013)

Paper III Julia Garbe, **Jonathan Sjögren**, Eoin Cosgrave, Weston Struwe, Marta Bober, Anders Olin, Pauline Rudd, Mattias Collin.
EndoE from *Enterococcus faecalis* hydrolyzes the glycans of the biofilm inhibiting protein lactoferrin and mediates growth
PLoS ONE 9(3), e91035 (2014)

Paper IV **Jonathan Sjögren**, Eoin Cosgrave, Maria Allhorn, Maria Nordgren, Stephan Björk, Fredrik Olsson, Sarah Fredriksson, Mattias Collin.
EndoS and EndoS2 hydrolyze IgG Fc-glycans with different glycoform selectivity and can be used to rapidly quantify oligomannose content on therapeutic antibodies
Submitted

Paper V Heike Danzer, **Jonathan Sjögren**, Karl Carlström, Andreas Nägeli, Rolf Lood, Arne Egesten, Maria Allhorn, Falk Nimmerjahn, Mattias Collin.
EndoS glycan hydrolysis of human IgE uncovers carbohydrate dependent interactions with FcεRI and inhibits IgE mediated activation of effector cells *in vitro* and *in vivo*
Manuscript

As part of the introduction the following review article is included.

Jonathan Sjögren & Mattias Collin
Bacterial glycosidases in pathogenesis and glycoengineering
Future Microbiology, 9(9), pp.1039–1051 (2014)

INTRODUCTION

BACTERIAL MODULATION OF HOST GLYCOSYLATION

The human defense systems are sophisticated networks of effector cells and molecules, aimed at tirelessly fighting off intruding pathogens. The addition of sugars to proteins, a process called glycosylation, is of key importance for the immune functions. The bacteria that surround us have, during co-evolution with humans, identified weak spots of our defense and display enzymes to modulate the glycosylation of the immune system. By studying this modulation we can gain new insight into how bacteria subvert the defense system and learn more about the importance of glycosylation of molecules in the immune system. The theme of this doctoral thesis work has been to investigate mechanisms by which bacteria modulate host glycosylation in order to favor bacterial survival, and to explore these mechanisms as therapy or biotechnology tools.

This introduction aims to provide the reader with insight into previous work on which the included articles are built, and to highlight key concepts and ideas later discussed in the presented articles. Chapter 1 discusses bacterial pathogenesis and introduces effectors of virulence of group A *Streptococcus* and *Enterococcus faecalis*. Chapter 2 presents the field of glycobiology, its biological impact and the methods used to study the structures of carbohydrates on proteins. Chapter 3 describes the structure and function of antibodies with a special focus on the glycosylation of IgG and discusses recent advances in therapies involving antibodies or modification of the IgG glycan. Chapter 4 is a published review article covering glycobiology and bacterial virulence factors. The role of glycosidases in pathogenesis of a range of species are discussed and combined with an update on recent advances of how these glycosidases have been applied as biotechnology tools.

CHAPTER 1 – BACTERIA AND VIRULENCE

PATHOGENESIS

The presence of bacteria has been known for hundreds of years; still, the global burden of bacterial infections remains a serious health issue. The rise of antibiotic resistance combined with poverty and limitations in treatment accessibility, contributes to the increased impact of infectious diseases. To combat this rising threat and aid the development of novel treatment and prevention strategies, a deeper understanding of the battle between bacterial pathogens and the human immune system is necessary. The ability of bacteria to cause disease is called pathogenesis or virulence and early efforts to define these properties were conducted by Koch in the late 19th century (Koch, 1876). Koch named four criteria that need to be fulfilled for a pathogenic microorganism in what was later denoted Koch's postulate:

I. The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.
II. The microorganism must be isolated from a diseased organism and grown in pure culture.
III. The cultured microorganism should cause disease when introduced into a healthy organism.
IV. The microorganism must be reisolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

The technical advances in genome sequencing and the tools of microbial genetics led to a molecular rephrase of Koch's postulate (Falkow, 1988). In this molecular definition, the disease-causing genetic elements from pathogenic bacteria must be isolated as distinct pathogenic genes and distinguished from common genetic elements using three criteria:

I. The phenotype should be associated with a pathogenic organism.
II. Specific inactivation of the gene should lead to reduced virulence.
III. Reversion or replacement should restore pathogenicity.

The development of genetic tools to manipulate bacteria led to a revolution of virulence studies, still ongoing today. However, the refined study of microbial pathogenesis called for even further stringent criteria, and less focus on phenotypic descriptions, from an academic perspective on microbial adaptation and evolution (Falkow, 2004). Falkow discussed the problem that some, or perhaps most, pathogens have two faces and that this complicates the definition of a pathogen. The Janus-faced properties of many pathogens are usually one asymptomatic state and one more aggressive and invasive. This is common for many asymptomatically carried pathogens such as *Streptococcus pyogenes*, *Staphylococcus aureus* and *Streptococcus pneumoniae* that many of us carry on the skin and in the pharynx, without severe symptoms.

In the case of *S. pyogenes,* an update on the two phased view has recently been suggested in a model applying evolutionary ecology models to the mechanistic understanding of streptococcal infections and proposing not two, but three distinctive infection patterns (Wollein Waldetoft and Råberg, 2014). These patterns are asymptomatic colonization, superficial symptomatic infection and invasive infection, where the two first are considered adaptations to maximize transmission and the latter a non-adaptive side effect of the effectors needed for superficial infections. This view on the pathogenesis of *S. pyogenes* moves away from reductionistic reasoning and paves the way for a more holistic view on pathogenesis that may be applied to other pathogens. Another proposed view on pathogenesis is to "ditch the term pathogen" and instead focus on the contextual interplay between host and the intruder and study the host damage (Casadevall and Pirofski, 2014).

Throughout this thesis work, we have used the human pathogens *Streptococcus pyogenes* (group A *Streptococcus*) and *Enterococcus faecalis* as model organisms. An introduction to these species and a brief overview of selected effectors of virulence are discussed.

GROUP A *STREPTOCOCCUS*

Over 500 000 deaths associated with group A streptococal infections are reported annually, which ranks this species as one of the 10 most deadly pathogens in the world (Carapetis et al., 2005). The disease manifestations of group A *Streptococcus*

span from mild and self-limiting diseases exemplified by pharyngitis and impetigo, to severe and life threatening invasive diseases such as necrotizing fasciitis and streptococcal toxic shock syndrome (Cunningham, 2000). Repeated infections with group A *Streptococcus* may also trigger autoimmune diseases such as acute poststreptococcal glomerulonephritis and acute rheumatic fever.

Group A *Streptococcus* is a Gram-positive bacterium arranged in chains of "cocci" (seed/berry) with β-hemolytic activity on red blood cells. The bacterial species was denoted group A *Streptococcus,* due to the antigenic group A carbohydrate classified by Rebecca Lancefield in the early 20th century. Today, diagnostic tests still uses this carbohydrate to detect the species. The carbohydrate called group A consists of a polyrhamnose backbone with an immunodominant N-acetylglucosamine (GlcNAc) side chain and the genes involved in the synthesis were recently discovered (van Sorge et al., 2014). By knocking out the enzyme adding the GlcNAc to the rhamnose chain, a decrease in virulence was detected in two infection models and this finding may aid developments of a group A streptococcal vaccine.

Group A *Streptococcus* is a heterogeneous species containing more than 200 subgroups called serotypes. The serotypes were historically typed based on serotype specific antibodies towards the immunodominant M protein. Today, the typing of group A streptococcal isolates is based on the sequence of the 5' variable region of the *emm* gene, encoding the M protein. The distribution of *emm* types differs around the globe and the disease manifestation of the *emm* types varies as well. In the industrial world the most prevalent group A *Streptococcus* serotypes belong to *emm* types 1, 3, 12, 28 while they are uncommonly isolated from human disease in Africa, the Pacific region and the Indian subcontinent (Steer et al., 2009). There are significant correlations between some streptococcal diseases and certain *emm* types. For example *emm* types 1 and 3 are associated with invasive disease, *emm* type 49 and 60 with acute poststreptococcal glomerulonephritis, while *emm* types 2, 4, 6, and 12 correlate with superficial infections (Johnson et al., 1992; Shea et al., 2011; Walker et al., 2014b).

EFFECTORS OF GROUP A STREPTOCOCCAL VIRULENCE

The arsenal of effectors involved in the pathogenicity of group A *Streptococcus* has been studied in detail and the contribution to virulence of many of the factors has been evaluated (Kwinn and Nizet, 2007; Nizet, 2007; Walker et al., 2014a). In this introduction, factors interacting with immunoglobulin G will be discussed, with a

patricular focus on EndoS, the enzyme that links streptococcal virulence to the glycobiology of antibodies (Table 1). The research focusing on the interaction between pathogens and immunoglobulins has for a long time focused on the nonimmune binding between bacterial proteins and antibodies. Many immunoglobulin binders have been discovered and applied in the biotechnology industry, an example being protein A from *Staphylococcus aureus,* used today routinely in industrial scale purification of antibodies (Forsgren and Sjöquist, 1966; Kim et al., 2012). Another example is protein G from group G *Streptococcus,* that binds IgG Fc and Fab fragments, and that is widely used as protein G columns for IgG affinity chromatography (Björck and Kronvall, 1984; Kronvall, 1973).

Immunoglobulin binding proteins from group A *Streptococcus* are numerous and many have been well characterized. The surface bound protein H binds IgG Fc-domain and inhibits bacterial killing through reduced opsonophagocytosis and complement activation (Åkesson et al., 1990; Berge et al., 1997). The classical M protein, the primary antigen of group A *Streptococcus*, has been shown to bind IgG Fc and contribute to phagocyte resistance (Fischetti, 1989; Metzgar and Zampolli, 2011; Navarre and Schneewind, 1999; Oehmcke et al., 2010). A specificity for IgG F(ab')2 fragments and not Fc domains, have been seen by the fibronectin binding protein I, SfbI (Medina et al., 2000). As the pharynx is a natural milieu for group A *Streptococcus* it is not surprising that the bacteria have several proteins that interact with the IgA molecule, which is abundant at mucosal surfaces. Protein Arp binds both serum and secretory IgA, not IgM, IgE or IgD, and shows limited binding to IgG at a separate site (Lindahl and Åkerström, 1989). Protein Sir binds both IgA and IgG as well as the complement regulator C4BP (Stenberg et al., 1994; Thern et al., 1995). Two secreted immunoglobulin binding proteins have been described; protein SibA and a leucine zipper protein (Lzp), both binding IgG, IgA and IgM (Fagan et al., 2001; Okamoto et al., 2008). Streptococcal enzymes binding or interfering with immunoglobulins have been summarized in Table 1.

Other important factors that mediate group A streptococcal virulence include Streptodornase 1 (Sda1), a DNAse breaking down neutrophil extracellular traps (Buchanan et al., 2006; Sumby et al., 2005; Walker et al., 2007), streptococcal inhibitor of complement (SIC) that binds and inhibits complement as well as anti-microbial peptides (Åkesson et al., 1996; 2010; Frick et al., 2003), hyaluronic acid capsule that mimics the human body to disguise the bacteria (Cole et al., 2010; Dale et al., 1996), the IL-8 protease SpyCEP that increases the resistance to neutrophil killing (Edwards et al., 2005; Zinkernagel et al., 2008) and, the family of streptococcal pyrogenic exotoxins (SPE) which are important superantigens (Commons et al., 2008), among others.

Table 1. Streptococcal effectors of immunoglobulin binding and interference.

Protein	Mode of Action	Reference
Protein H	Binds IgG Fc, inhibits opsonophagocytosis and complement.	(Åkesson et al., 1990; Berge et al., 1997)
M proteins	Bind IgG Fc, contributes to phagocytosis resistance.	(Fischetti, 1989; Navarre and Schneewind, 1999)
SfbI	Binds IgG F(ab')2 and fibronectin.	(Medina et al., 2000)
Protein Arp	Binds serum and secreted IgA, weak IgG binding.	(Lindahl and Åkerström, 1989)
Protein Sir	Binds IgA, IgG and complement regulator C4BP.	(Stenberg et al., 1994; Thern et al., 1995)
SibA	Secreted protein that binds IgG.	(Fagan et al., 2001)
Lzp	Secreted and surface bound protein that binds IgG.	(Okamoto et al., 2008)
Proteases		
IdeS	Cysteine protease, cleaves IgG in F(ab')2 and Fc/2.	(Björck et al., 1989; Pawel-Rammingen et al., 2002a)
SpeB	Cysteine protease, cleaves IgG in the hinge region.	(Nelson et al., 2011; Pawel-Rammingen and Björck, 2003)
Glycosidases		
EndoS	Endoglycosidase, hydrolyzes IgG Fc-glycans.	(Collin and Olsén, 2001a)
EndoS2	Endoglycosidase, hydrolyzes IgG Fc-glycans and biantennary glycans of α_1-acid glycoprotein.	(Sjögren et al., 2013)

IdeS

Group A *Streptococcus* has the ability to reduce neutrophil killing through interference with phagocytic functions. One of the mechanisms of phagocyte resistance is the IgG specific protease, IdeS. Immunoglobulin degrading enzyme from *S. pyogenes* (IdeS) is a cysteine protease and was discovered to cleave IgG below the hinge region and the cysteine bridge holding the heavy chains in place (Pawel-Rammingen et al., 2002a). The cysteine protease activity was first discovered in group A *Streptococcus* using a cysteine protease inhibitor peptide derived from, cystatin C (Björck et al., 1989). IdeS specifically hydrolyzed IgG molecules and did not cleave any other immunoglobulins (Vincents et al., 2004). The cleavage of IgG into two Fc/2 fragments and one F(ab')2 fragment inhibited the antibody from interacting with immune cells and abolished the IgG mediated killing by neutrophils (Pawel-Rammingen and Björck, 2003). At the time of the discovery of IdeS, another research group identified protein Mac, a protein identical to IdeS, and described that Mac mimicked parts of the CD11b/CD18 complement receptor and blocked interactions between FcγRIIIb and antibodies (Lei et al., 2001). However, the researchers failed to notice the proteolytic activity of this protein. The impact of receptor binding vs. proteolytic activity was compared by constructing an inactive form of IdeS that still bound polymorphonuclear cells but did not protect bacteria against phagocytic killing, arguing that the protease activity of IdeS/Mac is of greater importance, compared to receptor binding (Pawel-Rammingen et al., 2002b). Allelic replacement of IdeS did not reveal reduced virulence in an invasive mouse model of infection, explained by the fact that IdeS does not cleave murine IgG1 and IgG2b (Okumura et al., 2013). IdeS has been shown to cleave IgG bound to the classical antigen determinant, the M protein, that in a concentration dependent way binds IgG at the Fc region and allows cleavage of the Fab region by IdeS (Nordenfelt and Björck, 2013; Nordenfelt et al., 2012). IdeS has been applied as a tool for fragmentation of therapeutic antibodies and antibody-drug conjugates and is widely used in the characterization of biopharmaceuticals (An et al., 2014; Janin-Bussat et al., 2013; Wagner-Rousset et al., 2014). The clinical applications for an IgG specific protease are many and include autoimmune conditions, such as arthritis or neuromyelitis optica, and acute transplant rejection (Johansson et al., 2008; Nandakumar et al., 2007b; Tradtrantip et al., 2013). The first indication where the therapeutic use of IdeS is clinically evaluated, is in sensitized transplantation patients, where phase I and II trials conducted by Hansa Medical have shown promising results (Nandakumar and Holmdahl, 2008; Pawel-Rammingen, 2012).

SpeB

The presence of proteolytic activity in supernatants from group A *Streptococcus* has been known since studies on bacterial cultures in 1945 (Elliott, 1945). Although the methods were basic, the observations were accurate. Elliot and colleagues described a protein secreted by the bacteria similar to papain in its function, with reduction dependent activity that was inhibited by iodoacetic acid or thiol-inactivating compounds. More recent research has revealed the SpeB protein as a cysteine protease and the *speB* gene to be conserved in all serotypes and strains of group A *Streptococcus* (Nelson et al., 2011). In terms of substrates, it has been shown that SpeB is able to degrade immunoglobulins A, M, D and E into small fragments and to cleave IgG in the hinge region (Collin and Olsén, 2001a). However, the clinical relevance of this activity has been questioned, as it was shown that SpeB does not cleave IgG *in vivo* and the true interaction between SpeB and antibodies during an infection is unclear (Persson et al., 2013). The regulation and secretion of active forms of SpeB is complex and involves the production of a zymogen form of SpeB later autocatalytically processed to an active form (Carroll and Musser, 2011). Interestingly, the enzyme is down-regulated during CovRS mutations while Sda1 is upregulated during selection pressure in neutrophil extracellular traps (Walker et al., 2007).

EndoS

The IgG endoglycosidase, EndoS, was discovered by Mattias Collin and Arne Olsén in 2001 (Collin and Olsén, 2001a). A secreted enzyme in the supernatant of group A *Streptococcus* was seen to cause a shift of 3-4 kDa of the heavy chain of IgG on an SDS-PAGE gel and was identified using Edman degradation and denoted EndoS, endoglycosidase from *S. pyogenes*. Recombinant expression and insertional mutagenesis concluded that the gene *ndoS* was coupled to the glycan hydrolyzing activity on IgG. The specificity of the enzyme was analyzed on IgA, IgM, IgD, IgE and IgG, and it was found that EndoS specifically catalyzed the hydrolysis of the glycans on IgG (Collin and Olsén, 2001b). Studying the activity of EndoS on a temperature gradient of increasingly denatured IgG, it was found that EndoS only cleaved glycans from the native structure of IgG and differs from other known endoglycosidases by requiring a protein interaction for enzymatic activity on the carbohydrate. The role of EndoS in human blood was explored and it was found that both antibody mediated killing through immune effector cells and activation of complement was inhibited by EndoS and a significant increase in bacterial survival was observed (Collin et al., 2002).

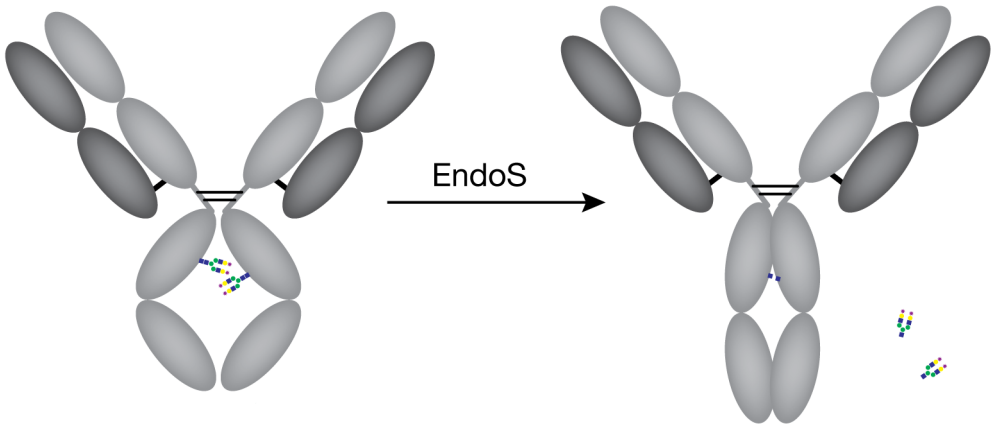

Figure 1. Cartoon of EndoS hydrolyzing the N-linked glycans of IgG, causing the Fc-fragment to collapse, leading to dramatically reduced Fc-receptor binding.

Incubating IgG with EndoS resulted in reduced binding of IgG to leukocytes as shown with flow cytometry, and abolished complement activation in an ELISA experiment. The analysis of EndoS activity on the FcγR/IgG interaction was studied using surface plasmon resonance and it was found that EndoS cleaved IgG (isotypes IgG1-4), causing a reduction in binding to FcγRIIa and FcγRIIb of isotypes IgG1, IgG2 and IgG4 (Figure 1) (Allhorn et al., 2008a). Interestingly, the hydrolysis of the Fc glycan on IgG2 caused significantly increased binding to the inhibitory FcγRIIb, but the observed effect may be due to aggregation of IgG2 in the ELISA experiment since no binding was seen in a surface plasmon resonance experiment. The active site of EndoS belongs to the family 18 of glycoside hydrolases (GHs) with the conserved motif DxxDxDxE (where x represents any amino acid). By site directed mutagenesis it was shown that glutamic acid at position 235 was crucial for the enzymatic activity (Allhorn et al., 2008b). In the same study, chemical modification of tryptophans was shown to dramatically reduce the glycan hydrolysis (Allhorn et al., 2008b).

Recently a number of studies have focused on the structural aspects of EndoS deglycosylation of IgG. By expressing fragments of EndoS, it was shown that regions outside the catalytic domain contributed to IgG deglycosylation (Dixon et al., 2014). In the same study it was shown that the isolated CH2 domain alone, was sufficient for EndoS activity on the N-linked glycan. Crystals of EndoS were obtained by liquid-liquid diffusion and diffracted at 2.6 and 1.9 Å resolution (Trastoy et al., 2013). The solved X-ray crystal structure of EndoS revealed five distinct protein domains; glycosidase, leucine-rich repeat, hybrid immunoglobulin, carbohydrate binding motif and a three-helix bundle (Trastoy et al., 2014).

ENTEROCOCCUS FAECALIS

Enterococcus faecalis is a Gram-positive human opportunistic bacterium that gained attention due to the increasing number of antibiotic resistant nosocomial infections. The bacteria primarily infect patients suffering from immune suppression, concurrent illness or prolonged hospitalization and may cause infectious endocarditis, urinary tract infections, bacteremia or sepsis (Hall et al., 1992; Rice et al., 1991; Sava et al., 2010). *E. faecalis* differ from *S. pyogenes* and *Staphylococcus aureus* by not producing potent pro-inflammatory toxins, instead the bacteria are equipped with several adhesive proteins (Arias and Murray, 2012; Fisher and Phillips, 2009). These factors are involved in the formation of biofilms such as in endocarditis or on plastic materials mediating the hospital transmitted infections (Joyanes et al., 1999; Mohamed and Huang, 2007). An example is the surface protein Esp that contributes to colonization of the urinary tract and confers the biofilm production capability (Shankar et al., 1999).

E. faecalis is known to secrete enzymes acting on host carbohydrates to promote bacterial growth and persistence. Due to its similarity to EndoS, the enterococcal endoglycosidase EndoE was discovered (Collin and Fischetti, 2004). The enzyme was expressed recombinantly and displayed activity on the glycoprotein IgG and high-mannose glycans on the bovine RNaseB glycoprotein. EndoE contains two active sites, one belonging to family 18 of glycoside hydrolases and the other to family 20. Site-directed mutagenesis located activity on IgG to the family 20 hydrolase domain and the activity on RNaseB to the family 18 glycoside hydrolase (Collin and Fischetti, 2004). Endo-β-N-acetylglucosaminidase activity was detected in 10 of 18 clinical enterococcal isolates and the hydrolyzed glycans supported bacterial growth (Roberts et al., 2001). These observations aligned with the previous finding of *E. faecalis* ability to grow on RNaseB and use its high mannose glycans as carbon source (Roberts et al., 2000).

VIRULENCE AND CARBOHYDRATE UTILIZATION

Microorganisms that colonize the human body face two major challenges, to avoid getting destroyed by the immune system and to obtain nutrients to support bacterial growth. To effectively colonize a host, group A *Streptococcus* present several toxins that cause a pro-inflammatory response, leading to vascular leakage of the nutrient rich plasma to the site of infection. This hypothesis has been supported by emerging evidence that regulation of bacterial virulence is linked to genes involved in complex carbohydrate utilization (Shelburne et al., 2008a). Historically, the study of bacterial

catabolism was confined to the area of basic bacterial physiology and did not attract major attention. However, transcriptome studies have revealed regulatory links between the nutrients available and production of virulence factors (Shelburne et al., 2008a). An example is direct link between metabolism and virulence through catabolite control protein A (ccpA), discovered in group A *Streptococcus* (Shelburne et al., 2008b). By comparing the transcript levels of 78 candidate genes of streptococci cultured in saliva or nutrient rich laboratory medium, a 10-fold increase of ccpA transcript levels could be detected in saliva. By knocking out the *ccpA* gene it was seen that the gene transcript levels of several virulence factors, including *ideS* and *speB*, were decreased and this explained the significant reduction in virulence seen in a murine model of infection.

Technological advancements have shed light on this concept in the lifestyle of intracellular pathogens *in vivo*. By high-throughput methods and radioactive carbon profiling, the metabolism of intracellular pathogens, such as *Listera monocytogenes* and *Shigella flexneri*, have successfully been studied. It has been found that the carbon metabolism may differ depending on what cellular compartment is infected (Eisenreich et al., 2010). To adapt to the intracellular life-style the bacteria depend on virulence factors that are co-regulated by global transcriptional regulators sensitive to complex carbohydrates.

Similarly to virulence genes, the metabolic genes are under selection pressure and it has been speculated that the evolutionary drive is the access to nutrients provided by the infected host (Laurence Rohmer, 2011). The adaptation to new niches in or at the host is demanding in terms of the harsh conditions in the infected milieu (e.g. low pH in the stomach, high pH in urine) but also in terms of the fierce competition for nutrients by already residing microorganisms.

The ability of pathogenic bacteria to interfere with host glycosylation is widely spread throughout a range of bacterial species (Garbe and Collin, 2012). This may indicate that there are more links between complex carbohydrate utilization and virulence to be discovered and could help explain the lifestyle of pathogens residing in nutrient poor milieus of the human body.

CHAPTER 2 – GLYCOSYLATION

GLYCOSYLATION

The paradigm of molecular biology is that information is stored in DNA, transcribed to RNA, and translated to a protein in a template-based fashion. The DNA starting block, ATG, transcribed into RNA becomes AUG that eventually translates to the first amino acid, methionine, in a newly synthesized protein. The linearity makes the system easy to understand and manipulate. However, the limited number of genes (human only have 25 000) and the enormous complexity of protein functions indicate that the picture requires more nuances.

Glycosylation is the most common and versatile post-translational modification that influences the functionality of proteins (Rudd et al., 2001). A majority of extracellular proteins, including surface and secreted proteins, are glycoproteins, meaning they carry carbohydrates covalently attached to the amino acid sequence. The sugar moiety, called "glycan", is attached to the protein as a post-translational modification, occurring in the endoplasmic reticulum and the Golgi apparatus of the cell (Figure 2). Other post-translational modifications that modify the properties of a protein include: phosphorylation, ubiquitination, acylation and many others.

The glycans attached to the glycoprotein are not modeled after a template, instead the levels of glycoactive enzymes in the biosynthesis of the N-glycan, determine the final structure of the carbohydrate. Glycans are attached to proteins via an amino-glycosidic bond at an asparagine (N-linked glycans) present as part of an Asn-X-Ser/Thr sequence, or serine/threonine (O-linked glycans) in a yet unknown sequence. At each glycosylation site of a glycoprotein, a variation of different sugars is present, called glycoforms. As we shall see, the function of the glycoprotein can be dependent on the glycoform present. The glycans are branched, and not linear, which complicates the study of glycan structures. Advances in glycan analysis have allowed a more detailed understanding of the contribution of sugars to biology and

eventually led to a new scientific field called "Glycobiology" (Rademacher et al., 1988).

> "One day, I received a knock on my door from a woman working for the Oxford English Dictionary. She informed me that she was responsible for the letter "G." The word glycobiology had been chosen, after many discussions and deliberations, for inclusion in the new addendum to the dictionary, which was to be published in 1992 with a reference to me as having coined the name. I subsequently attended a launch of the supplement to the Oxford English Dictionary and was assured by the vice chancellor of Oxford University that there was no higher honor for a scientist than to have his name and his word in so eminent a publication as the Oxford English Dictionary!"
>
> *Raymond Dwek* (Dwek, 2014)

BIOSYNTHESIS OF N-GLYCOSYLATION

How do different glycoforms arise and what enzymes are involved in the glycosylation of proteins? These questions are discussed in brief in this section. The addition of N-linked carbohydrates to proteins occurs in three major steps (Figure 2) (Bieberich, 2014; Taylor and Drickamer, 2011; Varki et al., 1998). First, the precursor glycan is synthesized on the cytoplasmic side of the endoplasmic reticulum (ER), linked to a dolichol lipid in the plasma membrane. At a certain stage in the build up, the growing glycoform is translocated to the lumen of the ER where additional sugars are added. This swopping of the glycan through the membrane is poorly understood but crucial for the steps to follow. Second, the precursor glycan is transferred *en bloc* to the glycosylation site at an asparagine residue on a newly translated protein. And third, the trimming and processing of the glycoform takes place. Exoglycosidases first trim down the glycan to the starting mannose residues and as the protein travels through the portions of the Golgi, transferases sequentially add different sugars until a complete biantennary and fully sialylated glycoform is present on the glycoprotein (Figure 2). The protein can now be secreted or attached to the cell wall and elicit its function.

Not all glycans reach the final fully sialylated form. This leads to the three major types of glycan structures; complex, high mannose, and hybrid type glycans (Figure 5). After the glucose residues have been cleaved off the precursor glycan by glucosidases I and II, the remaining 9 mannose residues are subject to the action of mannosidases in the ER and cis-Golgi.

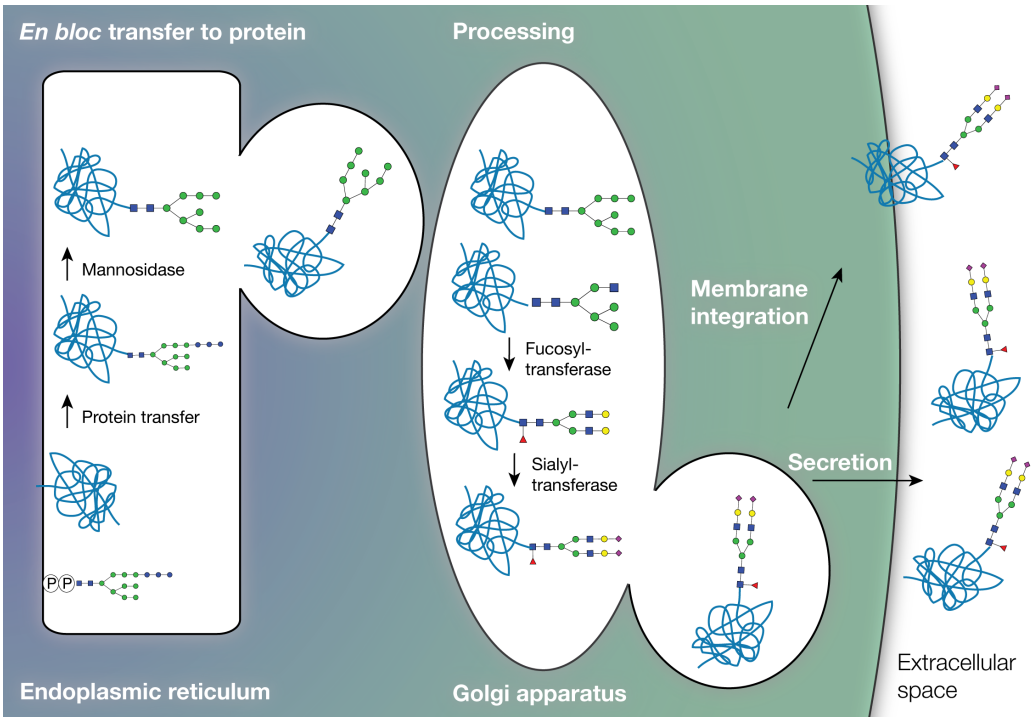

Figure 2. The process of N-glycosylating a protein is shown schematically. First, the precursor glycan is built up. Second, the precursor glycan is transferred to the protein as it is being synthesized by the ribosome. Third, the glycan is trimmed and processed by a range of glycosyltransferases, Finally, the glycoprotein is either secreted or integrated in the cell membrane to elicit its function.

High mannose glycans arise from an incomplete processing of the five to nine mannose residues that remain on the final glycoprotein. A type of intermediate form is the hybrid type glycans, where mannosidases do not act on the glycan after the addition of the first GlcNAc on the α-1,3 arm, leading to the α-1,6 arm being branched mannose residues while the other is accessible for addition of sugars. The complex type glycans are built on the core three mannose residues where both arms are modified with GlcNAc, galactose and sialic acid residues. Biantennary glycans are most abundant but additional GlcNAc residues attached to the mannose residues could give rise to tri- and tetra-antennary structures. Bisecting GlcNAc is a structure where the GlcNAc residue is attached to the core mannose. At an individual glycosylation site a variety of glycoforms can be present. These are the result of the accessibility of the enzymes in the ER and Golgi apparatus to modify the glycan at this particular site. The glycoforms present at an individual glycosylation site are quite few and the heterogeneity varies from protein to protein.

BIOLOGICAL IMPACT OF GLYCANS

The biological roles of protein glycosylation can be divided into three areas including; contributing to protein stability (Wormald and Dwek, 1999), modulating the properties of the glycoprotein, and recognition by glycan binding proteins (Dalziel et al., 2014). The human defense molecules, of both innate and adaptive immunity, are highly glycosylated, and examples that represent the main functions of glycans can be found here (Rudd et al., 2001). A majority of proteins in serum are glycosylated and their stability and circulation time is greatly enhanced due to the decoration with carbohydrates (Rudd et al., 2001). Probably the most studied glycans in the human body; the Fc-glycans of human IgG and their role in binding affinities with Fc receptors will be discussed in greater detail in the following chapter. An example of a glycan binding protein is mannose binding lectin (MBL) that binds to arrays of mannose residues, a characteristic of many bacterial and yeast species, and activates the complement system to induce killing of the intruding organism (Weis et al., 1998).

The glycosylation machinery is a delicate process involving a high number of enzymes that work in concert to add or remove the carbohydrates on proteins and when mutations arise in key enzymes the subsequent symptoms are many. This group of rare diseases is called congenital disorders of glycosylation (CDG) and manifestations may include severe psychomotor retardation (Jaeken, 2010). CDG contains disorders of both N- and O-linked glycosylation and the diagnosis for N-linked glycosylation disorders is carried out by isoelectric focusing of transferrin (Zühlsdorf et al., 2015).

Another field in which changes in glycosylation correlate with disease phenotypes is in different forms of cancer. It has been discovered that irregular glycan structures are present in serum for a number of cancer forms and that this could be utilized as diagnostic markers for disease using highly reproducible chromatographic analytical methods (Adamczyk et al., 2012). There is also hope that changes in glycosylation of a tumor cell can explain some of the phenotypic changes. For instance, tumor derived glycans have been seen to contribute to metastasis (Häuselmann and Borsig, 2014).

GLYCAN ANALYSIS

The progress of studies within the glycobiology of the immune system relies heavily on available methods to analyze the glycans and their structure. The complexity of a protein having several glycosylation sites, various glycoforms at each site and the branching of the glycans, make glycan analysis challenging. The development of liquid chromatography methods to effectively separate glycans has paved the way for structural analysis of glycans (Bones et al., 2010; Mariño et al., 2010). Releasing N-glycans from the first GlcNAc with the amidase PNGaseF and labeling of the glycan with a fluorescent dye, allows the glycoprofile of a glycoprotein to be studied using sensitive chromatography. By further sequentially digesting the profile in exoglycosidase arrays, structural and linkage information can be obtained (Guile et al., 1996; Royle et al., 2006; Tharmalingam et al., 2012). The reproducibility of the chromatographic separation allows retention times to be translated to glucose units using a dextran ladder. This has allowed the build up of databases of structural information linked to a certain glycoform's glucose unit value (Campbell et al., 2008; Gotz et al., 2014; Lieth et al., 2011). The development of high-throughput systems for glycan analysis has allowed the analysis of population wide studies on IgG glycome using this approach (Pučić et al., 2011). Today, a range of different methods are available for chromatographic separation of labeled N-glycans (Reusch et al., 2015). In our work we have relied on ultra-high performance liquid chromatography (UHPLC) with fluorescence detection of 2-AB labeled glycans, coupled by mass spectrometry of the individual glycoforms to confirm the masses.

The downside with the PNGaseF approach is the lack of site-specific analysis of the individual glycoforms. Mass spectrometry analysis of glycopeptides has proved effective in determining site-specific glycosylation using matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) or electrospray ionisation (ESI) MS/MS (Wada et al., 2007). In this thesis work we have combined chromatography analysis and exoglycosidase arrays with mass spectrometry to verify the detected structures. The structures in the presented articles are drawn according to the Oxford glycan nomenclature (Harvey et al., 2009).

CHAPTER 3 – GLYCOSYLATION OF ANTIBODIES

ANTIBODIES

Antibodies are proteins of the adaptive immune system that play an essential role in finding and highlighting foreign material (antigen) in the body and directing the immune response towards the antigen. Antibodies, also called immunoglobulins, are present in five different isotypes IgA, IgG, IgM, IgE, IgD. The high affinity and specific binding of an antibody to its target allows cells or molecules of the immune system to efficiently destroy or remove the intruding pathogen. This chapter gives a brief overview of the antibody isotypes and focuses on the structure, glycosylation and function of IgG and IgE.

Prior to maturation and class switching, all antibody-producing cells (plasma cells) produce the IgM molecule. This is a large pentameric complex with 10 antigen binding sites. The type of antigen the antibody is directed towards induces a class switch and the plasma cell adjusts the antibody isotype for best efficiency. IgA is by far the most produced antibody in the human body, as it is continuously secreted to cover mucosal surfaces such as the pharynx and the intestinal tract. At normally sterile sites, antibodies react to all foreign material and elicit a response. IgA is present at sites where the integrity of the microbial flora is important and it must therefore maintain a balance between responding fiercely to pathogens and mildly or not at all to commensal bacteria (Russell et al., 1999).

IgG is present in high concentration in human serum, ranging from 10-15 mg/mL, and it is secreted by plasma and memory B-cells. There are four different subclasses of IgG, denoted IgG_{1-4}, and they differ in structure as well as in what receptors they engage (Nimmerjahn and Ravetch, 2005). IgD is present in human plasma in concentration as low as 1 µg/mL (Arnold et al., 2004). The biological role of IgD is

uncertain, but early B-cell research found IgD bound to the B-cell receptor on immature B-cells (van Boxel et al., 1972). IgE is well known for its effector functions initiating type I immediate hypersensitivity, most known as the allergic response. The amount of free IgE in plasma is low (150-300 ng/mL) and the majority of IgE molecules are attached to the high affinity receptor FcεRI on mast cells, in tissue, and basophils, in the blood (Gould et al., 2003).

IMMUNOGLOBULIN G AND Fc-RECEPTORS

The IgG molecule is generally seen as two parts; the Fab region (fragment antigen binding) carrying the antigen recognition domain, responsible for detection of the foreign material, and the Fc region (fragment crystallizible) communicating with immune cells and directing downstream effector functions. This chapter provides the reader with an overview of IgG, focusing on its structure and function, and highlights recent advancements in this field.

The IgG molecule consists of two heavy chains (50 kDa) and two light chains (25 kDa) making up a total molecular weight of 150 kDa (Figure 3). The heavy chains consist of 3 constant domains (CH1-3) and one variable domain (VH) and the two are linked via two disulfide bonds in the hinge region (Hinge) of the antibody (depicted as black lines in Figure 3). In the antigen-binding fragment (Fab), the outer variable domain of both the heavy and the light chain contain the variable region responsible for the great variation and high specificity through the three complementary determining regions.

Fc-receptors

When an antibody has bound its antigen it communicates to cells and molecules of the immune system through binding of Fc-receptors (FcRs). The FcRs are divided into groups depending on their ability to bind the immunoglobulins and are assigned a greek letter corresponding to the antibody isotype, in the case of IgG; FcγR. In humans FcγR are further subdivided into four families I-IV and the affinities for IgG varies, as does the presence on immune cells and the impact on immune regulation (Nimmerjahn and Ravetch, 2008). For example, FcγRI is the only high affinity receptor and it is present on monocytes, macrophages and neutrophils and leads to phagocytosis or release of pro-inflammatory mediators. Another example is FcγRIIb, present on B-cells, myeloid and dendritic cells, the only inhibitory FcγR, causing a release of anti-inflammatory mediators and reducing cell activation (Smith and Clatworthy, 2010).

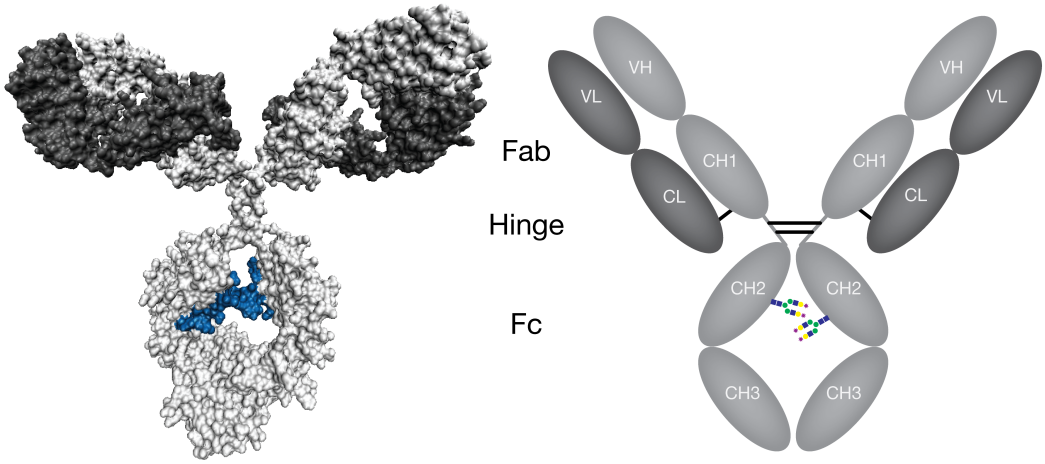

Figure 3. The crystal structure of IgG1 with its N-glycans depicted in blue (left) and a cartoon of IgG with structural assignments (right). V: variable, C: constant region, H: heavy chain, L: light chain, Fab: fragment antigen binding, Fc: fragment crystallizible. Disulfide bonds are shown as black lines in the cartoon. The model was generated from a deposition in the Protein Data Bank (M Clark, Cambridge University, UK) using VMD 1.9.1.

It has been proposed that the sialylation of the N-linked glycan of IgG gives rise to two distinct conformational states of the Fc region of the antibody, that in turn interact with two different sets of Fc receptors, I and II (Pincetic et al., 2014). Type I FcRs belong to the classical immunoglobulin receptor superfamily and include the activating and inhibitory FcγRs interacting with the non-sialylated IgG Fc. The type II FcRs consist of receptors belonging to the C type lectin group (DC-SIGN, CD23) and interact with sialylated Fc (Pincetic et al., 2014). The type I Fc receptors are glycosylated and binding studies have shown that the interaction between FcγRs and rituximab was decreased when the receptors were deglycosylated (Hayes et al., 2014).

The neonatal Fc receptor (FcRn) is actively transferring antibodies from the mother to the fetus and in this way establishing passive immunity of the unborn child (Roopenian and Akilesh, 2007). FcRn also contributes to the increased serum half-life of IgG through recirculation of IgG in a pH dependent manner (Lencer and Blumberg, 2005). Monocytes and endothelial cells continuously filtrate the blood through endosomes with low pH at which FcRn binds IgG. The content of the endosomes are degraded in the lysozyme while the receptor bound IgG is transported back to the blood stream where the pH of 7.4 causes the FcRn to release IgG. The FcRn has also been described to be involved in phagocytosis by neutrophils (Vidarsson et al., 2006).

New insight into antibody functions

The traditional view of an antibody as two parts, Fc and Fab, working independently of each other, one binding the antigen and the other communicating with the immune system, has for long been the dogma of antibody immunity. The traditional antibody functions include neutralization of toxins and viral particles, complement fixation, antibody-dependent cytotoxicity and opsonization. Research on antibody immunity has discovered novel antibody functions that include direct antimicrobial activity, alteration of microbial signaling and immunomodulation, all of which may have implications for infection medicine, vaccine design and monoclonal antibody development (Casadevall and Pirofski, 2012). A selection of updates on antibody functions is discussed in this chapter and under IgG glycosylation.

The molecular events leading to activation of complement by antibodies have, until very recently, remained a mystery. It was discovered that IgG forms hexamers after antigen binding on the surface of the target cell, caused by non-covalent interaction between the Fc-regions of the antibodies (Diebolder et al., 2014). The hexamer was found to bind C1q, the first component of the complement cascade, and triggered its activation. The Fc-Fc interaction could be manipulated to block, reconstitute, and enhance the complement-dependent cytotoxicity of therapeutic antibodies and will most likely have a major impact on the development of future candidate therapeutic antibodies with complement activating properties.

The most basic antibody function is to neutralize its target. In the case of toxins or other secreted microbial virulence factors, neutralization occurs when antibodies are bound to the molecule and prevent it from causing damage. The importance of FcRs in neutralization was highlighted in an animal model of HIV infection, where a neutralizing monoclonal antibody was engineered to loose its interaction with FcRs and complement, leading to dramatically reduced protective ability. However, when engineered unable to activate complement, no loss in antibody protection was observed, stressing the impact of Fc-receptors in neutralization and the importance of FcRs for antibody immunity (Hessell et al., 2007). A neutralizing monoclonal antibody was engineered to interact specifically with selected FcRs and in a mouse carrying human FcRs, it was shown that selectively interacting with activating FcRs increased the efficacy of the neutralizing antibody (Bournazos et al., 2014). A similar indication was observed, as it was discovered that an antibody with three variants of the constant region (IgG2a, IgG2b, IgG1), but with the exact same variable domain, showed different efficacy in antibody-mediated neutralization of bacterial toxins (Abboud et al., 2010). Passive immunization with these antibodies protected wild-

type mice against a *Bacillus anthracis* infection, but failed to do so in mice lacking FcRs.

GLYCOSYLATION OF ANTIBODIES

As the key mediator of adaptive immunity, antibodies play a crucial role in the defense of known intruding pathogens. Antibodies carry one or more glycosylation sites depending on the isotype, and at each site, the glycoforms can be many. The glycoforms present on antibodies are reproducible; meaning differences in glycoforms could be used as diagnostic biomarkers of disease (Arnold et al., 2007). A brief overview of antibody glycosylation is presented, while IgG glycosylation will be discussed in further detail.

The secreted forms of IgA, including the J chain and the secretory component, are heavily glycosylated and the glycans have been shown to be involved in binding of Gram-positive bacteria at mucosal surfaces (Mathias and Corthésy, 2011a; 2011b). Serum IgA carries a different glycosylation profile as compared to its secreted counterpart, and the glycans on serum IgA terminating in galactose have been shown to be ligands for the asialoglycoprotein receptor that regulates clearance of IgA from serum (Basset et al., 1999).

IgE has four constant ε-domains, of which Ce3 and Ce4 form a horseshoe-shaped Fc structure, similar to IgG, and together with Ce2, the whole Fc-region of IgE adopts a bent conformation (Woof and Burton, 2004). The IgE mediated activation of effector cells is carried out through two receptors, FcεRI and FcεRII (CD23). FcεRI is a high affinity receptor with IgE binding affinities down to $1e^{-10}$ M and this receptor is present on basophils and mast cells. The low affinity receptor FcεRII is present on monocytes, eosinophils, platelets and B-cells and has been proposed to be involved in regulation of IgE synthesis (Conrad et al., 2007). Initial characterization of the glycosylation sites of IgG, IgD and IgE hypothesized that a conserved glycosylation site pointing inwards in the Fc-region would be present in all three antibody classes, N297 on IgG, N354 on IgD and N275 on IgE (previously denoted N394) (Arnold et al., 2004). Structural studies indicated similarities between the Fc region of IgG and IgE in terms of orientation and conformation (Woof and Burton, 2004).

Whether the glycan present in the Fc-region is important for the interaction between IgE and FcεRI has been a hot topic of debate. Early work argued that glycosylation of IgE was not required for interaction with either receptor (Vercelli et al., 1989). A couple of years later, another study claimed the presence of N-linked glycans to be unnecessary for FcεRI interactions (Basu et al., 1993). However, a study added

PNGaseF to the binding experiments between IgE and FcεRI and noted a remarkable loss in binding, suggesting that N-glycans indeed are important for the interaction (Björklund et al., 1999). In 2013, the site specific glycosylation pattern of IgE was analyzed and published (Plomp et al., 2014). This study indicated six of seven glycosylation sites to be occupied with an N-linked complex glycan and noted the presence of high-mannose glycans at site N275 in the Fc-region.

Each γ-chain of the pentameric structure of IgM carries three N-linked glycosylation sites carrying complex glycans and a small population of hybrid structures (Arnold et al., 2007). It is speculated that the glycosylation of IgM contributes to agglutination of intruding microorganisms through the arrangement of glycans in the IgM complex. IgD, together with IgA1, are the only immunoglobulins to carry O-linked glycans in the hinge region. In addition to these, three N-linked sites are present on the H-chain, where site 354 is exclusively occupied with high-mannose glycoforms (Arnold et al., 2004). The glycan in this site is inaccessible for lectin interaction but elimination of this glycosylation site through mutagenesis led to incomplete assembly and failure of secretion of IgD (Gala and Morrison, 2002).

GLYCOSYLATION OF IgG

On each of the two heavy chains of IgG, an N-linked glycosylation site at asparagine (Asn) 297 is occupied by a complex biantennary glycan (Figure 3). The glycans of IgG are probably some of the most studied and their impact on IgG effector functions have implications in the field of infection biology, adaptive immunology and for development of therapeutic antibodies (Read et al., 2011). The glycans on IgG give the Fc region a shape of a horseshoe and it has been seen that truncations of the glycans changes the shape of the Fc fragment (Figure 1 and 3) (Krapp et al., 2003). The removal of the Fc glycan caused the CH2 domains to collapse in a closed state, contrary to the open state with the glycan required for FcγR binding. At the glycosylation site of IgG up to 30 different glycoforms can be present (Pučić et al., 2011). The N-glycan profile of IgG has been shown to vary with physiological and pathological status, such as age, disease, and pregnancy (Arnold et al., 2007). An overview of the individual carbohydrate moieties and their functional impacts are discussed in this chapter.

A crystal structure of IgG1 Fc fragment and the FcγRIII receptor showed electron density of the carbohydrates in the Fc, stabilizing the binding to the receptor through glycan-protein interactions although the carbohydrates are on the periphery of the binding site (Sondermann et al., 2000). Thermodynamics of the interaction between a homogenous glycoform IgG preparation and FcγRIIb showed that

truncations of the glycan led to a disarrangement of the CH2 domains and impaired binding (Mimura, 2001)

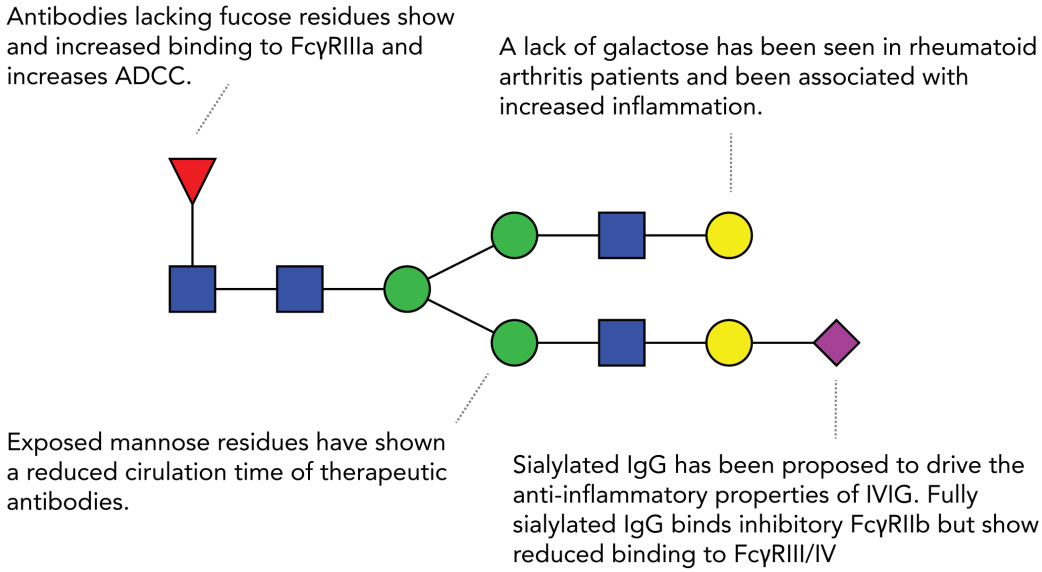

Figure 4. The N-linked glycan of IgG. A selection of functions associated with the individual carbohydrate structures are presented.

Not much is known about the control and regulation of glycosylation but there is emerging evidence that the glycosylation is adjusted depending on what antigen the antibody is directed towards, to maximize the immune response. As an example, a study of the glycosylation of IgG of elite controllers of HIV infections showed that individuals carrying effective anti-viral antibodies also showed a skewed profile towards IgG glycans lacking galactose residues, so called G0 (Ackerman et al., 2013a). Antibodies carrying G0 glycans showed increased anti-viral activities and this may be an active process from the plasma cells that produce the HIV specific antibodies. Also, external stimuli have been demonstrated to affect the glycosylation pattern of IgG1 by specifically changing the regulation of B-cells and not affecting other glycosylation machineries (Wang et al., 2011). It is an intriguing idea that the glycosylation of IgG is directed and specified depending on the type of antigen.

The sugar moieties that constitutes the Fc-glycans of IgG starts with the core GlcNAc (blue) with our without a core fucose (red), three mannose residues (green), one GlcNAc on each branch, galactose (yellow) and finally the sialic acid (purple) (Figure 4). A discussion of the individual sugars contribution to IgG functionality is presented below.

Core fucose

An intensive area of research has been the impact of core fucose on IgG functions, as it was discovered that lack of this sugar residue increased antibody dependent cytotoxicity (ADCC) (Shields et al., 2002). It was discussed whether the increased ADCC was due to lack of fucose or bisecting GlcNAcs, but a study showed clearly that the absence of fucose and not bisecting glycans or galactose increased ADCC (Shinkawa et al., 2003). A comparison of non-fucosylated glycoforms of hybrid, complex or high mannose type showed that the lack of this single fucose was the causative agent for the increased binding to FcγRIIIa and the improved effector functions of the antibody (Kanda et al., 2007). The glycan at site Asn-162 on the activating FcγRIIIa was shown to be stabilizing for the high affinity interaction between non-fucosylated IgGs and the receptor (Ferrara et al., 2006b). Fucosylation of the Fc glycan inhibited this interaction through steric hindrance and negatively impacted the interaction (Mizushima et al., 2011). Crystal structures and NMR analysis comparing the interaction between FcγRIIIa and fucosylated and non-fucosylated IgGs concluded that fucosylation of IgG caused a subtle conformational change of IgG-Fc leading to the increased binding (Matsumiya et al., 2007). These combined findings led to the development of glycoengineered therapeutic antibodies lacking fucose, and a detailed study of the interaction confirmed a unique carbohydrate-carbohydrate interaction between the glycans of the antibody and the glycans of FcγRIIIa (Ferrara et al., 2011).

Galactose

The most common glycoforms present on human serum IgG are glycans with none, one or two terminal galactose residues, called G0F, G1F or G2F. Glycans lacking galactose have been associated with pro-inflammatory activities, increased anti-viral functions and reduced complement dependent cytotoxicity (CDC) (Collin and Ehlers, 2013). In patients suffering from rheumatoid arthritis or systemic lupus erythematosus, the percentage of IgG without galactose (G0) was increased and correlated with disease severity and pro-inflammatory immune response (Collin and Ehlers, 2013). As have been discussed, the IgG glycosylation profile of HIV elite controllers was skewed towards more G0 antibodies and when these antibodies were tested for antiviral properties, they were found to more effective in killing of viruses (Ackerman et al., 2013a). This property relates to an increased binding of both HIV specific and bulk IgG antibodies to FcγRIIa/b and increased phagocytosis (Ackerman et al., 2013b). In a study with glycoengineered IgG, it was found that IgG carrying G0 glycans were linked to reduced CDC by approximately 50% compared to control antibodies and the study emphasizes the importance of controlling the glycoforms of IgG during therapeutic antibody development (Hodoniczky et al.,

2005). Many of the first monoclonal antibodies on the market were produced in murine cell lines that may add a non-human type of galactosylation, namely the addition of α-1,3-linked galactose. This linkage is not found in primates and it has been shown that the presence of this sugar is responsible for the hypersensitivity seen in some patients treated with cetuximab (Chung et al., 2008).

Mannose

Three mannose residues are present in all N-linked glycans but the high-mannose type of glycans may carry a range of 4-9 mannose residues. The presence of high-mannose glycans on monoclonal therapeutic antibodies leads to faster clearance of the antibody and a reduction of serum circulation time (Goetze et al., 2011). The mechanism behind this is not an altered binding to the FcRn, the receptor involved in recirculation of IgG, but rather the binding of mannose receptors that mediate clearance (Kanda et al., 2007).

Sialylation

The most terminal sugars in the full-length glycan on IgG are sialic acids. Addition of this glycan to IgG is rare and only a small percentage of the glycans carry one or two sialic acids. Although it is present in low quantities it has been ascribed some remarkable immune modulatory effects. High doses of pooled IgG, called intra venous immunoglobulin (IVIG), are administered to a broad spectrum of autoimmune disease patients where the exogenous IgG present anti-inflammatory activity (Gelfand, 2012; Schwab and Nimmerjahn, 2013). By studying the glycosylation of the exogenously added IgG, a suggested mechanism behind the observed effect was recently described (Anthony et al., 2008a). It was found that sialic acids present in the IVIG preparation constituted the anti-inflammatory properties, as a recombinant IgG Fc with full sialylation resembled the anti-inflammatory activity of IVIG.

The receptor interaction with sialic acid on IgG was identified to be SIGN-R1, a C-type lectin receptor on macrophages, while mice lacking this receptor were unable to produce an anti-inflammatory response (Anthony et al., 2008b). In humans the orthologous receptor DC-SIGN shows a similar binding specificity and leads to the up-regulation of the inhibitory FcγRIIb and likely accounts for the onset of the anti-inflammatory response (Anthony and Ravetch, 2010; Anthony et al., 2008b). The actual binding of sialylated IgG Fc to DC-SIGN has been questioned as serum IgG did not compete with known ligands for DC-SIGN and it was postulated that other cell bound receptors would be involved (Yu et al., 2013). Furthermore, whether the sialylation actually leads to structural changes in the Fc region has been debated. It has been shown that the structure of sialylated IgG Fc is no different from that of

galactosylated Fc and that this cannot explain the binding to DC-SIGN receptors (Crispin et al., 2013). Although debatable, still the anti-inflammatory effect of IVIG is ascribed to the sialic acid in the Fc region and could be explored to increase efficacy of IVIG treatments (Anthony and Ravetch, 2010; Anthony et al., 2012; Shade and Anthony, 2013).

Bisecting GlcNAc

Bisecting glycans carry one GlcNAc attached to the core mannose residue in a β-1,4-linkage. The presence of a bisecting GlcNAc makes it less likely to have a fucosylation at the first GlcNAc due to steric hindrance. It was thought that bisecting glycans were responsible for the increased ADCC, but as discussed above, the interaction between fucose and the FcγRIIIa proved to be responsible for the increased affinity (Ferrara et al., 2006a; Shinkawa et al., 2003). Any functional consequences of IgG carrying bisecting glycans remain to be discovered.

THERAPEUTIC ANTIBODIES

The concept of specifically targeting diseases through "Zauberkugeln" (magic bullets) was first envisioned by Paul Erlich in 1897 and has now, more than a hundred years later, become a reality (Ehrlich, 1897; Lerner, 2006). The "magic bullet" is now called a therapeutic antibody and since the late 1990s the development of therapeutic antibodies has grown rapidly. To date the regulatory authorities have approved over 30 monoclonal therapeutic antibodies (Beck et al., 2010). In 2010 there were over 240 antibodies in clinical trials and the field is rapidly growing as more sophisticated methods for developing safe and efficient antibodies are discovered (Chan and Carter, 2010).

Therapeutic antibody development has traditionally been focused on the binding of the antibody to the antigen, thus specificity and affinity were the most sought properties. Development of therapeutic antibodies has in the recent years focused more towards design of the downstream effector functions elicited by the antibody. The immune system relies on several steps to fine-tune an immune response including glycosylation. The mechanisms of regulation can be modulated to achieve a desired function of the antibody (Jefferis, 2005; 2009a; 2009b; Quast and Lünemann, 2014).

As we have seen, the N-linked glycan of IgG gives the Fc region its characteristic horseshoe shape and the present glycoform affects the binding to the FcγRs.

Glycoengineering of the therapeutic antibody has been shown to improve its efficacy. As an example, genetic engineering of the production cell line and removal of a fucosyltransferase has led to a therapeutic antibody carrying afucosylated complex glycans with bisecting GlcNAcs. The lack of core fucosylation increased the affinity for FcγRIIIa and in turn, led to increased levels of antibody dependent cytotoxicity in comparison to cetuximab, an antibody targeting the same receptor (Gerdes et al., 2013). The actual binding to the FcγRIIIa on neutrophils and monocytes were actually not that different. However when normal IgG was added the effect was pronounced indicating a competition scenario that favors the glycoengineered antibody (Herter et al., 2014). The analytical challenges of characterizing the post-translational modifications of IgG are many and regulators and manufacturers strive to control and describe the differences to obtain pure and safe therapeutics (Hmiel et al., 2015). The first glycoengineered antibody to be approved was mogamulizumab (Poteligeo®) that was approved by the regulatory authorities in Japan in March 2012 (Beck and Reichert, 2012). There are currently more than 15 other glycoengineered antibodies in clinical trials, and designed glycans on IgG have begun making an impact on patients' lives.

ANTIBODIES DEGLYCOSYLATED WITH ENDOS

Could removal of the Fc-glycan alleviate autoimmune diseases? The autoimmune diseases are many, and the common feature is the development of an immune response towards self-antigens, causing the immune system to attack the body itself, in many cases through the production of antibodies. EndoS has been tested as a potential therapy in many pre-clinical models of autoimmune disease. The specificity for IgG combined with the appreciation of the glycosylation impact of IgG has paved the way for EndoS as a promising therapeutic candidate. Still, the clinical development is lagging behind and a phase I trail has yet to be initiated. Nevertheless, if proven safe and efficient in humans, EndoS therapy may provide a novel treatment option for a number of autoimmune diseases discussed in this chapter (Collin, 2012; Dalziel et al., 2014).

The first autoimmune model where EndoS was explored as a therapeutic agent was a murine model of arthritis. A monoclonal antibody directed against collagen type II was injected into mice to establish arthritis, but when treated with EndoS, the onset of disease was inhibited (Nandakumar et al., 2007a). EndoS treated antibodies were still able to bind the collagen type II antigen and activate complement, but IgG engagement of FcγRs was reduced, as were the formation of immune complexes. Another model of arthritis is the transfer of serum from arthritogenic K/BxN mice to

wild-type C57BL/6 mice that quickly develop clinical symptoms of arthritis. When treating the arthritogenic serum with EndoS this led to significant reduction in joint swelling as well as a decrease in the recruitment of immune cells (Albert et al., 2008).

In a model of spontaneous lupus-like autoimmune syndrome (BXSB mice), the efficacy of EndoS treatment on an already established disease was tested. After 30 weeks, 80% of the mice in the non-treated group were dead whereas all mice treated with EndoS were alive. Development of anti-EndoS antibodies was detected but did not interfere with the enzymatic activity. This was first shown in a mouse model of immune (idiopathic) thrombocytopenic purpura (ITP), where rabbit anti-mouse platelet antibodies treated with EndoS rescued 100% of the mice in a survival experiment (Collin et al., 2008). After three injections, antibodies were developed against EndoS but this did not interfere with the enzymatic activity or the half-life of EndoS in serum.

Autoantibodies attacking red blood cells leading to hemolysis and anemia, represents a serious condition often treated with broadly acting corticosteroids. An alternative approach may be to utilize EndoS as a treatment strategy. Treatment of pathogenic antibodies did not affect the binding to the antigen but resulted in lowered cellular and complement mediated hemolysis (Allhorn et al., 2010). In the kidneys, anti-neutrophil cytoplasmic autoantibodies (ANCA) directed against myeloid peroxidase (MPO) may cause glomerulonephritis. Hydrolysis of the glycans of anti-MPO antibodies with EndoS reduced the protein leakage in the kidneys and the formation of glomerular crescents (van Timmeren et al., 2010). Antibodies that bind to the glomerular basement membrane of the kidneys may also lead to glomerulonephritis. Due to their specificity for IgG, EndoS and IdeS were evaluated as therapeutics in experimental glomerulonephritis. This resulted in a significant reduction in albuminuria when treated with EndoS and complete prevention of disease with the streptococcal protease IdeS (Yang et al., 2010).

Collagen VII is a structural protein of the dermal-epidermal junction and in different models of autoimmunity towards this protein, EndoS was shown to reduce pathogenicity, both when the antibodies were pretreated and also after injection of the enzyme into the mice (Hirose et al., 2012). Interestingly, administration of EndoS hydrolyzed the glycan of already bound autoantibodies thereby converting them to blocking antibodies, unable to interact with immune cells and complement. Also of interest, in this study, EndoS treatment caused a down-regulation of lesional expression of activating FcγRs and up-regulation of the inhibitory FcγRIIb. The reduction in pathogenicity was later ascribed to the reduction of neutrophil

activation through impaired binding of immune complexes to FcγRs on neutrophils (Yu et al., 2014).
In many autoimmune diseases a common feature is the formation of immune complexes. In systemic lupus erythematosus (SLE) the formation of immune complexes, consisting of IgG and autoantigens, deposits in local tissues leading to inflammation and recruitment of polymorphonuclear cells that contribute to tissue destruction. Purified immune complexes were treated with EndoS and this abrogated all pro-inflammatory manifestations including phagocytosis and production of TNF-α, complement activation, oxidative burst and chemotaxis (Lood et al., 2012).

The contribution of a humoral immune response in chronic neuroinflammatory demyelinating disorders of the central nervous system was thought to be of limited importance. However, in experimental autoimmune encephalomyelitis (a mouse model of multiple sclerosis) treatment with EndoS resulted in reduced serum complement activation and deposition in the spinal cord (Benkchoucha et al., 2012). This may indicate that EndoS impairs B-cell functions and the study suggests EndoS as a treatment strategy for antibody driven autoimmune diseases of the central nervous system. Inflammation and demyelination of the optic nerve and spinal cord is the pathogenicity behind neuromyelitis optica. The condition arises from the presence of autoantibodies against the membrane water transport protein, aquaporine 4. Treatment of patient serum with EndoS caused a 95% reduction of complement dependent cytotoxicity as well as antibody dependent cell mediated cytotoxicity (Tradtrantip et al., 2012). The autoantibodies bound to aquaporine 4 deglycosylated with EndoS, still bound their antigen and competitively displaced pathogenic antibodies.

The strategy of specifically targeting the glycosylation of antibodies is conceptually novel (Dalziel et al., 2014). A phase I clinical trial would shed light on the efficacy and safety of EndoS in humans. With the solid scientific evidence for EndoS efficiency presented here, the initiation of clinical studies should be imminent. Taken together, the pre-clinical data suggests that EndoS is a promising therapeutic candidate for a range of autoimmune disease.

CHAPTER 4 – BACTERIAL GLYCOSIDASES IN PATHOGENESIS AND GLYCOENGINEERING

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ABSTRACT

Glycosylation is a common post-translational protein modification and many key proteins of the immune system are glycosylated. As the true experts of our immune system, pathogenic bacteria produce enzymes that can modify the carbohydrates (glycans) of the defense mechanisms to favor bacterial survival and persistence. At the intersection between bacterial pathogenesis and glycobiology, there is an increased interest in studying the bacterial enzymes that modify the protein glycosylation of their colonized or infected hosts. This is of great interest in order to fully understand bacterial pathogenesis, but also presents itself as a valuable source for glycoengineering and glycoanalysis tools. This review article highlights the role of bacterial glycosidases during infections, introduces the use of such enzymes as glycoengineering tools, and discusses the potential of further studies in this emerging field.

INTRODUCTION

In the microenvironment where pathogenic bacteria reside within their host, immune effector molecules, immune cells, mucins, and epithelial cells are glycosylated to varying extent. The glycans play a fundamental role in many functions of the immune system, and it is therefore not surprising that these carbohydrates are targets for bacterial interactions and/or modifications. By studying the mechanisms by which pathogenic bacteria interact with host glycosylation, new insight can be gained into bacterial pathogenesis and into the importance of protein glycosylation in the immune system. Furthermore, discoveries of new glycan hydrolyzing enzymes could serve as a source for novel glycoengineering and glycan analysis tools.

Glycans in the immune system play a variety of different roles; to protect from proteases, to mediate protein interactions and to contribute to protein stability (Rudd et al., 2001). In humans, protein glycosylation is present in two major forms; N-linked and O-linked glycosylation. The consensus sequence for asparagine (N)-linked glycosylation is Asn-X-Ser/Thr, where X can be any amino acid except proline. O-linked glycosylation involves Ser or Thr residues but there is no consensus sequence. N-linked glycans can be high-mannose, hybrid, or complex type structures, all carrying the core Man3GlcNAc2 structure (Figure 5). The most common O-linked glycans are core 1-4, starting with an α-linked N-acetylgalactosamine (GalNAc) and additional galactose or N-acetylglucoasamines (GlcNAcs) (Figure 5). Recent advances in glycan analysis have revealed that not only eukaryote organisms, but also bacteria, and especially mucosal associated bacteria, have protein glycosylation machineries (Szymanski and Wren, 2005).

The well-studied glycans of the antibody IgG are located at the asparagine residue 297, on each of the two heavy chains of the Fc region (Figure 6). The glycans of IgG gives the Fc region its horseshoe-like structure and are of major importance for the antibody's interaction with the Fc receptors and for the communication with immune cells (Krapp et al., 2003). The impact of the IgG glycan is highlighted by the importance of the correct glycoform of therapeutic monoclonal antibodies where glycan engineering and selection is used to improve antibody based therapeutics (Jefferis, 2009a). The various glycoforms present on the heavy chains of IgG gives different Fc conformation and affects the binding affinities to the Fc-receptors leading to changes in the elicited effector functions. For example, it has been postulated that fully sialylated glycans on IgG increases the anti-inflammatory properties of IgG, while IgGs carrying agalactosylated (G0) glycans display increased anti-viral activities (Ackerman et al., 2013a; Anthony and Ravetch, 2010).

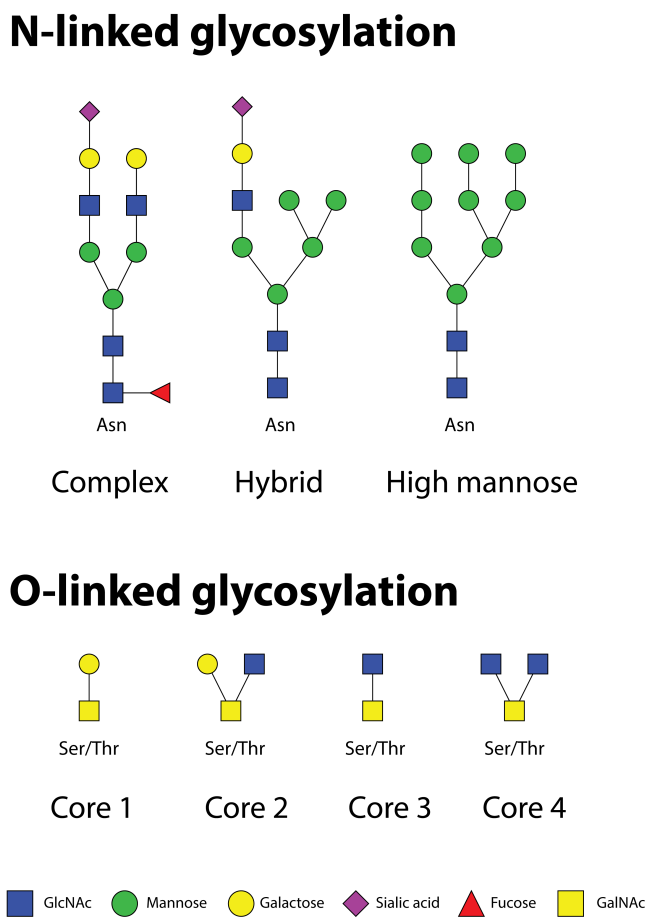

Figure 5. Types of glycans attached to asparagine (N-linked) and the four most common core structures attached to serine or threoinin (O-linked). GalNAc: N-acetylgalactosamine; GlcNAc: N-acetylglucoasamine.

Enzymes that catalyze the hydrolysis of carbohydrates on N- and O-linked glycans are either exo- or endo-glycosidases. Exoglycosidases act on the terminal residue of the glycan and cleave one specific residue whereas endoglycosidases have activity located within the glycan structure and can release several residues in one reaction. Peptide-N-glycosidase F (PNGaseF) is not a true glycosidase (rather an amidase), but is still a very important enzyme in this context since it catalyzes the hydrolysis of the amide bond between the innermost GlcNAc and the asparagine residue resulting in the release of the complete N-linked oligosaccharide from the glycosylation site on

the protein. Details on the mechanisms of action of exo- and endo-glycosidases are outside the scope of this review but can be found in a review by Bojarova and Kren (Bojarová and Kren, 2009). Enzymes that catalyze the reaction of hydrolysis of glycosidic bonds are called glycoside hydrolases (GHs) and are subdivided into families based on amino acid sequence similarities. Today there are more than 130 families described in the Carbohydrate Active Enzymes (CAZy) database (www.cazy.org).

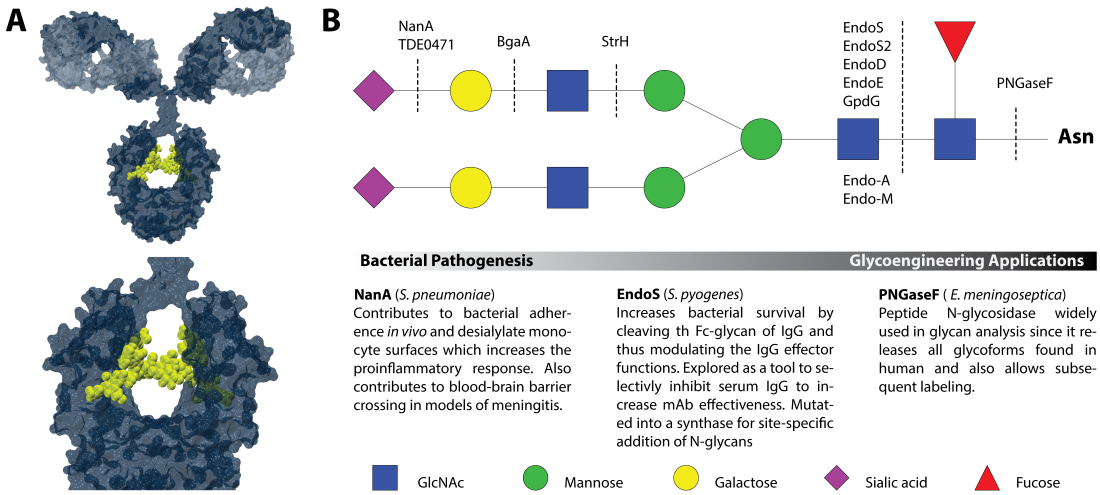

Figure 6. Overview of bacterial glycosidases interacting with the Fc-glycan of igG. (A) A model of IgG1 with the Fc-glycan depicted in yellow. The model was generated from a deposition in the Protein Data Bank by M Clark (Cambridge University, UK) using VMD 1.9.1. (B) A schematic picture of an N-linked glycan and examples of bacterial enzymes that cleave carbohydrate residues, along with three examples of enzymes in the transit from bacterial pathogenesis to glycoengineering applications. Endo: Endoglycosidase; GlcNAc: N-acetylglucoasamine; mAb: Monoclonal antibody; PNGaseF: Peptide-N-glycosidase F; VMD: Visual molecular dynamics.

The first section of this review will provide examples of involvement of bacterial glycosidase in bacterial pathogenesis, through immunomodulation, adherence, and acquirement of nutrients. The second section describes the applications for bacterial glycosidases in antibody glycan engineering and glycan analysis of glycoproteins. Following the concluding section, we present our future perspectives on bacterial glycosidases in infectious diseases and as tools for glycobiology research and drug development. We hope that this glimpse into the world of bacterial glycosidases will inspire to further inquiries within this expanding field of study.

GLYCOSIDASES IN BACTERIAL PATHOGENESIS

Immunomodulation

One prominent example of the ability of bacteria to interfere with host glycosylation and modulate the immune response is the endoglycosidase S (EndoS) from *Streptococcus pyogenes* (Figure 6). *S. pyogenes* (group A *Streptococcus*, GAS) causes diseases in humans ranging from relatively mild infections of the upper respiratory tract (pharyngitis) or skin (impetigo) to severe and life threatening necrotizing fasciitis and streptococcal toxic shock (Cunningham, 2000). The number of infections annually is estimated to over 700 million and the deaths per annum due to severe diseases, counts to over 500,000 globally (Carapetis et al., 2005). Extensive efforts are made to study the mechanisms by which the bacterium circumvents the immune system for a deeper understanding of the infection process that potentially could lead to new targets for antibacterial therapies (Nizet, 2007; Walker et al., 2014a).

One effector molecule in the arsenal of GAS that interacts with host glycosylation is EndoS. EndoS, a secreted endo-β-N-acetylglucosaminidase, was discovered to cleave the β-1,4 bond between the GlcNAcs in the core of the IgG glycan in human plasma (Collin and Olsén, 2001a). The enzyme did not act on major glycans on IgA or IgM and needed a native fold of IgG to hydrolyze the glycans (Collin and Olsén, 2001a; 2001b). Addition of recombinant EndoS and subsequent hydrolysis of the glycan of IgG was shown to increase bacterial survival in whole human blood (Collin et al., 2002). The mechanisms behind the increase in bacterial survival was revealed as a decreased binding of IgG to its Fc-receptors, thus limiting opsonophagocytosis, and also impaired activation of the classical pathway of complement (Allhorn et al., 2008a; Collin et al., 2002). Recently it was found that regions outside the catalytic domain of EndoS contribute to IgG deglycosylation and that the CH2 domain is sufficient for glycan hydrolysis (Dixon et al., 2014). The importance of this enzyme *in vivo* was studied in a mouse model of invasive GAS infection using an allelic replacement ndoS-knockout strain coupled to heterologous expression in a less virulent strain. The study showed that the ndoS-knockout was not significantly less virulent compared to wild type bacteria in the studied model, however, bacterial overexpression of EndoS led to significantly increased bacterial virulence (Sjögren et al., 2011). Interestingly, EndoS2, an EndoS homolog with 37% identity was discovered in strain NZ131 of serotype M49, a less virulent serotype of GAS. Despite the fairly week similarity, EndoS2 is still able to cleave all glycoforms of IgG and also specifically removes biantennary glycoforms from the acute-phase protein α_1-acid glycoprotein (Figure 6) (Sjögren et al., 2013). Another EndoS homolog has been

discovered in the related animal pathogen *Streptococcus equi* subsp. *equi* denoted EndoSe (Flock et al., 2012). Similarly to EndoS, this enzyme hydrolyzes the Fc-glycan on IgG and contributes to immune evasion by *S. equi*. Vaccination with EndoSe protected mice from infections with *S. equi* subsp. *equi* or subsp. *zooepidemicus* and this experiment highlights the potential importance of this enzyme in the infection scenario.

Enterococcus faecalis is a bacterium with a vast range of genes encoding enzymes with putative glycoside hydrolyzing domains, but to date very few have been characterized. EndoE was discovered due to its similarity to EndoS and was found to have two active sites, GH family 18 and GH family 20, responsible for the release of N-linked glycans of IgG and RNaseB (Figure 6) (Collin and Fischetti, 2004). Modulating the immune response by removing the glycan of IgG is an effective way of disrupting the IgG binding to its Fc-receptors, but the consequences of the activity on the bovine glycoprotein RNaseB is more difficult to interpret. Recently, activity of EndoE on human lactoferrin was discovered and was found to decrease the ability of lactoferrin to inhibit biofilm formation of *E. faecalis* as well as contribute to bacterial growth in a poor medium (Garbe et al., 2014). Another endoglycosidase from *E. faecalis* have been characterized, EfEndo18A. This enzyme shows glycosidic hydrolyzing activity between the two GlcNAcs in the core of N-linked glycans and cleaves hybrid and high mannose type glycans (Bøhle et al., 2011).

Two chitinases are present in *Listeria monocytogenes*, a gram-positive bacterium that adopts a pathogenic lifestyle when consumed by a susceptible host. Chitinases cleave the β-1,4 linkage between GlcNAc residues in chitin, a linear polymer of GlcNAcs. By knocking out the chitinase genes *chiA* and *chiB*, the bacteria showed normal growth in cultured cells but decreased persistence *in vivo*, especially in livers and spleens of the infected animals (Chaudhuri et al., 2010). The seen effect was later ascribed to the chitinases interfering with innate immunity by reducing the expression of inducible nitric oxide synthase (iNOS) and *chiA* mutant virulence was restored in NOS2 deficient mice, lacking iNOS (Chaudhuri et al., 2013). Although the substrates for the enzymes are unknown, this work describes a new type of mechanism in which bacterial chitinases can modulate an innate immune response.

The role of sialic acids in the immune defense is of great importance and in bacterial vaginosis, which is a polymicrobial imbalance of the vaginal flora, extensive sialidase activity was present in clinical samples (Lewis et al., 2012). It was shown that the sialidases in these clinical samples effectively removed the sialic acids from secretory IgA (SIgA) and exposed underlying mannose residues in the N-glycosylated secretory component of the antibody. This led to exposure of SIgA for further degradation by

other bacterial effectors such as exoglycosidases and proteases.

Adherence

A recurring theme of bacterial glycosidases is the contribution to colonization through increased adherence. Airborne pathogens first encounter with the human defense systems takes place in the airways where heavily glycosylated mucins, epithelial cells, immune molecules and cells serve as the first line of defense. A selection of examples of glycosidases and their role in adherence and colonization is presented below.

Streptococcus pneumoniae is a gram-positive bacterium that primarily resides asymptomatically in the nasopharynx but can cause pneumonia and, in immunocompromised patients or children, serious conditions such as meningitis and sepsis. The diseases are preceded by pneumococcal colonization and the mechanisms and complexity of this event is of great importance for the preventive strategies (Bogaert et al., 2004). *S. pneumoniae* carries a range of exoglycosidases that cleave host glycans and in this way provide carbon for growth, aid biofilm formation and mediate adhesion, combined these effects contribute to the colonization of the human airway (King, 2010).

Three cell wall-anchored exoglycosidases; neuraminidase NanA, α-galactosidase BgaA and β-N-acetylglucosaminidase StrH, have been shown to work in concert to cleave sialic acid, galactose and GlcNAc from human glycoproteins (Figure 6) (Cámara et al., 1994; Clarke et al., 1995; Zähner and Hakenbeck, 2000). The structure of StrH has been solved and revealed that the two GH family 20 sites showed different specificity on N-glycans and in the same study it was shown that an inhibitor of StrH increased opsonophagocytosis (Pluvinage et al., 2011). Unencapsulated pneumococcal strains lacking NanA or BgaA showed decreased adherence *in vivo*, as did a triple knockout of all three exoglycosidases but the StrH knockout on its own showed wild-type adherence levels (King et al., 2006). On its own, the neuraminidase NanA showed no decrease in bacterial adherence in capsulated pneumococci but still cleaved silalic acids from lactoferrin, human secretory component and IgA1 (King et al., 2004). *S. pneumoniae* need to cross the blood brain barrier endothelium to cause meningitis. By knocking out the sialidase NanA and establish heterologous expression of the protein, NanA was shown to be both necessary and sufficient for promoting *S. pneumoniae* adherence and invasion of human brain microvascular endothelial cells (Uchiyama et al., 2009). The same sialidase is also important for the leukocyte inflammatory response as a knockout decreased, and heterologous expressing NanA strains, increased the proinflammatory effect of THP-1 cells through desialylation of the monocyte's surface (Chang et al., 2012). In this study, NanA was

seen to increase the proinflammatory cytokine response *in vivo* in a murine intranasal challenge model of pneumococcal infection. The Ashwell-Morell receptor of hepatocytes detects changes in sialic acid present in the bloodstream and regulates the components of blood coagulation, including von Willebrand factor, and the receptor is a key factor for the development of glycosylated biopharmaceuticals. The receptor was discovered to be responsible for the thrombocytopenia seen during *S. pneumoniae* sepsis by eliminating platelets desialylated by the pneumococcal sialidase NanA (Grewal et al., 2008). In a following study this system could be preactivated and modulated to induce a protective response in pneumococcal sepsis by lowering the intravascular coagulation (Grewal et al., 2013).

In the 1970s it was shown that *S. pneumoniae* carries O-glycosidase activity, glycan hydrolysis between the GalNAc and serine or threonine in the O-glycosidic bond (Bhavanandan et al., 1976). The enzyme was later denoted SpGH101 and belongs to the recently established GH family 101 with endo-α-N-acetylgalactosamine activity (CAZy). The structure of this enzyme has been solved and the gene identified (Caines et al., 2008). In the airway, both N- and O-linked glycans are present and for this reason the potential contribution of this enzyme to *S. pneumoniae* colonization was studied *in vivo*. The cleavage of core 1 O-linked glycans was found to be dependent on the prior removal of terminal sialic acid by NanA and a SpGH101 knockout showed a reduction in adherence to epithelial cells as well as a decrease in upper respiratory tract colonization (Marion et al., 2009).

Pseudomonas aeruginosa is an opportunistic human pathogen causing serious infections in severe burns, chronic wounds and in the lungs of cystic fibrosis patients. Adhesion to epithelial cells is of major importance for bacterial virulence and has been tested in assays involving lung (A549), liver (HepG2), and colon (Caco-2) epithelial cell lines (Pastoriza Gallego and Hulen, 2006). The variations in adherence was associated with the levels of sialic acids in cell surface glycoconjugates that increased expression of a *P. aeruginosa* sialidase that is thought to unmask new binding sites and in this way contribute to bacterial adherence. Potentially, the host can adapt to the ongoing infection by altered protein glycosylation. This can be exemplified by cystic fibrosis, where the N-glycosylation of the airway is increased and leads to attenuated infection by *P. aeruginosa* (Martino et al., 2011).

Nutrition

The niche in which the bacteria colonize the host has likely driven the evolution to maximize the outcome of the metabolic processes for bacterial abundance (Rohmer et al., 2011). The obvious function of bacterial enzymes active on host sugars is for

carbon utilization. There is evidence that regulation of bacterial virulence is linked to genes involved in complex carbohydrate utilization (Shelburne et al., 2008a). The finding of a direct link of metabolism to virulence through catabolite control protein A in group A *Streptococcus* indicates that the two are intimately intertwined (Shelburne et al., 2008b). The concept of interaction with host glycosylation is common among bacterial pathogens and have been studied both as modulation of immune functions and as sources of nutrition (Garbe and Collin, 2012). The nutritional aspect of bacterial glycosidases has been explored in a range of model organisms of which a few examples are discussed here.

In the dog pathogen *Capnocytophaga canimorsus* a deglycosylation complex for N-linked glycans has been discovered (Renzi et al., 2011). This pathogen carries a complex consisting of a binding motif, an endo-β-N-acetylglucosaminidase (GpdG), a pore protein and sequential exoglycosidases that work together to catch, release, transport and digest complex N-glycans from its surroundings. The bacteria can cleave the glycan of IgG in this manner and use the released sugars as carbon source to support growth (Figure 6).

Streptococcus oralis is an oral commensal but causes infections in immune-compromised patients. The ability of this bacterium to gain nutrients from the human acute phase protein α_1-acid glycoprotein has been studied (Byers et al., 1999). It was concluded that S. oralis growth is accompanied by expression of sialidase, N-acetylglucosaminidase and β-galactosidase activities. Matrix-assisted laser desorption/ionization (MALDI) analysis showed that all glycans of α_1-acid glycoprotein were cleaved and a terminal GlcNAc remained at the N-glycosylation site after sequential degrading of the sialic acids, galactose, fucose, GlcNAcs and mannose residues. The released sugars were all metabolized during growth except fucose that remained in the culture supernatants.

One individual enzyme can sometimes elicit functions both in nutrient acquisition and immune evasion. An example from *Treponema denticola* is the cell surface exposed exo-neuraminidase TDE0471 (Figure 6). This enzyme removes sialic acids from human serum glycoproteins to support bacterial growth and, at the same time, the enzyme protects from killing by the complement system through prevention of the deposition of membrane attack complex on the bacterial surface (Figure 6) (Kurniyati et al., 2013). In a mouse model lacking the complement system, the sialidase knockout strain caused a similar level of tissue damage as the wild type but in the normal mice it showed decreased virulence due to the inhibition of complement.

The genome of the gut commensal *Bifidobacterium longum* subsp *infantis* carries a

vast number of genes encoding putative enzymes with glycan hydrolyzing activities and three endo-β-N-acetylglucosaminidases have been characterized, EndoBI-1, EndoBI-2 and EndoBB (Garrido et al., 2012). The enzymes EndoBI-1 (GH family 18) and EndoBI-2 (GH family 18) were active on N-linked glycans after treatment at 95°C for 5 min, indicating that the enzymes potentially are heat resistant. EndoBI-1 was found to cleave N-linked glycans from a range of glycoproteins including IgG, IgA and human lactoferrin and also bound to $Man_3GlcNAc_2$, the common core of all N-linked glycans, in a glycan array experiment. The authors indicate that *B. longum* subsp *infantis* respond to glycoproteins in a similar manner as to human milk oligosaccharides and the clear interpretation is that these enzymes are involved in nutrient acquisition, although experimental evidence is lacking. It is believed that the composition of the intestinal bacterial flora, where *B. longum* subsp *infantis* reside, is of great importance for immunocompromised patients, elderly and infants, the latter being exemplified by the human milk that contains sugars that the digestive system cannot digest without help of commensal bacteria (Dallas et al., 2012).

Pathogenic bacteria have enzymes to modulate host glycosylation for various purposes, but compared to commensal bacteria, the glycoside hydrolase arsenal of pathogens are very limited. This can be exemplified by comparing the number of glycosyl hydrolases present in commensal and pathogenic bacterial genomes. For instance, *S. pneumoniae* carry 44 GH genes, *S. pyogenes* 25 GH genes while the commensal *Bacteroides thetaiotamicron* has 171 GH genes and *Bacteroides vulgatus* has 184 GH genes (CAZy). There is recent evidence that vancomycin-resistant enterococci hijack the commensal's ability to gain nutrition from complex carbohydrates to promote its own survival and persistence (Pultz et al., 2006). The same concept has been seen with *Clostridium difficile* and *Salmonella enterica* serovar *Typhimurium* that uses mucosal carbohydrates hydrolyzed by the microbiota for its own expansion within the gut (Ng et al., 2014). In inflammatory bowel disease, an increase in mucolytic bacteria led to a wider utilization of mucin by other bacterial species (Png et al., 2010). The commensal interaction with host glycosylation is potentially of great interest, both for bacterial pathogenesis since they may serve nutrients to pathogens, but also as a source for more advanced tools to study glycans. These three examples demonstrate that not only humans have co-evolved with our normal flora, bacterial pathogens have too.

GLYCAN ENGINEERING TOOLS

Studying bacterial effectors that interact with glycosylated players of the human immune system not only leads to a better understanding of the infection process, but

may also lead to applications of the proteins as biotechnology tools. Although the primary focus of research in the field of pathogenesis is to understand the infection process better, there are a number of examples of bacterial glycosidic enzymes being explored as tools for glycan engineering, site-specific glycosylation or glycan analysis.

IgG glycan engineering

The streptococcal endoglycosidase EndoS is currently being explored as a biotechnology tool to specifically modify the Fc-glycan of IgG. In neuromyelitis optica, an inflammatory demyelinating disease of the central nervous system, EndoS was used to convert pathogeneic antibodies to blocking antibodies that prevented neuromyelitis optica pathology in the spinal cord (Tradtrantip et al., 2012). Other biotechnology applications addressed the issue of serum IgG being an inhibitor of monoclonal antibodies by occupying the cell surface Fc-receptors. In an attempt to enhance the efficacy of monoclonal antibody therapy, EndoS was used to selectively deactivated serum IgG while a therapeutic antibody, resistant to the enzyme, was administered and showed dramatically increased binding to Fc-receptors compared to administration in normal serum (Baruah et al., 2012). The important glycan of IgG cannot only be removed but also replaced using EndoS. In a recent study, site-directed mutagenesis was applied to substitute amino acids D233A and D223Q, mutations that transformed EndoS to a glycosynthase and in this way reversing the hydrolytic reaction. Rituximab, carrying a mix of glycoforms (G0F, G1F, G2F), was first deglycosylated with EndoS and later predefined fully sialylated N-glycans were efficiently transferred through the mutated EndoS without product hydrolysis (Huang et al., 2012). This approach can be used to selectively engineer the wide variety of glycoforms on intravenous immunoglobulin (IVIG) to a fully sialylated Fc-glycan, to take full advantage of the postulated anti-inflammatory effects of glycans with terminal sialic acid (Anthony and Ravetch, 2010). This concept was further developed into being able to design glycoforms on IgG without the need of the cell glycosylation machinery. This was carried out by replacing the N-linked site at Asn297 with an amino acid sequence that was enzymatically modified to aldehyde groups that could react with GlcNAc and with an EndoS-glycosynthase, a defined glycoform was added to the IgG molecule (Smith et al., 2014). EndoS is not only explored as a tool for glycoengineering but has shown to be a promising biotherapeutic in a number of autoimmune conditions. The antibody driven autoimmune models where EndoS-treatment has been used to deglycosylate IgG and alleviate the symptoms include cellular and complement mediated hemolysis (Allhorn et al., 2010), encephalomyelitis (Benkchoucha et al., 2012), anti-collagen autoimmunity of the skin (Hirose et al., 2012), systemic lupus erythematosus (Lood et al., 2012), rheumatoid arthritis (Nandakumar et al., 2007a), and glomerulonephritis (Collin, 2012; van

Timmeren et al., 2010; Yang et al., 2010). Recently the crystal structure of EndoS was solved and revealed a five domain organization consisting of a endoglycosidase domain, a leucine rich repeat domain, a hybrid immunoglobulin domain, a carbohydrate binding domain, and a three helix bundle domain (Trastoy et al., 2014). The structure of EndoS could potentially aid further efforts in protein engineering of EndoS for both biotechnology and therapeutic applications.

Reversing the action of bacterial glycosidases has been applied to peptide glycosylation using a two step process. In the study an initial glucose residue was attached to the peptide backbone by the glycosyl transferase from *Actinobacillus pleuropneumoniae* (ApNGT) and transfer of a complex glycan to the specific site by either Endo-A from *Arthrobacter protophormiae* or Endo-M from *Mucor hiemalis* (Figure 6) (Lomino et al., 2013). The resulting glycan attached to the peptide was resistant to peptide-N-glycosidase F (PNGaseF) hydrolysis and also showed that the approach is potentially useful for glycosylating peptides to gain novel properties.

A therapeutic oriented application of PNGaseF (discussed later) utilizes the fact that the neonatal Fc receptor (FcRn) binds IgG independent of the glycosylation whereas the interactions of IgG to the other Fc receptors are glycan dependent (Radaev and Sun, 2001; Roopenian and Akilesh, 2007). In the study, PNGaseF deglycosylated monoclonal antibodies was administered to the mother and transferred to the fetus via FcRn and when in circulation it bound the antigen and blocked further interaction with effector cells of the immune system, in this case alleviating the autoimmune disease (Bakchoul et al., 2013). This study elegantly shows how the functions of a monoclonal therapeutic antibody can be fine tuned by modification of the Fc-glycan.

An enzyme from *S. pneumoniae* that has been used in a variety of applications is EndoD (Figure 6). The activity of this enzyme was first described in the 70s as an activity on radiolabeled γ-globulin and peptides thereof (Muramatsu, 1971). The enzymatic activity of EndoD can be reversed and acting as a glycosynthase, the enzyme has shown efficiency in specifically transferring mannose glycans with oxazolines to IgG pretreated with EndoS (Fan et al., 2012). It was found that EndoD preferred hydrolyzing core-fucosylated structures and that transglycosylation was more efficient on non-fucosylated GlcNAc. The mutants EndoD-N322Q and EndoD-N322A were able to transfer complex glycans after sialic acid, galactose and terminal GlcNAcs had been removed. The usefulness of transferring mannose glycans to IgG is however questionable since mannosylation has been shown to increase clearance of IgG in serum of humans (Goetze et al., 2011). EndoD was found to be specific for IgG carrying high-mannose glycans in the Fc-region possibly due the open nature of the putative carbohydrate binding site (SpCBM32) in the enzyme (Abbott and

Boraston, 2011).

Glycan analysis

The discovery of the peptide-N-glycosidase F (PNGaseF) has led to the emerge of the entire field of glycan analysis that today is a cornerstone in glycobiology and have contributed to making biological therapeutics safer (Figure 6). The peptide-N4-(N-acetyl-β-glucosaminyl)asparagine amidase activity was detected in supernatants from *Flavobacterium meningosepticum* (today known as *Elizabethkingia meningoseptica*) and was later defined to two enzymes, endo-F being a endoglycosidase and PNGaseF the true peptide-N-glycosidase (Elder and Alexander, 1982; Plummer et al., 1984). PNGaseF is today widely used in glycan analysis since it releases all glycoforms found in humans and allows fluorescent labeling of glycans for quantification in liquid chromatography (Ruhaak et al., 2010). Enzymatic release of intact N-glycans is a crucial step in the analysis of glycans and has supported profiling, characterization and structural analysis of the N-linked glycans (Tharmalingam et al., 2012). PNGaseF has also been applied in high-throughput analysis that allowed population wide studies of the human plasma glycome (Pučić et al., 2011).

Although PNGaseF was found in the supernatants of a pathogen and its activity on a broad range of glycoproteins described in detail, no study so far have investigated the contribution of this enzyme to the pathogenesis of *Elizabethkingia meningoseptica*. Recent reports indicates emerging nosocomial infections in developing countries with this opportunistic pathogen and future work on the impact of PNGaseF on pathogenesis would be of tremendous interest (Chang et al., 2014; Ratnamani and Rao, 2013).

Endo H is an endoglycosidase from *Streptomyces plicatus* that specifically cleave high-mannose and some hybrid N-linked glycans in the chitobiose core (Robbins et al., 1984). Since high-mannose glycans are a part of the biosynthetic pathway of all N-linked glycans the enzymes has been used to study the basic processes of glycosylation. Another example of EndoH use, is a study where the glycosylation machinery of yeast was modified to add unnatural sugars to the GlcNAc in the chitobiose core of glycoproteins and to analyze the efficiency of this modification, Endo H was used to digest the glycopeptides prior mass spectrometry analysis (Breidenbach et al., 2010).

The analysis of O-linked glycans is to date carried out using β-elimination, a process that may cause glycans to further degrade, called peeling (Wada et al., 2010). Efforts have been made to find a broad O-glycanase to facilitate the characterization of O-

linked glycans and allow fluorescent labeling prior to liquid chromatography. *E. faecalis* has been shown to carry endo-GalNAcase activity on non-sialylated core 1 and core 3 O-linked glycans (Goda et al., 2008; Koutsioulis et al., 2008). The O-GalNAcase SpGH101 from *S. pneumoniae* has not only been studied in bacterial adherence but also explored as a tool for O-glycan release. It specifically releases core 1 O-linked glycans and thus the usefulness is limited by the narrow specificity (Bhavanandan et al., 1976; Marion et al., 2009). Other bacteria that have enzymes with characterized O-glycosidase activity include: *B. bifidum, Clostridium perfringes, Propionibacterium acnes, Alcaligenes* sp., *Bacillus* sp., and *Streptomyces* sp. (Ashida et al., 2000; Fan et al., 1988; Fujita et al., 2005; Goda et al., 2008; Iwase et al., 1988; Koutsioulis et al., 2008; Yamamoto et al., 1987). The specificity of these enzymes for core 1, non-sialylated O-linked structures (in some cases also core 3) makes it difficult to use the enzymes for characterization of the complete O-linked profile on glycoproteins, but the enzymes have nonetheless been brought to the market; O-glycosidase from *S. pneumoniae* (Sigma Aldrich), O-glycosidase from *E. faecalis* (New England Biolabs) and Glyko-O-glycanase from *S. pneumoniae* (Prozyme).

CONCLUSION

To this date a number of bacterial glycosidases have been discovered and characterized. The impact of these enzymes on the pathogenesis of the bacteria involves nutrient acquisition, increased adherence, modification of glycoprotein effector functions, and modulation of the immune response. The concept that bacteria are able to modify glycans of their host is widespread among pathogens and commensals and most likely reflects an adaptation to the niche in which the bacteria reside. The study of bacterial enzymes has shed light on bacterial pathogenesis but has also led to important advances in glycoengineering and glycan analysis. The findings include site-specific glycosylation, high-throughput glycan analysis, deactivation of serum IgG, and tools to construct blocking antibodies. The advances are of substantial impact on the biotherapeutic industry and research community. However, some of the enzymes applied in glycoengineering have not at all been studied in their true environment as virulence factors and as mediators of bacterial infections. An example is PNGaseF that is widely used as an effective tool in analyzing N-glycans throughout the biotherapeutic industry but the understanding of this highly active peptide-N-glycosidase in the infection scenario of *E. meningoseptica* remains to be studied. Without doubt, such a broadly active enzyme could have a major impact on the pathogenesis of the bacteria and could lead to novel findings of the importance of glycans in the immune system. As we learn more about the known enzymes and continue to identify new ones, new applications and concepts will arise.

For instance on how to develop specific tools for further improvement of therapeutic antibodies or other glycoengineering applications. This will most likely have a major impact on the rapidly expanding biotherapeutic market.

Future perspective

The field of bacterial glycosidases is a growing area of research. New enzymes are continuously discovered and characterized and have played, and will continue to play a major role in the understanding of bacterial pathogenesis and aid the development of glycan analysis and glycoengineering techniques. The recent advances in glycan analysis as well as the integration of glycobiology to various disciplines will allow systematic and thorough analysis of bacterial modulation of host glycosylation. There are many bacterial sources that could be explored to study the interaction with host carbohydrates and that potentially may lead to novel approaches in glycan engineering and analysis. The bacterial commensals present themselves as a gold mine when it comes to enzymes with sophisticated and specific activities. The increasing availability of genomic information greatly facilitates rational screening for activities. However, in order to identify truly novel types of enzymes, traditional culturing under diverse conditions, genetic approaches such as random mutagenesis in combination with biochemical methods, will most likely be needed. As we learn more about the enzymes and their activity on new substrates, new insights may be gained to the importance of glycosylation of the host. The glycan of IgG is of crucial importance for the structural integrity of the antibody and it is likely that glycans are equally important for the function of other proteins. Through evolution, the bacteria have found the Achilles heels of the host and likely have ways of manipulating the most important glycosylation sites on proteins in the immune system.

The contribution of bacterial glycosidases to infection processes is of great interest but also challenging. The expression level of bacterial glycosidases *in vivo*, substrate differences between the human host and the model organism, and the interplay of pathogens with commensal bacteria, are issues that complicate the studies. New models to study the activity of these enzymes are therefore needed. The availability of glycoanalytical core facilities will allow a more systematic approach to finding the actual substrates for the enzyme of study and this will allow a more precise methodology to elucidate the virulence contribution. Taken together, the field of bacterial glycosidases will continue to influence the glycoengineering efforts, the study of site-specific glycans on host glycoproteins, and, most importantly, potentially give new insights to bacterial pathogenesis that may support the combat against the rising pressure of infectious diseases.

EXECUTIVE SUMMARY

Glycosidases in Bacterial Pathogenesis

- Immunmodulation
    - EndoS cleaves glycans from IgG and contributes to immune evasion
    - EndoE from *E. faecalis* cleave glycans of IgG, RNaseB and lactoferrin for nutritional and immunomodulatory purposes
    - Chitinases from *Listeria monocytogenes* reduces iNOS production *in vivo* and leads to increased bacterial survival
    - Sialidases in bacterial vaginosis makes SIgA susceptible for further degradation
- Adherence
    - *S. pneumoniae* have sialidase NanA, galactosidase BgaA, and β-N-GlcNAcase StrH. NanA and BgaA contributes to adherence *in vivo*.
    - NanA contributes to blood brain barrier crossing and desialylates the surface of THP-1 cells to increase the proinflammatory effect
    - The pneumococcal enzyme SpGH101 cleaves core 1 O-linked glycans and when knocked out the bacteria show reduced adherence.
    - The expression levels of a sialidase from *Pseudomonas aeruginosa* is responsible for variations in adherence seen among strains.
- Nutrition
    - The regulation of bacterial virulence is linked to genes involved in complex carbohydrate utilization.
    - *Capnocytophaga canimorsus* has a deglycosylation complex that binds, cleaves and, transports N-linked glycans as carbon source.
    - In *Treponema denticola* a cell surface neuraminidase cleave off sialic acids for growth and at the same time contributes to decreased complement activation.
    - Pathogenic bacteria hijacks the metabolism of commensals by utilizing carbohydrates released by mucolytic bacteria.

Glycosidases as Glycan Engineering Tools

- IgG Glycan Engineering
    - EndoS treated pathogenic antibodies turned therapeutic in neuromyelitis optica
    - IgG inhibition of a monoclonal antibody therapy can be helped with selected deactivation of serum IgG with EndoS
    - Peptide glycosylation can be achieved using a two step process involving Endo-A or Endo-M
    - PNGaseF deglycosylated monoclonal antibodies can be transported across the placenta and act blocking in the fetus
- Glycan Analysis
    - PNGaseF is widely used for glycan analysis since it releases all glycoforms of N-glycans in humans and has enabled high throughput analysis of population wide studies of the glycome
    - O-linked glycans are still released with chemical methods and no broad enzyme releasing all core types of O-glycans is present.

CHAPTER 5 – PRESENT INVESTIGATION

AIM OF THIS THESIS

The work presented in this thesis lies at the intersection of infection medicine and glycobiology. The foundation of this work has been the fact that glycosylation has fundamental functional roles in the immune system and that bacterial effectors interact with glycans to modify host defense mechanisms. This thesis aims to explore the above stated hypothesis, to investigate novel aspects of bacterial modulation of host defense in infection biology, and to explore bacterial enzymes for applications in biotechnology or as therapy. The reports presented in this thesis starts off studying endoglycosidases from gram-positive pathogens in infection (Paper I-III) and continue with a proposal of a biotechnology application using EndoS and EndoS2 (Paper IV). Finally, a potential therapy using EndoS modulation of antibody glycans is presented (Paper V).

PAPER I

The discovery of EndoS as a streptococcal effector with the ability to hydrolyze glycans on IgG, led to *in vitro* studies of the role of this enzyme in the pathogenesis of streptococci (Collin and Olsén, 2001a). However, the contribution of EndoS to virulence *in vivo* had not been studied. Therefore, we set out to study the importance of EndoS *in vitro* and *in vivo* using allelic replacement knockout bacteria coupled with heterologous expression in a less virulent strain. *In vitro* we saw that pretreating plasma with exogenous EndoS prior to opsonization of bacteria led to increased resistance to killing by neutrophils and monocytes. *In vivo* the EndoS knockout strain showed no significant difference in virulence as compared to the wildtype strain. In the same invasive model of infection, the strain with heterologous expression of EndoS showed a significant increase in virulence. We concluded that EndoS does not significantly contribute to group A streptococcal virulence in the invasive infection model, but that heterologous expression of EndoS augmented virulence.

PAPER II

The presence of EndoS was thought to be conserved throughout all group A streptococcal serotypes. But in strain NZ131 of serotype M49 a homologous gene was discovered and we aimed at characterizing this unknown enzyme. We found that this enzyme was 37% sequentially identical to EndoS and we denoted the enzyme EndoS2, considering the same location in the genome, the conserved active site, and the enzymatic activity on the N-glycan of IgG. Furthermore, we discovered activity on the acute phase protein α_1-acid glycoprotein, where EndoS2 selectively cleaved biantennary structures. In conclusion, EndoS2 is a unique, conserved endoglycosidase in serotype M49 of group A *Streptococcus* that cleaves glycans from IgG and α_1-acid glycoprotein.

PAPER III

EndoE is an endoglycosidase with sequence similarities to EndoS and the gene was discovered in the genome of *Enterococcus faecalis* (Collin and Fischetti, 2004). This enzyme is known to cleave glycans from IgG and the bovine glycoprotein RNaseB. We set out to test the activity of this enzyme on other important molecules of the immune system and characterize the functional aspects of potential glycan hydrolysis. We found EndoE to hydrolyze glycans from lactoferrin, a glycoprotein involved in biofilm inhibition and with general antibacterial properties. The hydrolysis of glycans on lactoferrin led to a decrease in biofilm inhibition and contributed to supporting to the growth of *E. faecalis*. To summarize, lactoferrin is a new substrate for EndoE and glycan hydrolysis of lactoferrin modulated its role in defense mechanisms and supported bacterial growth.

PAPER IV

Although EndoS and EndoS2 only hold 37% identity, both enzymes have enzymatic activity on the glycan on IgG. In paper IV we aimed to characterize and compare the enzymatic activities of EndoS and EndoS2 and used a selection of therapeutic antibodies as substrates. We found that both enzymes are active on the Fc glycan, and not on Fab glycosylation and that EndoS2 to a higher degree than EndoS hydrolyzes glycans with high mannose or hybrid structures. The difference in glycoform selectivity was utilized to generate a new enzymatic method for rapid determination of high mannose content on therapeutic antibodies, without the need for mass-spectrometry. We concluded that EndoS and EndoS2 are active on the Fc glycan on monoclonal antibodies, that they differ in glycoform selectivity, and that

they could be used in combination to analyze high mannose content, an important quality parameter of therapeutic antibodies.

PAPER V

The glycan in the Fc region of IgG is conserved and shared with other immunoglobulins, such as IgE and IgD. The significance of this glycan in IgE effector functions and receptor binding is debated. In paper V, we hypothesized that EndoS interacts with the conserved Fc glycan in IgE and that the lack of this glycan would impact the IgE mediated immune effector functions. We coupled biochemical analysis of the interaction between EndoS and IgE with *in vitro* and *in vivo* experiments to analyze the effect. Our results suggest that EndoS cleaved glycans of IgE and that this caused a reduction in IgE mediated immune cell activation. When EndoS was administered *in vivo* we saw a dramatic decrease in IgE mediated immune cell activation. Our conclusion is that the Fc-glycan of IgE is important for IgE mediated effector functions and that this could be a target using EndoS as therapy in severe IgE mediated allergies.

DISCUSSION AND FUTURE DIRECTIONS

The research on bacterial glycosidases has been carried out for decades, but the recent advent of tools to characterize these enzymes in detail has allowed an expansion in the field. The work in this thesis begun in the infection biology of group A *Streptococcus* and we brought the knowledge of bacterial endoglycosidases to potential therapeutic use and as tools for the biotechnology industry.

The contribution of glycoactive enzymes to bacterial pathogenesis may be of major impact as the presence of these type of enzymes is widespread throughout bacterial species, as discussed in chapter 4. Our data suggests that the role of EndoS in the pathogenesis of streptococci may be linked to the expression levels of the enzyme, since heterologous expression of EndoS increased virulence (Sjögren et al., 2011). In the discovery of EndoS and in our work on EndoS2, it became clear that expression and secretion are linked to the carbohydrates present in the culture media. To further shed light on the role of EndoS and EndoS2 during infection, future studies could focus on the regulation of expression to find in what phase of the infection the enzymes are secreted. It is unlikely that highly specific enzymes such as EndoS and EndoS2 are directly involved in gaining nutrients, as seen with the activity of EndoE on lactoferrin, but they are rather modulators of host defense. A way to explore if this occurs during a severe infection in humans would be to study streptococcal

sepsis patient material and look at the glycosylation profile of IgG in serum or specifically search for deglycosylated IgG. The amounts of IgG present in serum is very high and it would be interesting, for both infection medicine and therapy applications, to study the efficacy of very limited amounts of EndoS on such an enormous amount of IgG.

The key to understanding the role of streptococcal endoglycosidases in infection is not further detailed biochemical analysis but a more integrative approach. To reduce the problem of virulence to a single enzyme is rather simplistic and a more complex approach is required to deepen the understanding of bacterial pathogenesis. One approach could be to consider host damage instead of bacterial virulence, since the damage during an infection is normally not caused by the bacteria or its enzymes and toxins, but rather by the body itself (Casadevall and Pirofski, 2014).

The glycans on IgG have attracted major attention due to their influence over receptor binding and thus direction of the immune response. There are probably other glycans with similar impact on the function of the glycoprotein where they are attached. As we demonstrate in Paper V, the Fc-glycan N275 of IgE may be important for IgE to be able to activate basophils and mast cells. The glycans on antibodies are only partly studied and many more secreted host defense molecules are glycosylated and could be targets for bacterial enzymes to dampen the immune response. As novel functions of glycans in the immune system are being discovered, bacteria likely have evolved ways to modulate the defense response. The Fc-glycosylation of IgE is likely just one of the many cases where glycans have a profound impact on the function of their glycoprotein. Another example is the activation of neutrophils to form NETs via sialyl-lewis X structures on mucins in the saliva, which likely could be modulated by bacterial sialidases (Mohanty et al. submitted).

A vast number of bacterial glycosidases remain to be discovered. The challenge is not to discover new enzymes, but to find specific enzymes with activities that are of major impact for the infection, interest for the biotech industry, or targeting therapeutically relevant glycans or glycoproteins. A potential goldmine for novel glycoactive enzymes is the gut-bacteria, which have evolved to survive on complex carbohydrates. Potentially, the long sought broadly acting O-glycosidase could be discovered in this milieu. The interaction surfaces between bacteria and the host are covered by a thick layer of mucins, which are heavily O-glycosylated, and bacteria have developed ways to penetrate this layer (Hansson, 2012). A closer look at the enzymes involved in this penetration would be interesting both for infection biology and as a source for novel glycoactive tools.

The study of the activity of EndoE on human lactoferrin is an example of how a bacterial enzyme may increase the understanding of the role that glycans play in human defense. Incubating lactoferrin with EndoE reduced the anti-bacterial properties of this glycoprotein, and part of the activity can be ascribed to the glycosylation. Not all glycans on glycoproteins are equally important, and bacterial enzymes could discriminate between the more important glycans. As an example, researchers focusing on α_1-acid glycoprotein may find it interesting that EndoS2 cleaves only a selection of the carbohydrates on this extensively glycosylated protein and it can be speculated that these glycans are important for the functions of this acute-phase protein.

For the biotechnology industry there is an emerging need for tools to characterize and analyze present and future biopharmaceuticals. As the complexity of the therapeutic molecule increases, so does the challenge of characterization. EndoS and EndoS2 have already reached the market as tools to deglycosylate IgG and our study shows that the enzymes may also be used for the rapid determination of high-mannose glycans on therapeutic antibodies (Paper IV). From a structural point of view, the low amino acid identity of EndoS and EndoS2 while having similar activity on IgG is interesting and could be explored. A crystal structure of EndoS2 would provide an interesting template to discuss structure and function relationships of glycosidases.

CONCLUDING REMARKS

In this thesis we explored the hypothesis that glycans of the host defense can be modulated by bacterial enzymes and that these enzymes are useful both as tools for the biotechnology industry and as potential therapies.

Our approach to studying the glycobiology of bacterial infections in detail have resulted in characterization of a novel bacterial enzyme, EndoS2, that cleaves all IgG Fc-glycans and that has been developed into a product for the biotechnology industry. Furthermore, we have gained a deeper understanding of the importance of endoglycosidases for invasive infections of group A *Streptococcus* and found new targets for EndoE from *E. faecalis*. Finally, the hypothesis that the Fc-glycan of IgE is important for immune activation is supported by our research, showing that EndoS may be developed into a therapy for severe IgE mediated allergies.

We demonstrate that glycans are an integral part of the immune system, and that the study of bacterial effectors of glycosylation paves the way for a deeper understanding of infections, for novel tools supporting the biotech arena, and for new therapeutic strategies.

SAMMANFATTNING

Hur gör bakterier för att komma undan immunförsvaret? Vi har studerat hur en vanligt förekommande bakterie, grupp A streptokocker, gör för att undvika kroppens immunförsvar vid infektion. Vi har upptäckt enzymer som bidrar till bakteriens förmåga att undvika immunförsvaret genom att avväpna antikroppar. De bakteriella enzymerna kan användas i bioteknisk industri och eventuellt som framtida läkemedel.

Alla kroppens celler innehåller proteiner och många proteiner i kroppen är glykosylerade och kallas glykoproteiner. Det betyder att proteinerna bär kedjor av socker som är viktiga för proteinets struktur och funktion. I immunförsvarets finns interaktioner som helt beror av proteins sockerkedjor.

Kroppens försvar är uppdelat i ett medfött och ett specifikt (adaptivt) immunförsvar. Delarna av immunförsvaret kompletterar varandra när kroppen utsätts för en bakterie eller virus. Det medfödda immunförsvaret har inget immunologiskt minne utan attackerar strukturer som är gemensamma för flera mikroorganismer. Det adaptiva immunförsvaret har förmågan att utveckla immunologiskt minne i form av celler som producerar antikroppar specifikt riktade mot något främmande.

Antikroppar i blodet är glykoproteiner som kallas immunoglobulin G, IgG. Tidigare forskning har visat att sockerkedjorna på IgG har stor betydelse för antikroppens funktion. Om sockerkedjan saknas kan inte antikroppen binda till immunceller och då förstörs inte den främmande mikroorganismen.

Grupp A streptokocker är en vanlig bakterie hos människor. Vi bär den på huden och i svalget men den kan även orsaka halsfluss eller andra mycket allvarliga infektioner. Hos grupp A streptokocker har forskare upptäckt enzymet EndoS. Ett enzym är ett protein som förändrar andra proteiner och just enzymet EndoS klipper bort sockerkedjan på antikroppen IgG. Antikroppen förlorar då sin förmåga att tillkalla immunceller och förstöra bakterien. Streptokocker har flera andra enzymer och proteiner som bidrar till att undkomma det mänskliga försvaret. Vad har egentligen EndoS för betydelse vid infektioner av streptokocker?

I **artikel 1** förändrade vi generna hos grupp A streptokocker och genererade en EndoS-mutant, en bakterien som saknar EndoS men som har alla andra enzymer och proteiner. Vi jämförde vår EndoS-mutant mot ursprungsbakterien och studerade hur

sjuka möss blev i en modell av allvarlig streptokockinfektion. Det visade sig att mössen blev lika sjuka och vi drog slutsatsen att EndoS inte ökar bakteriens förmåga att orsaka sjukdom i möss. Vi genererade även en bakteriestam som utrycker stora mängder av EndoS och vi såg då att mössen blev sjukare och bakterien mer aggressiv. Vi tror att halten av enzymet EndoS i den lokala infektionsmiljön har betydelse för hur allvarligt sjukdomsförloppet.

I **artikel 2** upptäckte vi ett nytt enzym hos streptokocker som inte har studerats tidigare. Genom att jämföra det okända proteinet med EndoS fann vi att proteinerna vad 37% identiska, vilket är en låg siffra. Vi såg också att genen för det okända enzymet fanns på exakt samma plats som EndoS i bakteriens DNA, och kallade därför enzymet för EndoS2. Vi studerade enzymets aktivitet på IgG och upptäckte att det klippte bort alla typer av sockerkedjor från antikroppen. Till skillnad från EndoS, så klipper EndoS2 även bort socker från ett glykoprotein som är involverat i inflammation. Slutsatsen blev att EndoS2 är ett nytt enzym från streptokocker som klipper bort alla sockerkedjor från IgG.

Artikel 3, beskriver en studie där vi studerade en annan bakterie, *Enterococcus faecalis*, och enzymet EndoE som liknar EndoS, men som klipper bort sockerkedjor från flera glykoproteiner. Vi upptäckte att EndoE klippte bort socker från laktoferrin, ett protein som finns i modersmjölk och som har antibakteriella egenskaper. Laktoferrin utan sockerkedjor var mindre antibakteriellt och bakterierna överlevde bättre i närvaro av laktoferrin. Vi såg också att enterokocker kunde använda det bortklippta sockret som näringskälla.

Många nya läkemedel som utvecklas idag är biologiska läkemedel som består av specifika antikroppar, oftast IgG. I **artikel 4** jämförde vi den enzymatiska aktiviteten av EndoS och EndoS2 på fyra antikroppar som används som läkemedel. Vi upptäckte att EndoS2 klippte loss alla typer av socker medan EndoS lämnade en typ av socker som kallas hög-mannos. Det finns indikationer på att antikroppar med mannossocker fungerar sämre som läkemedel i kroppen. I artikeln presenterar vi en metod som använder EndoS och EndoS2 för att snabbt och enkelt bestämma halten av mannos på antikroppsläkemedel.

Artikel 5 handlar om en annan typ av antikropp, immunoglobulin E (IgE) och EndoS. IgE är en typ av antikropp som har stor betydelse vid allergiska reaktioner. IgE är precis som IgG, ett glykoprotein och bär flera sockerkedjor. Våra experiment visade att EndoS även klipper bort socker från IgE och att antikroppen då fungerar sämre. I en musmodell för allergi kunde vi genom att injicera EndoS, kraftigt minska den immunaktivering som genom IgE leder till en allergireaktion. Våra resultat är

preliminära, men kan tyda på att EndoS skulle kunna användas som läkemedel för kraftiga allergier.

I denna avhandling visar vi att bakterier använder enzymer som är aktiva på sockerkedjor på antikroppar för att undkomma immunförsvaret. Vi har upptäckt ett nytt enzym hos streptokocker, EndoS2, och studerat betydelsen av EndoS vid allvarlig streptokockinfektion. Vi visar att bakteriella enzymer kan användas i bioteknisk industri för utveckling av antikroppsläkemedel. Slutligen, har vi upptäckt att sockerkedjor på IgE har stor betydelse vid allergi och att EndoS skulle kunna användas som läkemedel vi svåra allergiska reaktioner.

Bakteriers smarta sätt att undkomma immunförsvaret kan lära oss mycket om hur immunförsvaret fungerar och hur bakterier orsakar infektion. Upptäckten av nya enzymer kan även leda till applikationer i bioteknisk industri och eventuellt till framtida läkemedel.

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PAPER I

BMC Microbiology

RESEARCH ARTICLE Open Access

Study of the IgG endoglycosidase EndoS in group A streptococcal phagocyte resistance and virulence

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Abstract

Background: The secreted enzyme EndoS, an endoglycosidase from *Streptococcus pyogenes*, hydrolyzes the *N*-linked glycan of the constant region of immunoglobulin G (IgG) heavy chain and renders the antibody unable to interact with Fc receptors and elicit effector functions. In this study we couple targeted allelic replacement mutagenesis and heterologous expression to elucidate the contribution of EndoS to group A *Streptococcus* (GAS) phagocyte resistance and pathogenicity *in vitro* and *in vivo*.

Results: Knocking out the EndoS gene in GAS M1T1 background revealed no significant differences in bacterial survival in immune cell killing assays or in a systemic mouse model of infection. However, exogenous addition and heterologous expression of EndoS was found to increase GAS resistance to killing by neutrophils and monocytes *in vitro*. Additionally, heterologous expression of EndoS in M49 GAS increased mouse virulence *in vivo*.

Conclusions: We conclude that in a highly virulent M1T1 background, EndoS has no significant impact on GAS phagocyte resistance and pathogenicity. However, local accumulation or high levels of expression of EndoS in certain GAS strains may contribute to virulence.

Background

Group A *Streptococcus* (GAS, *S. pyogenes*) is a human-specific pathogen producing diseases ranging from pharyngitis and impetigo to severe, invasive conditions such as necrotizing fasciitis and streptococcal toxic shock syndrome [1]. Causing an estimated 500,000 deaths annually [2], GAS is one of the world's most important pathogens, reflecting its wide repertoire of virulence factors that interfere with host immune clearance mechanisms [3]. A hypothesized GAS immune evasion factor is the secreted enzyme EndoS, an endoglycosidase possessing a highly specific hydrolyzing activity toward the *N*-linked glycan of immunoglobulin G (IgG) [4]. The IgG heavy chain is *N*-glycosylated at asparagine 297 with a complex biantennary oligosaccharide that is crucial for the interaction with Fc gamma receptors (FcγRs) on phagocytic cells [5-7]. Experimentally, enzymatic deglycosylation of murine IgG can decrease complement activation, binding of IgG to FcγRs on macrophages, and antibody-mediated cytotoxicity [5].

EndoS is specific to native IgG, which is in contrast to many related endoglycosidases that requires denaturation of their glycoprotein substrates [8,9]. Furthermore, pretreatment of IgG with recombinant EndoS diminishes its ability to opsonize bacteria and interact with FcγRs on leukocytes [10,11]. The activity of EndoS on IgG heavy chain glycans is well characterized and conserved among GAS serotypes [12]. However, a potential role of endogenous EndoS expression by the GAS bacterium in phagocyte resistance and virulence has not been elucidated. We hypothesize that EndoS contributes to GAS virulence by hydrolyzing the *N*-linked glycan on IgG and thereby impairing antibody mediated functions in the immune system. Here we couple targeted allelic replacement mutagenesis and heterologous gene expression to study EndoS activity during bacterial-host cell interaction *in vitro* and *in vivo*.

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Results

Generation of EndoS mutants and heterologous expression

To investigate the contribution of EndoS to GAS and host-cell interactions an allelic replacement knockout in the M1T1 background was constructed and denoted 5448 Δ*ndoS*. Heterologous expression of EndoS in a non-native EndoS producing GAS strain, NZ131 (serotype M49), was established by transformation of the EndoS expressing plasmid pNdoS. Loss- and gain-of-function was confirmed by Western immunoblot (Figure 1A) and IgG glycan hydrolysis assays (Figure 1B) [8]. As suspected no detectable EndoS was identified in the supernatants of the 5448Δ*ndoS* strain, and heterologous expression of EndoS in NZ131 was successful. In addition, higher levels of EndoS were observed in the overexpressing strain NZ131 [pNdoS] compared to the wild-type M1 strain 5448.

Neutrophil killing assay

The phagocytic resistance of GAS with and without EndoS contribution was investigated in a human neutrophil killing assay with GAS strains 5448Δ*ndoS* and wild-type 5448. Loss-of-function did not reveal significant difference in GAS resistance to phagocyte killing in the M1T1 background (Figure 1C). In the same M1T1 background, exogenous recombinant EndoS, rEndoS, or PBS was used to pretreat plasma to investigate phagocytic resistance contribution of the enzyme itself. It was found that rEndoS increases GAS survival in the presence of neutrophils and plasma containing GAS antibodies (Figure 1D). The contribution of EndoS to GAS virulence was also studied in the less virulent strain NZ131 (serotype M49) in gain-of-function analysis. The results reveal that heterologous overexpression of EndoS in M49, NZ131[pNdoS] increased GAS resistance to killing by human neutrophils (Figure 1E).

Monocyte killing assay

As with neutrophil killing assays, no significant difference in bacterial survival was detected in the monocytic killing assays when comparing M1T1 GAS strain 5448 to the isogenic *ndoS* knockout strain (Figure 2A). Pretreatment of plasma with exogenous rEndoS resulted in a significant increase in GAS resistance to killing by monocytes (Figure 2B), as did heterologous expression of EndoS in the less virulent strain NZ131 (Figure 2C).

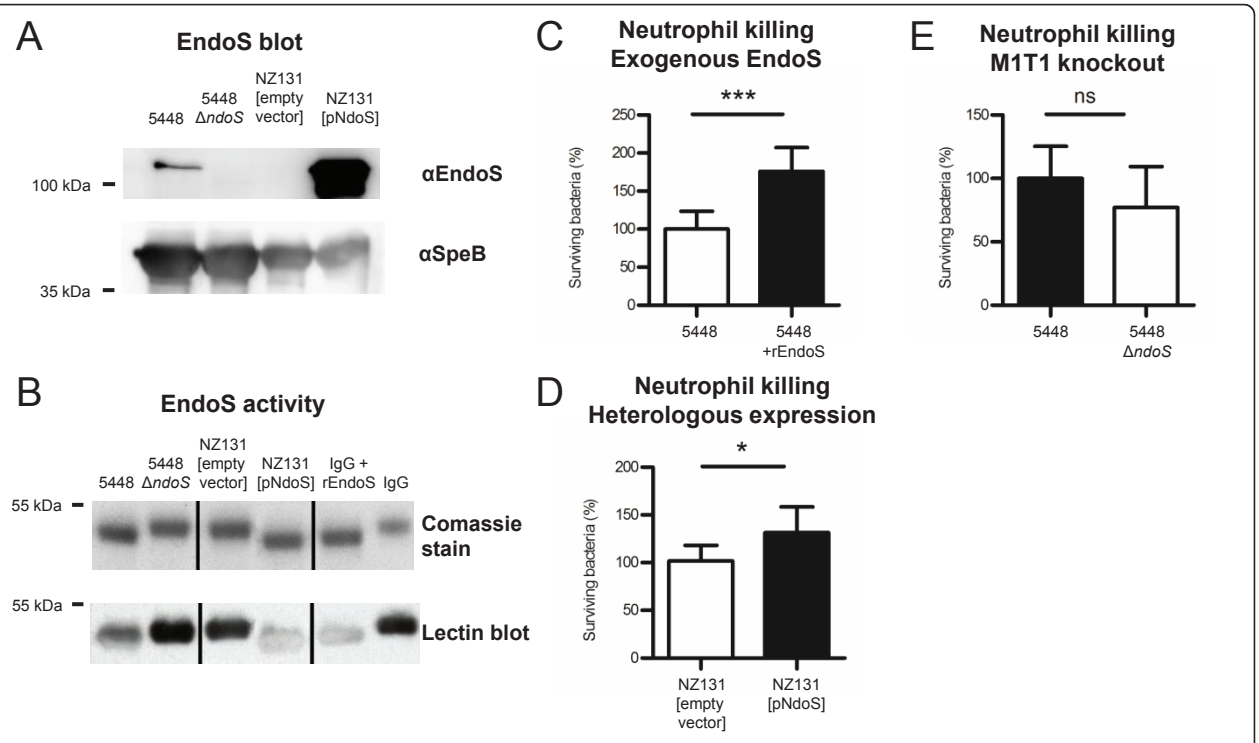

Figure 1 EndoS expression and activity, and neutrophil killing assays. (A) Western immunoblot showing EndoS expression in bacterial supernatants. SpeB is shown as a loading control. (B) Lectin blot analysis of murine IgG incubated with bacterial supernatants or rEndoS as a positive control. Opsonized bacterial survival in the presence of human neutrophils: (C) M1T1 GAS strain 5448 and isogenic *ndoS* knockout, 5448Δ*ndoS*. (D) Exogenous treatment of plasma with rEndoS prior to opsonization of GAS. (E) Heterologous expression of EndoS in NZ131 (serotype M49). Error bars indicate standard deviation from the mean. Experiments were performed in triplicate. * indicates $P < 0.05$, *** indicates $P < 0.001$, ns indicates no significant difference.

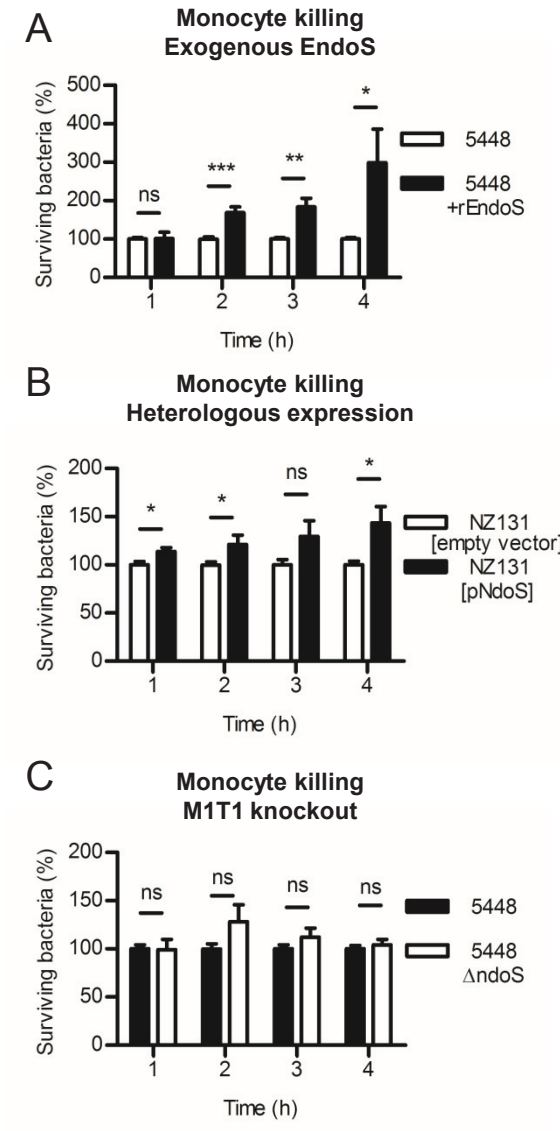

Figure 2 Opsonized bacterial survival in U937 monocytic cell killing assays. (A) M1T1 GAS strain 5448 and isogenic *ndoS* knockout, 5448Δ*ndoS*. (B) Exogenous pretreatment of plasma with rEndoS prior opsonization of GAS. (C) Heterologous expression of EndoS in NZ131 (serotype M49). Error bars indicate standard deviation from the mean. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, ns indicates no significant difference.

In vivo mouse model

Many major GAS virulence factors have been shown to decrease overall virulence when knocked out and studied in murine infection models [13-16]. It has also been shown that EndoS has activity on all subclasses of murine IgG [17]. Taken together, this led us to believe that the contribution of EndoS to GAS virulence could be studied *in vivo*. However, in this murine model of infection GAS strain 5448Δ*ndoS* showed no significant difference in virulence compared to wild-type 5448 (Figure 3A).

However, when we studied the less virulent GAS strain NZ131 (serotype M49) overexpressing EndoS, it was found that strain NZ131[pNdoS] showed increased virulence *in vivo* (Figure 3B) compared to wild-type NZ131[empty vector]. This may be a function of the relatively high level of expression of EndoS in NZ131 [pNdoS] compared to 5448 (Figure 1A).

Discussion

A single clone of the M1T1 serotype has disseminated globally during the last few decades to represent the leading cause of severe, invasive GAS infections [18]. The unique virulence of the M1T1 clone has been associated with many factors including the phage-encoded DNAse Sda1, allowing escape from neutrophil extracellular traps [13,19,20], the streptococcal inhibitor of complement (SIC) protein, promoting serum and antimicrobial peptide resistance [14,21], pro-inflammatory and phagocyte resistance properties of the M1T1 protein [15,22], high level expression of the pore-forming cytotoxin streptolysin O (SLO) [16], and a propensity for genetic mutations in the *covR/S* regulatory locus promoting hypervirulence [23,24]. There exist many inherent limitations of modeling a secreted bacterial virulence factor *in vitro* and of the mouse as a surrogate host for GAS infection studies. However, our studies do strongly suggest that the endogenous expression of EndoS may be redundant or dispensable for M1T1 GAS phagocyte resistance and pathogenicity, since targeted mutation of the other factors described above do yield clear attenuation of virulence phenotypes in similar *in vitro* and *in vivo* assay systems.

Conversely, pretreatment of plasma containing antibodies against GAS with recombinant EndoS reduced opsonphagocytic killing of GAS, and heterologous overexpression of EndoS in a less virulent M49 GAS strain conferred increased phagocyte resistance and increased lethality in the mouse infection model. These results suggest that high level expression or local accumulation of EndoS in tissues could contribute to virulence in certain GAS strain backgrounds or infection scenarios, a subject that could merit future analysis in larger clinical or molecular epidemiologic surveys.

EndoS is highly conserved among GAS serotypes and can also be found in *Streptococcus equi* and *zooepidemicus* [12]. Therefore, it was somewhat surprising that we could not detect a significant contribution to GAS virulence *in vivo*. This may be due to the limitations of the mouse model used, and the expression levels of EndoS during the murine infection. The expression level of this enzyme during a human infection could have an impact on GAS immune cell killing resistance but this remains

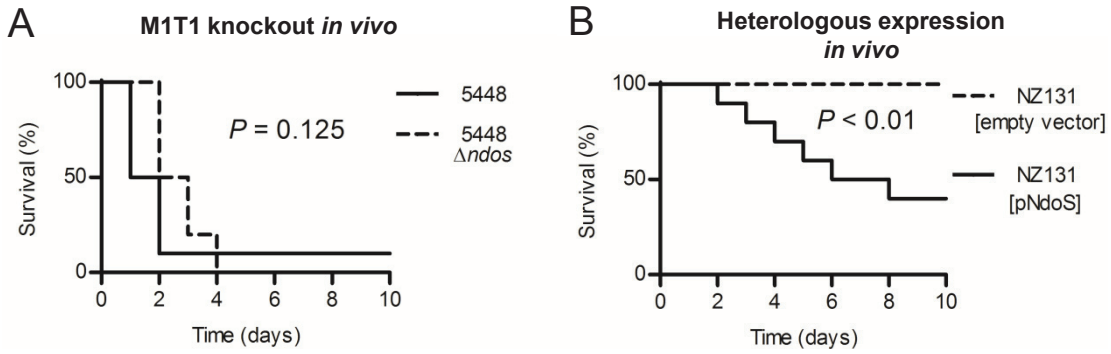

Figure 3 Survival curves of female CD-1 mice following intraperitoneal challenge with GAS. (A) M1T1 GAS strain 5448 and isogenic *ndoS* knockout, 5448Δ*ndoS*, at 2×10^7 cfu with 5% mucin (n = 6). (B) Heterologous expression of EndoS in NZ131 (serotype M49) at 5×10^8 cfu with 5% mucin (n = 10).

to be investigated. The specificity of EndoS activity towards IgG suggests that the enzyme may have an important role in the pathogenesis of GAS, yet to be discovered.

Finally, whether or not GAS can effectively deploy this unique enzymatic activity targeted IgG *N*-glycosylation to promote its own survival in the host (as is intuitively appealing), the enzyme itself has already proven a promising lead biotherapeutic for treatment of antibody-mediated inflammatory pathologies [17,25-29].

Conclusions

We conclude that in a highly virulent M1T1 background, EndoS has no significant impact on GAS phagocyte resistance and pathogenicity. However, our overexpression experiments could indicate that local accumulation or high levels of expression of EndoS can contribute to virulence in certain GAS strains, or in other infection scenarios than the systemic infection model used in this study.

Methods

Bacterial strains and growth

GAS strain 5448 (serotype M1T1, *ndoS*-positive) and GAS strain NZ131 (serotype M49, *ndoS*-negative) are well-characterized and were selected for use in this study [30,31]. *Escherichia coli* MC1061 was used as cloning tool [32]. The streptococcal and *E. coli* strains were propagated on Todd-Hewitt agar (THA). For selection, erythromycin (*erm*) was used at 5 μg/mL (5448), 2 μg/mL (NZ131) and 500 μg/mL (MC1061). GAS and its isogenic mutant were grown in Todd-Hewitt broth (THB (Difco, Detroit, MI)) at 37°C without shaking. For *in vitro* and *in vivo* experiments, fresh overnight cultures were diluted 1:10 in THB and grown to mid logarithmic phase (OD_{600} = 0.4) and resuspended in PBS, or in mid-log supernatants for neutrophil assays with NZ131. For analysis of streptococcal supernatants, strains were grown in C-medium (0.5% (w/v) Proteose Peptone no. 2 (Difco), 1.5% (w/v) yeast extract, 10 mM K_2HPO_4, 0.4 mM $MgSO_4$, 17 mM NaCl pH 7.5) to maximize EndoS expression.

GAS mutants

EndoS is encoded by the gene *ndoS*. A precise, in-frame allelic replacement of *ndoS* with chloramphenicol transferase, *cat*, was created in M1T1 GAS strain 5448 by a method previously described [13] and was denoted 5448Δ*ndoS*. Briefly, a 798 bp fragment upstream, and 987 bp fragment downstream of *ndoS* was amplified using polymerase chain reaction, PCR, using primers *ndoS*-up-F-XbaI (GCATCTAGAGCTTGTCGGTCTT GGGGTAGC), ndoS-up-R (GGTGGTATATCCAGT-GATTTTTTTCTCCATTTGGACACTCCTTATTTTT GGTACTAAGT C) and *ndoS*-dn-F (TACTGCGATG AGTGGCAGGGCGGGGCGTAAACAAAGTAACTT TCTTAGATAGCAACATT CAG), *ndoS*-dn-R-Bam HI (GCGGATCCGTTCTTGCGCCATGACACCTCC) respectively. The primers adjacent to *ndoS* contained 30 bp overhang of the *cat* gene corresponding to the 5' and 3' ends of *cat*, respectively. The upstream and downstream fragments were combined with the 650 bp *cat* gene in a fusion PCR using primers *ndoS*-up-F-XbaI and *ndoS*-dn-R-BamHI. This triple fragment was digested using restriction enzymes *Xba*I and *BamH*I and ligated using T4 ligase into the temperature sensitive vector pHY304, bearing erythromycin resistance, to generate the knockout plasmid pHY-*ndoS*-KO. pHY-*ndoS*-KO was transformed into GAS 5448 by electroporation and transformants were grown at the permissive temperature of 30°C with erythromycin. Transformants were then grown at the non-permissive temperature of 37°C with erythromycin present to select for homologous recombination and integration of the plasmid into the genome. Single crossovers were confirmed by PCR analysis. Relaxation of the plasmid was carried out at 30°C with

no antibiotic selection to allow the plasmid to reform, outside the chromosome. Growing the bacteria at 37°C without antibiotic pressure resulted in loss of the plasmid. Finally, screening for erythromycin sensitive colonies was used to identify double crossover events and allelic replacement mutants were confirmed by PCR. In frame allelic replacement *ndoS* mutant, 5448Δ*ndoS*, was confirmed by multiple PCR reactions showing the insertion of the *cat* gene and absence of the *ndoS* gene in the genome. Heterologous expression of EndoS in M49 GAS strain NZ131 was established by transformation with the EndoS expression plasmid pNdoS. *ndoS* was amplified from the M1 genome using primers *ndoS*-F-EcoRI (GCGAATTCATGGATAAACATTTGTTGGTAAAAAGAAC) and *ndoS*-R-BamHI (GCGGATCCTTATTTTTTTAGCAGCTGCCTTTTCTC), digested with *EcoR*I and *BamH*I prior to T4-ligation into the expression vector pDCerm, denoted pNdoS. As a control, GAS strain NZ131 was transformed with the empty vector pDCerm to generate NZ131[empty vector].

Western blot

Supernatants from stationary phase (16 h) GAS strains 5448, 5448Δ*ndoS*, NZ131[empty vector] and NZ131 [pNdoS] were precipitated with 5% final concentration of trichloroacetic acid and separated on a 10% SDS-PAGE gel and blotted onto a methanol activated PVDF membrane. The membrane was blocked in 5% skimmed milk (Difco) for 1 h and washed 3 × 10 minutes in phosphate buffered saline, PBS (137 mM NaCl, 2.7 M KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The membrane was then incubated with polyclonal rabbit antiserum against rEndoS at 1:2000 dilution in 0.5% skimmed milk and incubated for 1 h at 37°C. The membrane was washed as before and incubated with goat anti-rabbit IgG conjugated with Horse radish peroxidase (Bio-Rad), at 1:5,000 in 0.5% skimmed milk for 1 h at 37°C. After washing, the membrane was developed using Supersignal West Pico Chemiluminescent (Thermo Scientific, Rockford, IL) and analyzed on a Chemidoc XRS (Bio-Rad, Hercules, CA).

Lectin blot

Supernatants from GAS strains 5448, 5448Δ*ndoS*, NZ131[empty vector] and NZ131[pNdoS] at stationary phase (16 h) was incubated with 1 μg murine IgG (mIgG) for 2 h at 37°C at static conditions. As a positive control, IgG was incubated with 1 μg rEndoS. The glycan hydrolyzing activity was analyzed with SDS-PAGE and lectin blot using biotinylated *Lens culinaris agglutinin* (LCA) (Vector Laboratories, Burlingame, CA). LCA lectin recognizes the α-1,3 mannose residue found on the *N*-linked glycan on IgG. Briefly, the supernatants and mIgG were separated on 10% SDS-PAGE gels, onestained with Coomassie blue and the other blotted onto Immobilon PVDF membranes (Millipore, Bedford, MA). The membrane was blocked in lectin buffer (10 mM HEPES, 0.15 M NaCl, 0,1% Tween 20, 0.01 mM $MnCl_2$, 0.1 mM $CaCl_2$, pH = 7.5) for 1 h. 10 μg LCA in lectin buffer was incubated with the membrane for 1 h at RT. The membrane was then washed for 3 × 10 min in lectin buffer and incubated with 2 μg streptavidin linked HRP (Vector Laboratories) for 1 h. After washing as above the blot was developed using Supersignal West Pico Chemiluminescent (Thermo Scientific) as described for Western blots.

Neutrophil killing assay

Neutrophils were purified from healthy donors using PolyMorphPrep-kit (Axis-Shield, Oslo, Norway) and RBCs lysed with sterile H_20 as previously described [33]. Neutrophils were seeded at 2 × 10^5 cells/well in 96-well microtiter plates in RPMI.

Plasma was obtained from healthy volunteers as previously described [33]. All neutrophil and plasma donors exhibited high serum titer (>1:20,000) against serotype M1 and M49 GAS (Additional file 1 Table S1). GAS strains were grown as described and opsonized for 1 h at 37°C in 80% plasma, with or without pretreatment using recombinant EndoS (rEndoS) under rotating conditions. For pretreatment, 1 mL of plasma was incubated with 50 μg of rEndoS or PBS (control) at 37°C for 2 h with rotation. The bacteria were then diluted to the desired concentration in RPMI with a final concentration of 2% plasma and added to the neutrophils at a multiplicity of infection (MOI) of 10 bacteria per cell. Control wells contained GAS in RPMI and 2% plasma without neutrophils. The plate was centrifuged at 500 × *g* for 10 min and incubated for 30 min at 37°C with 5% CO_2 before being serially diluted in sterile H_2O and triplicate wells were plated on Todd-Hewitt agar (THA) plates for enumeration. Percent survival of the bacteria was calculated relative to control wells. Data from three separate experiments were normalized to 5448 or NZ131[empty vector] and combined.

Monocyte killing assay

The human monocytic cell line U937 was seeded at 5 × 10^5 cells/well in RPMI supplemented with 10% fetal bovine serum (FBS) in 24-well plates. GAS was grown and pre-opsonized in human plasma with or without rEndoS treatment, as described above. Bacteria were grown as described above and added to the U937 cells at MOI = 10 and incubated at 37°C with 5% CO_2. Samples were collected at 1, 2, 3 and 4 h when monocytes were lysed with 0.025% Triton X-100 (MP Biomedicals, Aurora, OH) and triturated vigorously. Surviving bacteria from triplicate wells were plated on THA for

enumeration. Percentage of surviving bacteria was calculated relative to the initial innoculum. Data from at least three separate experiments were normalized to 5448 or NZ131[empty vector] and combined.

Determination of donor serum titers

Blood from healthy human donors was collected in glass venous blood collection tubes with no additives (BD Biosciences, San Jose, CA) and clotted at room temperature for 15 min. Blood was centrifuged at 3,200 × g for 10 min at 4°C. The serum fraction was collected and stored at -80°C.

GAS strains NZ131 (serotype M49) and 5448 (serotype M1) were grown to mid-log phase in THB. Bacteria were resuspended in PBS and heat-killed at 95°C for 10 min. Heat-killed bacteria were mixed with a final concentration of 0.1 M NaHCO3 pH 9.6 and 10^6 bacteria per well were coated to 96-well high-bind ELISA plates (Costar, Cambridge, MA) at 4°C overnight. Plates were washed with PBS + 0.05% Tween (PBS-T) and blocked with 4% BSA + 10% FBS in PBS-T for 1 h at 37°C. Serum samples were diluted in blocking solution and incubated for 2 h at 37°C. Plates were washed with PBS-T and incubated with 1:5000 dilution of HRP-conjugated goat anti-human IgG antibody (Promega, Madison, WI) for 1 h at room temperature. Plates were washed five times with PBS-T and incubated with TMB substrate reagent (BD OptEIA TMB Substrate Reagent Set, BD Biosciences) at room temperature for 30 min. The reaction was stopped with an equal volume of 0.2 N sulfuric acid, and the plate was read at 450 nm. End point titer was determined as the dilution giving signal above a calculation cutoff determined using a mouse serum negative control and the calculation method described in [34].

In vivo mouse model

To evaluate the contribution of EndoS to GAS virulence *in vivo*, we utilized a murine model of systemic infection. GAS strains were grown as described and resuspended in PBS with 5% mucin for an inoculum of 2 × 10^7 cfu for WT M1T1 strain 5448 and isogenic mutant 5448Δ*ndoS*, and 5 × 10^8 cfu for NZ131[empty vector] and NZ131[pNdoS]. 8-10 week old female CD-1 mice (n = 6 for 5448, n = 10 for NZ131) were infected intraperitoneally with GAS strains and mortality was monitored daily for 10 days.

Statistical analysis

Cfu enumeration in neutrophil and monocyte killing assays were statistically analyzed by unpaired Student's *t*-test. Differences were considered significant if $P < 0.05$. The *in vivo* results were evaluated with log-rank (Mantel-Cox) test for comparison of survival curves. Differences in survival were considered significant if $P < 0.05$. All statistical analysis was performed using GraphPad Prism v.5 (GraphPad Software).

Ethical approval

Permission to collect human blood under informed consent was approved by the UCSD Human Research Protections Program. All animal use and procedures were approved by the UCSD Institutional Animal Care and Use Committee.

Additional material

Additional file 1: Table S1.

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Authors' contributions

JS participated in the design of the study, performed experiments and drafted the manuscript. MC and VN conceived of the study. CO performed experiments. AH designed the study and performed experiments. All authors read and approved the final manuscript.

Conflicts of interests

Patents for the *in vitro* and *in vivo* use of EndoS have been applied for by Genovis AB and Hansa Medical AB, respectively. MC is listed as inventor on these applications that are pending. Hansa Medical AB in part funded this study, but had no influence on the design of study, interpretation of data, or the final form of the manuscript. MC is a part time scientific consultant for Hansa Medical AB.

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Table S1: GAS serum titers of donors

Serum titer against heat-killed GAS (serotype M1 and M49) was determined for all donors whose plasma was used in neutrophil (Fig 1) and monocyte (Fig 2) killing assays.

Donor	M1 (strain 5448) titer	M49 (strain NZ131) titer
1	1:100,000	1:80,000
2	1:200,000	1:200,000
3	1:200,000	1:100,000
4	1:100,000	1:50,000
5	>1:500,000	1:200,000
6	1:20,000	1:20,000

PAPER II

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online data

EndoS$_2$ is a unique and conserved enzyme of serotype M49 group A *Streptococcus* that hydrolyses N-linked glycans on IgG and α_1-acid glycoprotein

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Many bacteria have evolved ways to interact with glycosylation functions of the immune system of their hosts. *Streptococcus pyogenes* [GAS (group A *Streptococcus*)] secretes the enzyme EndoS that cleaves glycans on human IgG and impairs the effector functions of the antibody. The *ndoS* gene, encoding EndoS, has, until now, been thought to be conserved throughout the serotypes. However, in the present study, we identify EndoS$_2$, an endoglycosidase in serotype M49 GAS strains. We characterized EndoS$_2$ and the corresponding *ndoS2* gene using sequencing, bioinformatics, phylogenetic analysis, recombinant expression and LC–MS analysis of glycosidic activity. This revealed that EndoS$_2$ is present exclusively, and highly conserved, in serotype M49 of GAS and is only 37 % identical with EndoS. EndoS$_2$ showed endo-β-N-acetylglucosaminidase activity on all N-linked glycans of IgG and on biantennary and sialylated glycans of AGP (α_1-acid glycoprotein). The enzyme was found to act only on native IgG and AGP and to be specific for free biantennary glycans with or without terminal sialylation. GAS M49 expression of EndoS$_2$ was monitored in relation to carbohydrates present in the culture medium and was linked to the presence of sucrose. We conclude that EndoS$_2$ is a unique endoglycosidase in serotype M49 and differs from EndoS of other GAS strains by targeting both IgG and AGP. EndoS$_2$ expands the repertoire of GAS effectors that modify key glycosylated molecules of host defence.

Key words: α_1-acid glycoprotein, endo-β-N-acetylglucosaminidase, host–pathogen interaction, IgG glycosylation, *Streptococcus pyogenes*.

INTRODUCTION

Glycosylation is a common post-translational modification, and almost all key molecules in the immune system are glycosylated [1]. IgG is the most abundant antibody in serum with the capacity to bind and neutralize antigens, facilitate antibody-dependent cytotoxicity, opsonize antigens and initiate phagocytosis. IgG is composed of two light and two heavy chains, of which the latter are glycosylated with complex N-linked glycans at Asn^{297}. The presence and structure of this glycan is of major importance for the interaction of the antibody with FcγRs (Fcγ receptors) and for the subsequent effector functions elicited by the antibody [2–4]. The glycan is present in a pocket of the two heavy chains of the IgG molecule, where it has been shown to be flexible and dynamic allowing it to influence the glycan–protein interaction with FcγR [5]. IgA, IgD, IgE and IgM each carry several occupied N- and O-linked glycosylation sites, and the study of the glycan's impact on the effector functions of these immunoglobulins has only begun [6].

Streptococcus pyogenes [GAS (group A *Streptococcus*)] is a leading Gram-positive bacterial pathogen exhibiting a wide array of immune evasion mechanisms, including interference with host glycosylation [7]. Every year, this bacterium causes over 500 000 deaths due to severe infections and post-infectious immunological disorders: invasive infections, rheumatic fever, glomerulonephritis and hundreds of millions of cases of milder and self-limiting infections, such as pharyngitis and impetigo [8]. GAS is subdivided into serotypes on the basis of the antigenic M-protein on the bacterial surface and there are currently over 100 serotypes described [9].

An endoglycosidase from *S. pyogenes*, EndoS, was discovered in serotype M1 of GAS and found to hydrolyse the N-linked glycan on the heavy chain of native human IgG and in this way modulate the binding of IgG to FcγR [10–12]. EndoS (EC

Abbreviations used: 2-AB, 2-aminobenzamide; ABS, *Arthrobacter ureafaciens* sialidase; AGP, α_1-acid glycoprotein; AMF, almond meal α-fucosidase; BEH, bridged ethane–silicon hybrid; BKF, bovine kidney α-fucosidase; BTG, bovine testes β-galactosidase; CM, C-medium; CcpA, catabolite control protein A; FcγR, Fcγ receptor; FLD, fluorescence detection; GAS, group A *Streptococcus*; GH18, family 18 of glycoside hydrolases; HILIC, hydrophilic interaction liquid chromatography; HRP, horseradish peroxidase; LCA, *Lens culinaris* agglutinin; 4MU-GlcNAc, 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide; MWCO, molecular-mass cut-off; NAN1, neuraminidase/sialidase 1; PNGase F, peptide N-glycosidase F; r, recombinant; UHPLC, ultra-HPLC.

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[2] Patents for the use of EndoS$_2$ have been applied for by Genovis AB. Jonathan Sjögren, Andrew Hollands, Victor Nizet, Maria Allhorn and Mattias Collin are listed as inventors on the application that is pending.

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The sequences of *Streptococcus pyogenes ndoS2* will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession numbers KC155346 (strain 3487-05), KC155348 (strain AP49), KC155347 (strain ACN49), KC155349 (strain AW1) and KC155350 (strain AW2).

3.2.1.96) belongs to GH18 (family 18 of glycoside hydrolases) and has endo-β-N-acetylglucosaminidase activity (CAZy, 2012; http://www.cazy.org). Enzymes in the family GH18 hydrolyses β-1,4-linked GlcNAc and this group of enzymes contains both chitinases (EC 3.2.1.14) hydrolysing the carbohydrate chitin and endo-β-N-acetylglucosaminidases (EC 3.2.1.96) with described endoglycosidase activity on the chitobiose core of N-linked complex glycans (CAZy, 2012).

EndoS is expressed in late stationary phase during streptococcal growth and the catalytically active glutamate residue (Glu^{235}) and several tryptophan residues are required for enzymatic activity [13]. Different from other described bacterial endoglycosidases, EndoS hydrolyses the N-linked glycan only on native and not denatured IgG [14]. Complement activation by the classical pathway was reduced when antibodies were treated with EndoS [11]. In human blood, the recombinant enzyme has been shown to deglycosylate IgG, and, in an opsonophagocytic killing assay, recombinant EndoS was shown to increase bacterial survival [11]. The contribution of EndoS to GAS virulence has been studied in a mouse model of invasive infection, and, although of minor importance in the wild-type M1 bacteria, it increased virulence of other GAS strains when heterologously expressed [15]. As a strategy to treat autoimmune diseases, EndoS has shown promise as a biotherapeutic in a number of animal models of autoimmunity [16–21]. For the biotechnology industry, the enzyme has applications both as a tool in the analysis of monoclonal antibodies (Genovis AB) and potentially for chemoenzymatic glycoengineering [22,23].

In the genome of GAS strain NZ131 of serotype M49, we have identified the gene *ndoS2* encoding the enzyme $EndoS_2$ [24]. *ndoS2* holds 53 % identity with *ndoS* and the proteins $EndoS_2$ and EndoS are 37 % identical. The GAS strain NZ131 is a clinical isolate from a case of acute post-streptococcal glomerulonephritis in New Zealand [24]. Serotype M49 belongs to a serotype grouping of GAS associated with skin infections and glomerulonephritis, group II (M2, M42, M49, M56, M57 and M60), rather than throat infections and rheumatic fever (M1, M4, M12 and M25) that define group I [24,25].

In the present study, we characterize $EndoS_2$ using bioinformatics, recombinant expression and LC–MS analysis to study the glycosidic activity.

MATERIALS AND METHODS

Bacterial strains and growth

The genome of *S. pyogenes* GAS strain NZ131 of serotype M49 has been sequenced and this strain was therefore selected as the reference strain in the present study [24,25]. GAS was propagated on blood agar, *Escherichia coli* strains Top10 (Invitrogen) and BL21 pLysS (Invitrogen) were propagated on lysogeny broth agar and used for cloning and recombinant expression. All strains used are summarized in Supplementary Table S1 (http://www.biochemj.org/bj/455/bj4550107add.htm). For selection in *E. coli* Top10 cells, carbenicillin was used at 100 $\mu g \cdot ml^{-1}$ and, for *E. coli* BL21 pLysS, 100 $\mu g \cdot ml^{-1}$ carbenicillin and 34 $\mu g \cdot ml^{-1}$ chloramphenicol were used. Overnight cultures of *E. coli* were carried out in lysogeny broth at 37 °C with aeration. Genomic DNA preparation of GAS strain NZ131 was performed using Puregene DNA Purification Kit (Qiagen). Transformation was carried out using heat-shock at 42 °C for 30 s. Plasmid preparations from *E. coli* were performed using Plasmid Miniprep Kit I (Omega Bio-Tek). All primers used are listed in Supplementary Table S2 (http://www.biochemj.org/bj/455/bj4550107add.htm). Expression of $EndoS_2$ was studied using growth of NZ131 in 50 % CM (C-medium) [0.5 % Proteose Peptone, 1.5 % (w/v) yeast extract, 10 mM K_2PO_4, 0.4 mM $MgSO_4$ and 17 mM NaCl (pH 7.5)].

Sequencing of *ndoS2*

Five GAS serotype M49 strains were selected for sequencing of the *ndoS2* gene; 3487-05, AP49, ACN49, AW1 and AW2. Sequencing was carried out using primers ndoS2-out-R, seq38-R, seq42-R, seq54-R, seq15-F, seq17-F, seq24-F and seq28-F and the Lightrun sequencing service of GATC Biotech (Konstanz, Germany). All primers used for sequencing are summarized in Supplementary Table S2. The sequences have been deposited in GenBank® with accession numbers as follows: KC155346 (strain 3487-05), KC155348 (strain AP49), KC155347 (strain ACN49), KC155349 (strain AW1), KC155350 (strain AW2) (Supplementary Table S2).

Recombinant expression of $EndoS_2$

Recombinant expression of $EndoS_2$ in *E. coli* was established by PCR amplification of the *ndoS2* gene from GAS NZ131 with the primers ndoS2-F-BamHI, 5′-CTGTAAGGATCCAGGAGAAGACTG-3′, and ndoS2-R-XhoI, 5′-GAAACCTCGAGTCTTTGTAATCGTAGGACTT-3′. The *ndoS2* fragment was digested with restriction enzymes BamHI and XhoI (restriction sequences are underlined) and ligated into the expression vector pGEX-5X-3 (GE Healthcare) using DNA ligase T4 (Thermo Fisher Scientific) creating the plasmid pGEX-ndoS2. The expression vector was transformed into *E. coli* Top10 chemically competent cells and screened with PCR using primers ndoS2-F-BamHI and ndoS2-R-XhoI. Positive clones were isolated and the pGEX-ndoS2 plasmid was purified and transformed into the *E. coli* expression strain BL21 pLysS. One recombinant clone was grown overnight at 37 °C with antibiotics, diluted 1:20 in lysogeny broth medium with antibiotics and grown for 3 h to mid-exponential phase. The expression of the protein GST–$EndoS_2$ was induced with 0.1 mM IPTG for 3 h. The cells were harvested and lysed with BugBuster Protein Extraction Reagent (Novagen/Merck). Recombinant GST–$EndoS_2$ was purified on a column with glutathione–Sepharose 4B (GE Healthcare) and eluted with reduced glutathione. The GST tag was cleaved off using Factor Xa (New England BioLabs). Site-directed mutagenesis was performed on pGEX-ndoS2 using a QuikChange® II Site-Directed Mutagenesis Kit (Agilent) with primers ndoS2(E-L)-F and ndoS2(E-L)-R, to exchange the glutamate residue (E) in the active site to leucine (L). Recombinant $EndoS_2$(E186L) was cloned and expressed in a similar way to $EndoS_2$.

Phylogenetic analysis

We searched non-redundant protein databases at NCBI with the BLASTP algorithm, submitting the $EndoS_2$ sequence of GAS strain ACN49 (M49). We retrieved similar protein sequences, setting a cut-off of the expect value at $<10^{-10}$. All but two sequences belonged to GH18, except for two sequences of other hypothetical proteins. All sequences were included in phylogenetic analyses of the proteins, and the non-GH18 proteins served as outgroup. A total of 101 protein sequences were aligned in Geneious version 6.0.3 (Biomatters Ltd, available from http://www.geneious.com/) using the ClustalW algorithm (Supplementary Table S3 at

http://www.biochemj.org/bj/455/bj4550107add.htm). From the alignment of 1817 amino acids, a region of generally high coverage comprising 1099 amino acids was extracted and analysed in BEAST version 1.7.4 [7,26]. We used the Blosum62 substitution model [8,27] with no site heterogeneity specification, set a strict molecular clock, selected the Yule process [9,28] for tree prior and ran Bayesian MCMC sampling every 1000 generations for 10 million generations. The output was examined with Tracer version 1.5 (A. Rambaut and A.J. Drummond, available from http://beast.bio.ed.ac.uk/Tracer) in order to ensure that likelihood scores were stationary and that effective sample sizes were adequate (>500), setting burnin to 25 %. A maximum clade credibility tree was calculated with TreeAnnotator version 1.7.4 [1,26]. 16S rRNA (RNA or cDNA) sequences were retrieved from the Ribosomal Project Database (http://rdp.cme.msu.edu) and GenBank® for taxonomic analyses of the corresponding taxa/strains in the EndoS dataset (Supplementary Table S3). In case a specific strain was lacking for taxa more distantly related in the EndoS protein tree dataset, another strain was chosen if available. This resulted in a total of 51 representative sequences, which were aligned according to the procedures described for the EndoS protein dataset above. From the resulting alignment of 2172 nt, a high coverage region comprising 1576 positions was extracted and analysed in BEAST version 1.7.4 [2–4,26] with similar parameter settings except for the substitution model [HKY with rate variation across sites following a discrete gamma distribution (G) with four rate categories]. Verification and tree calculation followed the procedures above, and the tree was rooted using the fungal sequences, which were a part of the dataset, as outgroup.

EndoS$_2$ expression analysis

Overnight cultures of NZ131 in CM were diluted 1:50 and grown for 16 h at 37 °C with 5 % CO_2 and the cysteine protease inhibitor E-64 at 20 μM. Glucose, galactose, sucrose, acetylglucosamine and mannose were added at 0.01 % concentration when indicated. Supernatants were concentrated using precipitation with 0.3 mM TCA (trichloroacetic acid). The samples and 0.5 μg of recombinant EndoS$_2$ were resuspended in SDS/PAGE loading buffer and loaded on to a 10 % Bis-Tris gel. The electrophoresis was performed at 180 V for approximately 60 min and stained with PageBlue Protein Staining Solution (Fermentas). Blotting on to a PVDF membrane was performed according to the manufacturer's instructions using Trans-Blot Turbo (Bio-Rad Laboratories) equipment. The membrane was blocked in 5 % (w/v) dried skimmed milk powder (Difco) and incubated with 10 μl of rabbit polyclonal anti-EndoS$_2$ for 1 h at 37 °C with rotation. Washing was consistently carried out in PBST (PBS with 0.05 % Tween 20) three times for 10 min. The membrane was washed and incubated with 2.5 μl of HRP (horseradish peroxidase)-conjugated goat anti-(rabbit IgG) (H + L) (Bio-Rad Laboratories), washed and developed using Supersignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Antiserum against EndoS$_2$ was obtained using 1 mg of recombinant EndoS$_2$ to immunize rabbits following standard protocols by Davids Biotechnologie, Regensburg, Germany.

N-glycan hydrolysis assay

A sample of 1 μg of recombinant EndoS$_2$, EndoS$_2$(E186L) or EndoS or 500 units of PNGase F (peptide N-glycosidase F) was incubated with 3 μg of IgG or 5 μg of AGP (α_1-acid glycoprotein) (Sigma–Aldrich) in PBS at 37 °C for 2 h. For PNGase F, the substrate was denatured according to the manufacturer's instructions (New England BioLabs). Human IgG subclasses IgG$_{1–4}$ (Calbiochem/Merck) were incubated with recombinant EndoS$_2$, EndoS$_2$(E186L) or PBS under the reaction conditions described above. All reactions were separated on a 10 % Bis-Tris gel as described above. Lectin blotting was performed on a PVDF membrane (Millipore). The membrane was incubated in lectin blot buffer (10 mM Hepes, 0.15 M NaCl, 0.1 % Tween 20, 0.01 mM $MnCl_2$ and 0.1 mM $CaCl_2$) for 1 h and incubated with 5 μg of biotinylated LCA (*Lens culinaris* agglutinin) (Vector Laboratories) in the same buffer. The membrane was washed three times for 10 min in lectin blot buffer and subsequently 2.5 μg of HRP coupled to streptavidin (Vector Laboratories) was added for 1 h. The membrane was developed as described above. The 16 h bacterial supernatants were concentrated using 10 kDa MWCO (molecular-mass cut-off) spin columns (Pall) and a functional assay on the activity of the supernatants on 3 μg of human serum IgG was performed. The reaction mixture was incubated at 37 °C overnight and analysed by SDS/PAGE (10 % gel) and a subsequent LCA lectin blot as described above.

Chitinase assay

4MU-GlcNAc (4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide) (Sigma–Aldrich) was incubated at 0.2 mM with 0.3 m-unit of chitinase from *Streptomyces griseus* (Sigma–Aldrich) or 2 μg of rEndoS$_2$ (where r denotes recombinant) or 2 μg of rEndoS or PBS in 100 μl of PBS. The reactions were incubated at 37 °C for 1 h. Then, 100 μl of 0.1 M glycine (pH 10) was added to stop the reaction. Absorbance at 355/445 nm was measured in a black 96-well plate using a spectrophotometer. The experiments were carried out using five replicates and results are shown as means±S.D. The response in absorbance was analysed statistically by an unpaired Student's *t* test, where differences were considered significant if $P < 0.05$. ****$P < 0.001$.

Glycoprotein denaturing

A 4 μg amount of IgG or AGP was incubated in 10 μl of PBS at 37 °C, 40 °C, 50 °C, 60 °C, 70 °C or 80 °C for 30 min. After the incubation, the samples were kept at 37 °C. Then, 2 μg of rEndoS$_2$ was added to each reaction mixture and incubated further at 37 °C for 2 h. The samples were analysed on a SDS/PAGE gel and for IgG with LCA lectin blotting as described above. For analysis of EndoS$_2$ specificity, 4 μg of α_2-macroglobulin, ovalbumin, human lactoferrin, RNase B and fetuin (all Sigma–Aldrich) were incubated with 2 μg of EndoS$_2$ at 37 °C overnight and subsequently analysed on SDS/PAGE gel as described.

LC–FLD (fluorescence detection)–MS

Online coupled LC–MS with FLD was performed using a Waters Xevo G2 QTof with Acquity UPLC and BEH (bridged ethane–silicon hybrid) glycan column (1.0 mm×150 mm, 1.7 μm particle size). MS data was acquired in negative mode with the following conditions: 2500 V capillary voltage, 50 V cone voltage, 280 °C desolvation temperature, 600 l·h^{-1} desolvation gas and 100 °C source temperature. The analyser was set to sensitivity mode. The fluorescence data rate was 1 point·s^{-1} and a PMT gain of 10 with excitation and emission wavelengths set at 320 nm and 420 nm respectively. Samples were in 80 % acetonitrile with an injection volume of 10 μl. The flow rate was 0.150 μl·min^{-1}. Solvent A was 50 mM ammonium formate (pH 4.4) and solvent

Figure 1 Genetic context analysis of *ndoS* and *ndoS2*

The genetic context of *ndoS2* (from NZ131/M49) and *ndoS* (from MGAS5005/M1) was analysed by aligning and comparing the identity of the sequences in MacVector.

B was acetonitrile. A 40 min linear gradient was used and was as follows: 28–43 % solvent A for 31 min, 70 % solvent A for 4 min and 28 % solvent A for 4 min.

Exoglycosidase digestion arrays

Analysis of glycan sequence, composition and linkage specificities was facilitated by the use of exoglycosidase digestion arrays. All digestion reactions were performed with enzymes from Prozyme. Fluorescently labelled glycans were digested in 50 mM sodium acetate (pH 5.5) at 37 °C overnight using a panel of enzymes with each digestion reaction brought to a final volume of 10 μl using double-distilled water. Digested glycans were then separated from the enzyme mixtures using 10 kDa MWCO centrifugal filters. Digested 2-AB (2-aminobenzamide)-labelled glycans were then prepared for separation on UHPLC (ultra-HPLC) with fluorescence detection using a BEH glycan column as described previously [5,29]. Specific non-reducing end monosaccharides were removed as follows: terminal sialic acid in all linkages was removed with 1 m-unit·μl^{-1} ABS (*Arthrobacter ureafaciens* sialidase); terminal galactose monosaccharides were removed using 0.5 m-unit·μl^{-1} BTG (bovine testes β-galactosidase), which releases both β(1,3)- and β(1,4)-linked galactose; terminal GlcNAc monosaccharides were released with 40 m-unit·μl^{-1} GUH (*Streptococcus pneumoniae* hexosaminidase), capable of cleaving β-linked GlcNAc moieties; core α(1,6)-fucose was selectively removed using 1 m-unit·μl^{-1} BKF (bovine kidney α-fucosidase), (2,3)-linked sialic acid was removed using 10 m-unit·μl^{-1} recombinant *Streptococcus pneumoniae* NAN1 (neuraminidase/sialidase 1), and AMF (almond meal α-fucosidase) at 6 m-unit·μl^{-1} was used to release (1,3)- and (1,4)-linked non-reducing terminal fucose residues.

Activity of EndoS$_2$ on free N-glycans

N-glycans present on 80 μg of bovine fetuin (Sigma–Aldrich) were released using 2500 units of PNGase F (New England BioLabs), and labelled with 2-AB (Ludger). The labelled fetuin 2-AB glycan pool was then incubated at 37 °C for 16 h in the presence of 80 μg·ml^{-1} EndoS$_2$ in PBS to determine the activity of EndoS$_2$ on free N-glycans. The resulting EndoS$_2$-digested glycan pool was then relabelled with 2-AB. Each glycan preparation was separated using a 1.7 μm BEH glycan column (2.1 mm×150 mm, Waters) and analysed by UHPLC–FLD–MS using a Waters ACQUITY UPLC® H-Class Bio with fluorescence detection coupled to a Waters Xevo G2-S Q-ToF mass spectrometer. The column temperature was 40 °C with a flow rate of 0.4 ml·min^{-1} using a linear gradient of 50 mM ammonium formate (pH 4.4) against acetonitrile with ammonium formate increasing from 30 % to 47 % over a 32 min period. Fluorescence detection was achieved using excitation and emission wavelengths of 330 nm and 420 nm respectively. Eluting glycans were detected in positive mode with the following settings: cone voltage of 80 V, capillary voltage of 3.0 kV, source temperature of 120 °C, desolvation temperature of 300 °C, and desolvation gas flow of 800 l·h^{-1}. Mass data were acquired using sensitivity mode with a mass range of 750 *m/z* to 2000 *m/z* with a 1.0 s scan time. Both LC–FLD and LC–MS data were acquired and processed using Waters UNIFI version 1.6.

RESULTS

Identification of EndoS$_2$ from GAS serotype M49

In the sequenced genome of GAS strain NZ131 (serotype M49), we identified *ndoS2,* a gene harbouring a GH18 domain [6,24]. *ndoS2* from GAS serotype M49 was found in the same genetic context as *ndoS* from GAS serotype M1, but showed only 53 % nucleotide identity with *ndoS* (Figure 1). The surrounding genes, i.e. *scrb, scra, scrk* and *pmi*, showed a high degree of nucleotide identity when comparing the chromosomal context between strain NZ131 and serotype M1 strain MGAS5005 (Figure 1). One genome of serotype M49 is available to the public (NZ131, GenBank® accession number NC_011375) and therefore *ndoS2* was sequenced in five M49 strains of different origin and isolation year (3487-05, ACN49, AP49, AW1 and AW2). The comparison revealed 100 % identity of *ndoS2* in the five selected strains compared with *ndoS2* found in NZ131. The *ndoS2* sequences have been submitted to GenBank® (Supplementary Table S1). The deduced amino acid sequence of EndoS$_2$ and EndoS revealed 37 % identity when aligned using ClustalW (Figure 2). The signal peptide was conserved, but three major sections of the EndoS amino acid sequence were lacking in EndoS$_2$; at positions 45–83, 535–561 and 933–986, gaps can be seen in the alignment. A comparison of the active site of EndoS$_2$ and EndoS revealed the GH18 motif (DXXDXDXE) with glutamate at position 186 as the catalytic amino acid to be conserved (Figure 2). Specific tryptophan residues have previously been shown to be important for the enzymatic activity of EndoS, and when EndoS$_2$ was aligned and compared with EndoS, tryptophan residues at positions 121, 164, 332, 361, 391, 809, 828 and 907 were found to be conserved [10–13].

In order to evaluate the evolutionary history of EndoS$_2$, we reconstructed a protein specific phylogenetic tree, using BEAST version 1.7.4, on 101 protein sequences selected with the BLASTP algorithm on EndoS$_2$. EndoS$_2$ (depicted in blue) was found to be unique to GAS serotype M49 and relatively different from EndoS found in other serotypes of GAS as well as EndoS-like proteins in other *Streptococcus* species (Figure 3). This can be contrasted with the taxonomic phylogeny of the 16S rRNA sequences (Supplementary Figure S1 at http://www.biochemj.org/bj/455/bj4550107add.htm).

EndoS$_2$ hydrolyses the N-linked glycan on the heavy chain of IgG

Previous work has concluded that EndoS hydrolyses the N-linked glycan on IgG [10,13]. Although the enzymes are different, we tested whether IgG is a substrate for EndoS$_2$. A comparison of

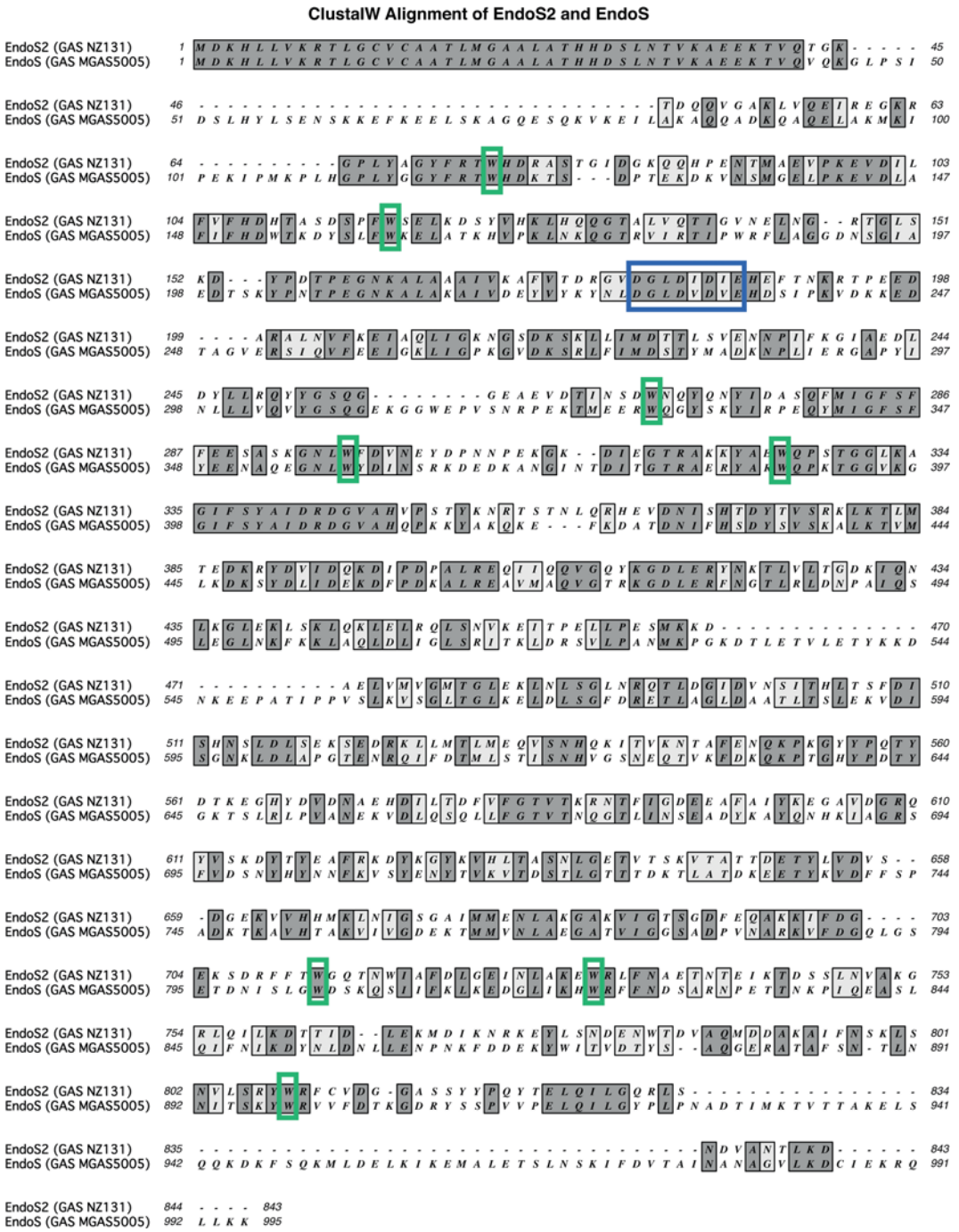

Figure 2 ClustalW alignment of EndoS$_2$ and EndoS

EndoS$_2$ from GAS strain NZ131 and EndoS from GAS strain MGAS5005 was aligned using ClustalW. Depicted in blue is the GH18 active site (DXXDXDXE) and in green are conserved tryptophan residues.

the hydrolysis of the N-linked glycan on the heavy chain of IgG was carried out using EndoS$_2$, EndoS and PNGase F as positive control [14,30]. PNGase F from *Elizabethkingia meningoseptica* cleaves between the GlcNAc and the asparagine residue of N-linked glycans, whereas EndoS cleaves between the two GlcNAc moieties in the chitobiose core of N-linked glycans [10,11]. EndoS$_2$ was mutated in the active site through site-directed mutagenesis where the catalytically active glutamate residue was mutated to leucine, creating the enzyme EndoS$_2$(E186L). EndoS$_2$, EndoS$_2$(E186L), EndoS and PNGase F were incubated with human IgG in PBS at 37 °C overnight, and analysed by SDS/PAGE and a subsequent LCA (recognizing α-linked mannose) lectin blot (Figure 4A). The gel shows a ~4 kDa shift of the heavy chain of IgG and a corresponding lack of LCA lectin signal when incubated with EndoS$_2$, EndoS or PNGase F, but not with EndoS$_2$(E186L) or PBS (Figure 4A). This result indicates that EndoS$_2$ hydrolyses the N-linked glycan on the heavy chain of IgG and confirms the glutamate residue at position 186 of EndoS$_2$ to be the catalytically

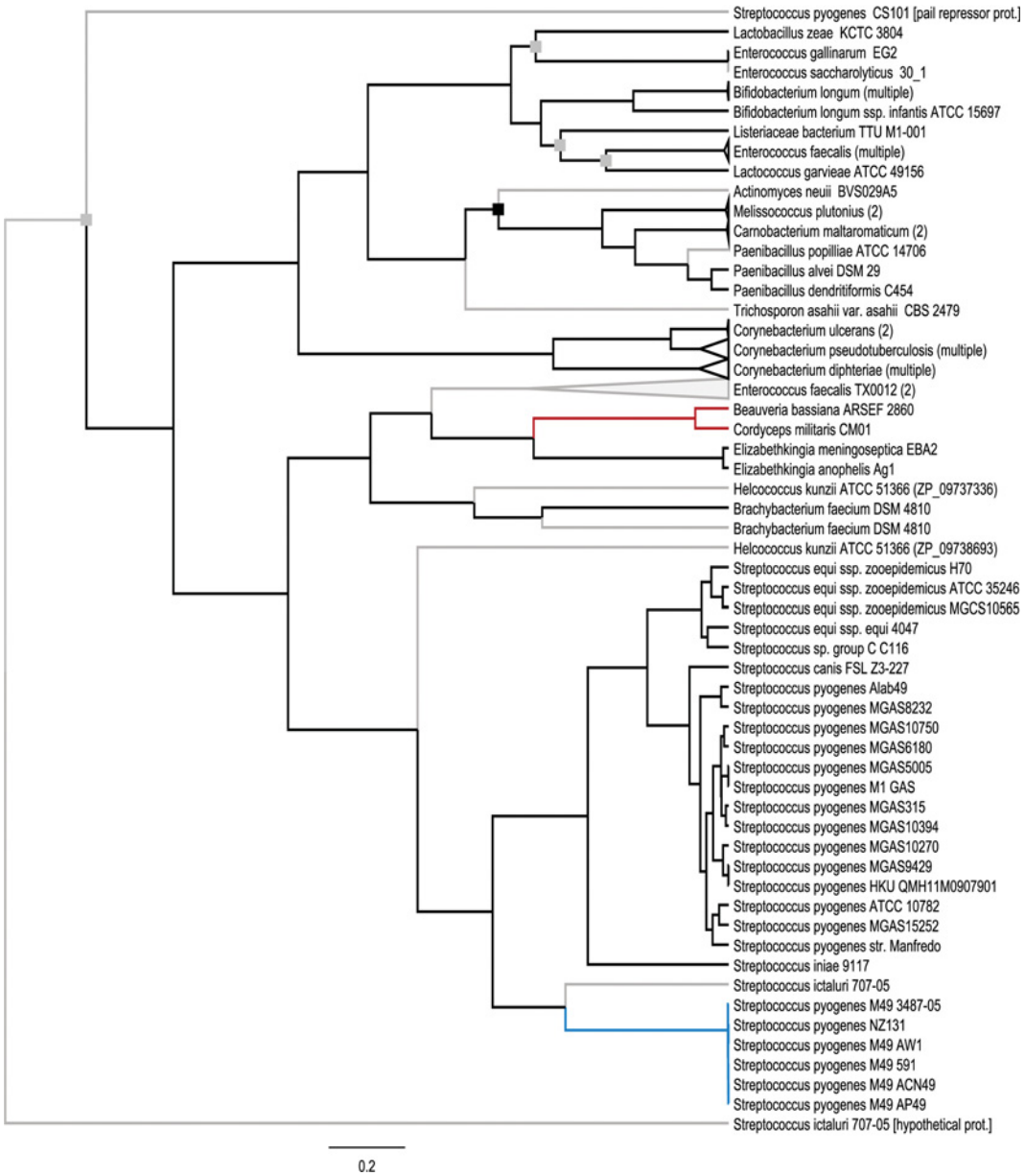

Figure 3 Phylogenetic reconstruction of the protein-specific tree for EndoS-like proteins, based on 1099 amino acids

All internal nodes were supported by a Bayesian posterior probability (PP) of 0.99–1.0 except for nodes highlighted with grey squares ($0.80<PP<0.95$) or black squares ($PP<0.80$). The scale bar indicates genetic distance. Non-verified hypothetical proteins predicted from gene models are indicated with grey branches, the $EndoS_2$ clade is highlighted in blue and the fungal clade is highlighted in red. For accession numbers, see Supplementary Table S3 at http://www.biochemj.org/bj/455/bj4550107add.htm.

active amino acid. To evaluate enzymatic activity of $EndoS_2$ on the subclasses of IgG, recombinant $EndoS_2$ and $EndoS_2$(E186L) were incubated with human IgG subclasses 1–4 and showed activity on all four human subclasses as analysed by SDS/PAGE and LCA lectin blot (Figure 4B). The glycan-hydrolysing activity of $EndoS_2$ on animal IgG was found for the following species: mouse, rat, monkey, sheep, goat, cow and horse. To investigate glycan specificity of $EndoS_2$, the composition of the released glycans from pooled human serum IgG was analysed by HILIC (hydrophilic interaction liquid chromatography)–UHPLC–FLD–MS and compared with the glycan profile of IgG generated by PNGase F (Figure 5). The HILIC–UHPLC–FLD–MS revealed $EndoS_2$ to cleave between the two GlcNAc residues in the chitobiose core of the N-linked glycan and thus leaving a single GlcNAc residue with or without α(1,6)-linked fucose attached to the protein backbone. All peaks present in the PNGase F chromatogram could be found in the glycan profile of IgG released by $EndoS_2$ with the difference of one GlcNAc with or without α(1,6)-linked fucose.

$EndoS_2$ releases biantennary and sialylated glycans on AGP

AGP, also known as orosomucoid, is a 41–45 kDa human plasma glycoprotein, a major positive acute-phase protein, up-regulated severalfold during inflammation and a member of the lipocalin family [11,31]. The immunomodulatory effects of AGP is linked to the carbohydrate composition of the five N-linked

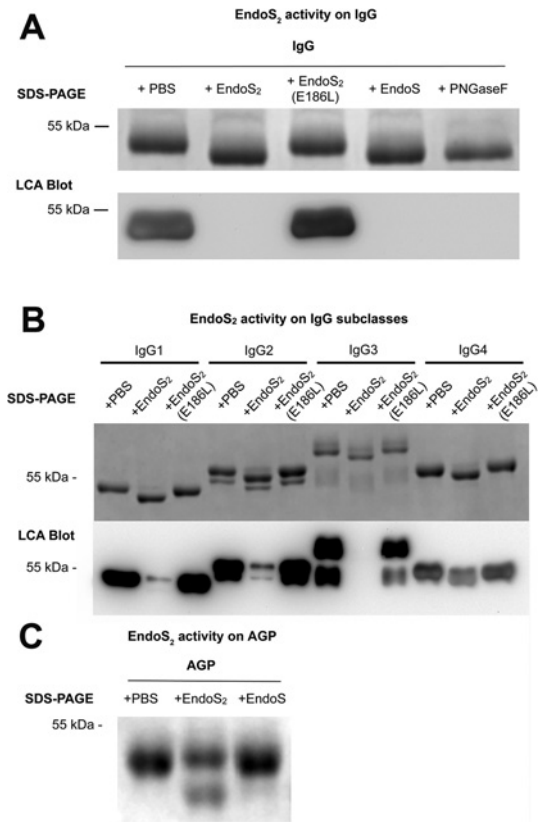

Figure 4 Activity of EndoS$_2$ on IgG, IgG subclasses and AGP

(**A**) Human serum IgG was incubated with recombinant EndoS$_2$, EndoS$_2$(E186L), EndoS, PNGase F or PBS at 37 °C in PBS overnight and analysed by SDS/PAGE (10 % gel) and a subsequent LCA blot. The gel and lectin blot shows the γ heavy chain of IgG at 50 kDa. (**B**) Human subclasses of IgG, IgG$_{1-4}$, were incubated with recombinant EndoS$_2$, EndoS$_2$(E186L) or PBS in PBS at 37 °C overnight and analysed by SDS/PAGE (10 % gel) and a subsequent LCA lectin blot. (**C**) AGP was incubated with recombinant EndoS$_2$, EndoS or PBS at 37 °C in PBS overnight and analysed by SDS/PAGE (10 % gel).

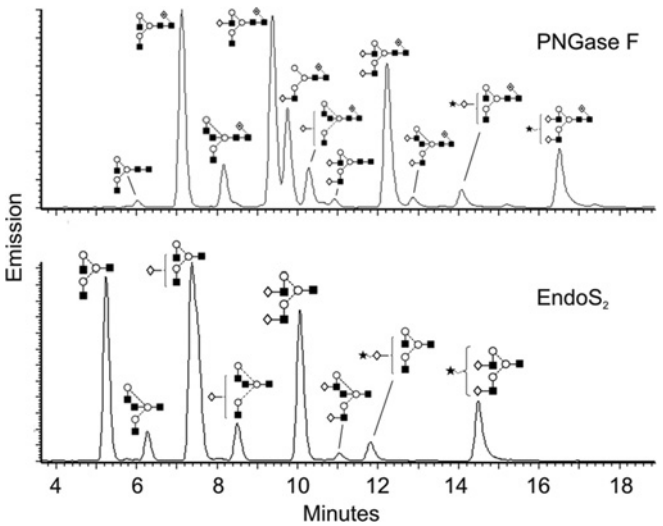

Figure 5 Glycan fluorescent profiles from human IgG released by EndoS$_2$ and PNGase F

HILIC–FLD–MS of 2-AB-labelled glycans released from human serum IgG by EndoS$_2$ (**A**) and PNGase F (**B**) respectively. Identified glycan structures are presented using the Oxford glycan nomenclature [45].

glycans (Asn^{33}, Asn^{56}, Asn^{72}, Asn^{93} and Asn^{103}) that make up 45 % of the molecular mass [15,31]. When incubating AGP with recombinant EndoS$_2$, subsequent SDS/PAGE revealed a new band at ~38 kDa and a decrease in the intensity of the band at 45 kDa (Figure 4C). No activity was detected with EndoS or PBS in the same assay (Figure 4C). To elucidate the enzymatic activity of EndoS$_2$ on AGP in detail, we analysed the glycans released from AGP by EndoS$_2$ using HILIC–UHPLC–FLD–MS and exoglycosidase arrays in UHPLC (Figure 6). The sequence, composition and linkage specificities of all glycoforms of AGP released by PNGase F were determined in the same way to serve as control. EndoS$_2$ was found to cleave only biantennary and sialylated structures of AGP, whereas the glycan profile from PNGase F contained sialylated bi-, tri- and tetra-antennary structures with or without outer arm fucosylation. The cleavage site of EndoS$_2$ was confirmed to be between the two GlcNAcs in the chitobiose core of the glycan. The glycan profiles were digested with NAN1 to remove α(2,3)-linked sialic acids and ABS to remove α(2,3)-, α(2,6)- and α(2,8)-linked sialic acid residues. The resulting bi-, tri- and tetra-antennary structures from AGP were identified as $M - 2H^{2-}$ ions (m/z 879.3, 1061.9, 1135.5, 1244.5 and 1317.5). Furthermore, glycans were digested with linkage-specific exoglycosidases to verify the presence of outer arm fucosylation. These enzymes were BTG, BKF and AMF (Supplementary Figure S2 at http://www.biochemj.org/bj/455/bj4550107add.htm). AMF digestion removed α(1,3) non-reducing terminal fucose linked to galactose residues and not core α(1,6)-fucose. BKF treatment, which is specific for core α(1,6)-linked fucose residues, did not result in glycan digest products.

EndoS$_2$ is specific for IgG and AGP and not a general chitinase

Previous work on EndoS has shown that the enzyme is specific for the native form of IgG [14]. To test whether this is valid for EndoS$_2$, IgG and AGP were incubated at temperatures ranging from 37 to 80 °C or 37 to 70 °C for 30 min before the addition of EndoS$_2$ or PBS and a 2 h incubation at 37 °C. SDS/PAGE analysis revealed a shift of IgG incubated at 37–50 °C and loss of signal was seen in a corresponding LCA lectin blot, whereas only partial shift could be seen at 60 °C and no shift and intact LCA signal at temperatures 70 °C and 80 °C (Figure 7A). Glycans from AGP were hydrolysed at 37 °C, but not at 40–70 °C (Figure 7B). The activity of EndoS$_2$ was tested further on a range of glycoproteins, i.e. α_2-macroglobulin, ovalbumin, lactoferrin, RNase B and fetuin, but no activity could be detected (Figure 7C). To study whether EndoS$_2$ shows general chitinase activity, we employed the substrate 4MU-GlcNAc, which fluoresces when cleaved, to compare the enzymatic activity of EndoS$_2$ and EndoS with that of a chitinase from *S. griseus*. The results indicate that neither EndoS$_2$ nor EndoS has a general chitinase activity compared with the positive control (Figure 7D).

EndoS$_2$ hydrolyses free biantennary glycans

Following the findings of the specificity of EndoS$_2$, we asked the question whether the enzyme is substrate-specific and/or has glycoform selectivity. To test this, we analysed the activity of EndoS$_2$ on free glycans. All glycoforms from bovine fetuin were released using PNGase F and were 2-AB-labelled; in a secondary

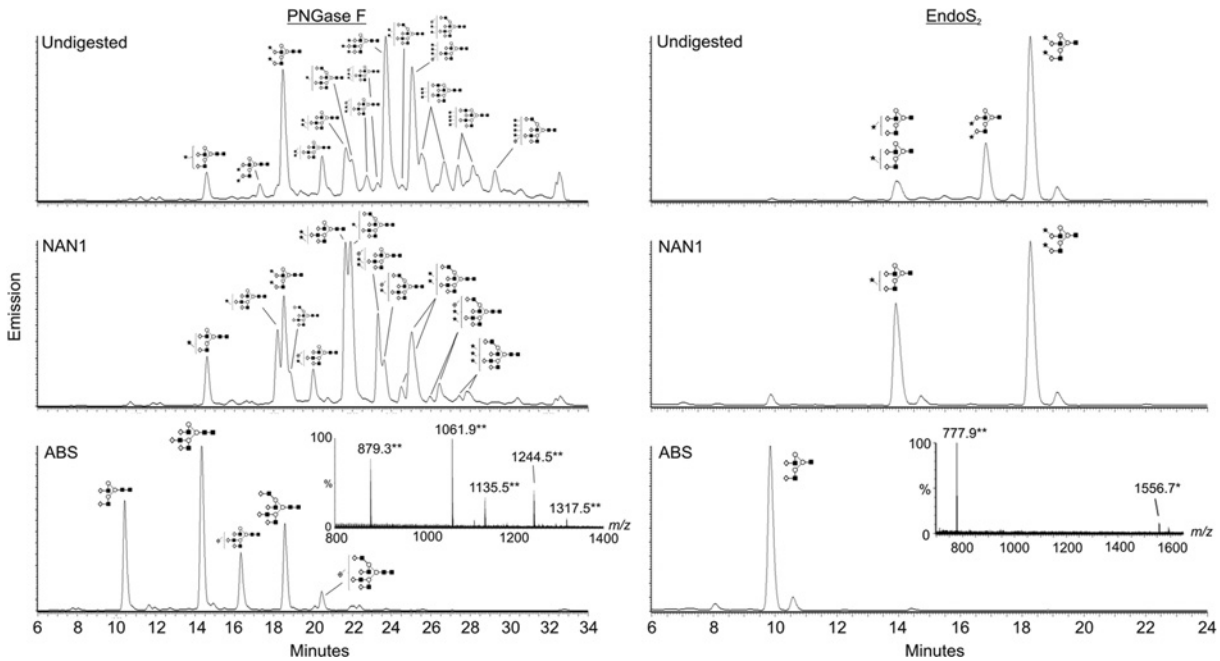

Figure 6 Glycan fluorescent profiles from human AGP released by PNGase F and EndoS$_2$

HILIC–FLD–MS of 2-AB-labelled glycans released from human AGP by PNGase F (left) and EndoS$_2$ (right) respectively. 2-AB-labelled glycans were digested further with NAN1 and ABS, and subsequent bi-, tri- and tetra-antennary structures are indicated using the Oxford glycan nomenclature [45]. Ions were detected as $[M-2H]^{2-}$ (**) and $[M-H]^{-}$ (*) species.

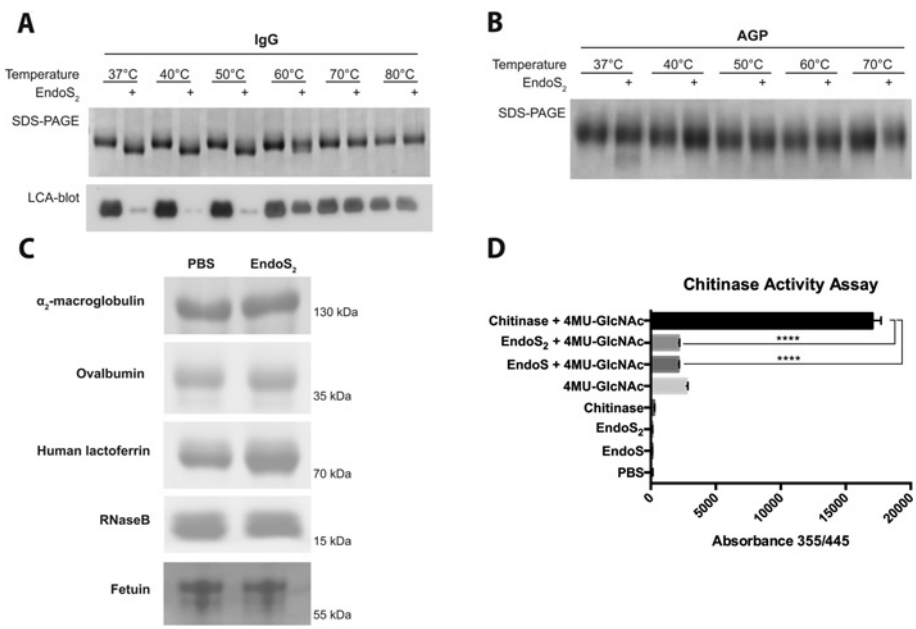

Figure 7 Activity of EndoS$_2$ on native and denatured IgG and AGP, other glycoproteins and chitinase assay

(**A**) IgG was incubated at temperatures ranging from 37 to 80 °C for 30 min followed by incubation with EndoS$_2$ at 37 °C for 2 h and analysis by SDS/PAGE and LCA lectin blot. (**B**) AGP was incubated at 37–70 °C followed by incubation with EndoS$_2$ at 37 °C for 2 h and analysis by SDS/PAGE. (**C**) EndoS$_2$ was incubated with α_2-macroglobulin, ovalbumin, human lactoferrin, RNase B and fetuin at 37 °C overnight and analysed by SDS/PAGE. (**D**) EndoS$_2$, EndoS and a chitinase from *S. griseus* was incubated with the fluorescent substrate 4MU-GlcNAc for 1 h and fluorescence was measured at 355/445 nm. The experiments were carried out using five replicates and results are means±S.D. The response in absorbance was analysed statistically by an unpaired Student's *t* test, where differences was considered significant if $P<0.05$. ****$P<0.001$.

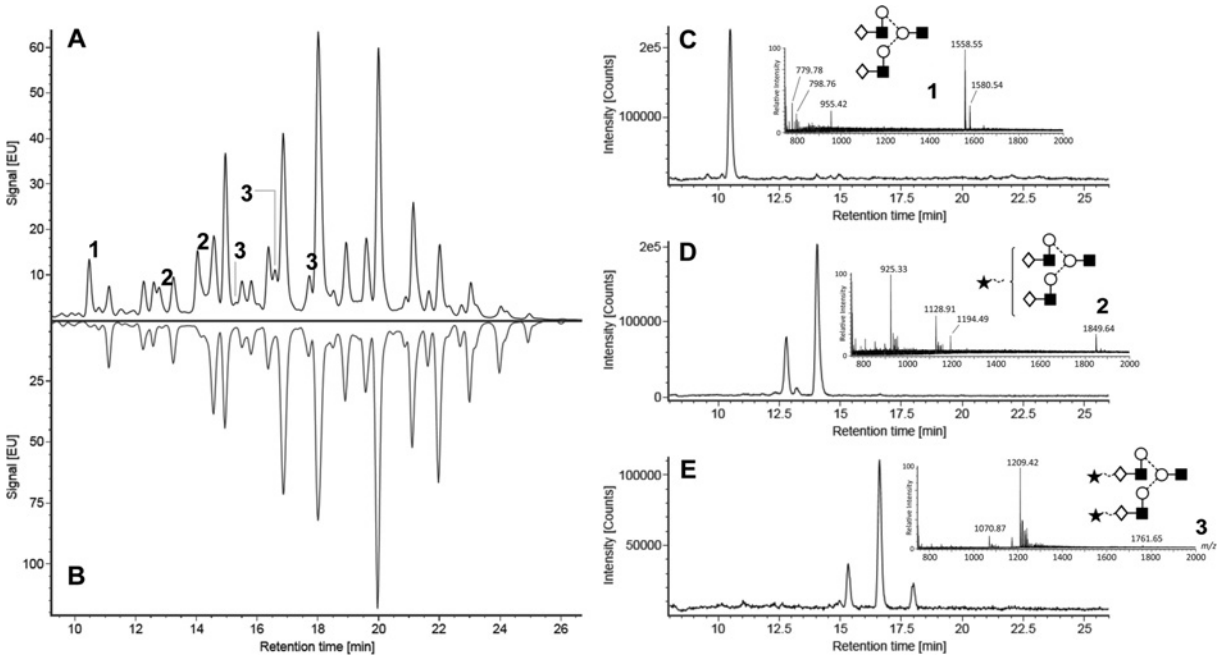

Figure 8 EndoS$_2$ hydrolyses biantennary free glycans

Bovine fetuin N-glycans were released with PNGase F, labelled with 2-AB, and analysed by HILIC–UHPLC–FLD–MS. Released N-glycans were digested further with EndoS$_2$ to determine enzymatic activity on free glycans. Comparison of the fluorescent chromatograms of glycans after PNGase F (**B**) and subsequent EndoS$_2$ digestion (**A**) identified three unique peaks (labelled 1, 2 and 3). These peaks correspond to three isomeric structures (A2G2, A2G2S1 and A2G2S2) and were detected primarily as *m/z* 1558.55 $[M+H]^+$, 925.33 $[M+2H]^{2+}$ and 1070.87 $[M+2H]^{2+}$ ions respectively (**C**)–(**E**). Extracted ion chromatograms of A2G2S1 and A2G2S2 precursor ions identified structural isomers, presumably from variation in sialic acid linkages.

reaction, the free glycans were incubated with EndoS$_2$, relabelled with 2-AB and analysed using HILIC–UHPLC–FLD–MS. Three structures in the chromatogram (labelled 1, 2 and 3) were modified by EndoS$_2$ compared with the PNGase F glycan pool (Figure 8A and 8B). The *m/z* of the $[M+H]^+$ ions of these structures were identified and revealed structures for A2G2 (1558.5513), A2G2S1 (925.3294) and A2G2S2 (1070.8700) less one GlcNAc residue (Figures 8C–8E). The results indicate that EndoS$_2$ specifically hydrolyses free biantennary glycoforms with or without terminal sialylation.

Expression of EndoS$_2$ is linked to carbohydrate utilization

To confirm the findings with recombinant EndoS$_2$, the expression levels and enzymatic activity of EndoS$_2$ were analysed in GAS supernatants. The expression of EndoS in serotype M1 of GAS is maximized in the nutrient-poor CM [10,16–21]. Therefore expression of EndoS$_2$ was analysed by Western blotting of 16 h bacterial supernatants grown in CM. However, EndoS$_2$ could only be detected in the supernatant when GAS was cultured in 50 % diluted CM, when the bacteria are starved (Figure 9A). In a subsequent functional assay incubating IgG with the bacterial supernatant, loss of the N-linked glycan on IgG was visualized as a 4 kDa shift of the heavy chain on the electrophoresis gel and corresponding lack of signal in the LCA lectin blot (Figure 9B). This experiment confirmed the activity of the native protein in the bacterial supernatant. The expression of EndoS$_2$ in poor medium led us to believe that the expression of EndoS$_2$ was linked to the carbohydrate utilization of the bacteria [22,23,32]. To address this, a selection of carbohydrates was added to the bacterial culture medium and the expression of EndoS$_2$ was studied. Adding glucose, galactose, GlcNAc or mannose to 50 % CM inhibited EndoS$_2$ expression, whereas additional sucrose increased the amount of EndoS$_2$ in the supernatant (Figure 9A).

DISCUSSION

The study of bacterial glycosidases has emerged as a field at the intersection of microbial pathogenesis and glycobiology. By studying the mechanisms by which bacteria interfere with host glycosylation, new insight can be gained into both bacterial pathogenesis and the impact of glycosylation of the immune system. Interfering with the glycosylation of the host defence is widespread among pathogenic bacteria for modulation of the functions of the immune system or as a way of utilizing the glycans of glycoproteins as nutrients [24,33].

For example, *Enterococcus faecalis*, a Gram-positive gut bacterium and opportunist, secretes EndoE, an endoglycosidase with activity on the Fc-glycan on IgG and on the glycoprotein RNase B that promotes bacterial growth when nutrients are scarce [24,34]. The endoglycosidases EndoF$_{1–3}$ from *E. meningoseptica* and EndoH from *Streptomyces plicatus* has been shown to be glycan-specific: high-mannose and hybrid oligosaccharides are cleaved by EndoF$_1$ and EndoH, whereas complex biantennary and bi- and tri-antennary glycans are released by EndoF$_2$ and EndoF$_3$ respectively [24,25,35–38]. An N-glycan deglycosylation complex in *Capnocytophaga canimorsus* has been found to cleave off N-linked glycans from IgG and to transport the glycans across the cell membrane for glycan catabolism [24,25,39]. *S. pneumoniae* has three surface-anchored exoglycosidases that work in concert to remove sialic acid, galactose and GlcNAc on human glycoproteins [40]. GAS EndoS was thought to be conserved throughout the GAS serotypes, and only minor variations are found when comparing *ndoS* among the sequenced GAS strains. It was therefore surprising to find that GAS strain

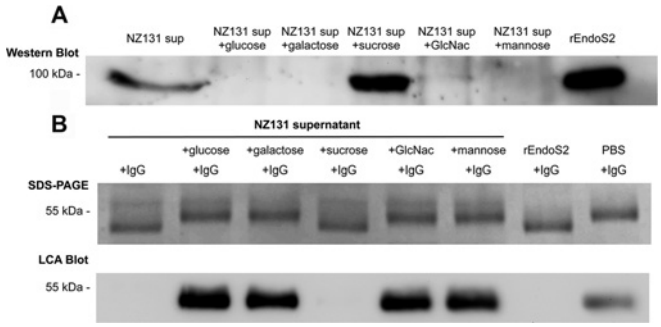

Figure 9 $EndoS_2$ expression and activity in GAS strain NZ131

(**A**) Western immunoblot showing $EndoS_2$ in bacterial supernatants with or without added carbohydrates. $rEndoS_2$ was used as a positive control. (**B**) Lectin blot analysis of human IgG incubated with bacterial supernatants with or without carbohydrates, or $rEndoS_2$ as a positive control.

NZ131 harboured *ndoS2*, with 53 % identity with *ndoS*. The sequenced *ndoS2* in five different M49 strains revealed high identity, arguing that this gene is conserved throughout the serotype.

In the phylogenetic protein tree, the $EndoS_2$ group is relatively different from EndoS in both *S. pyogenes* and in other *Streptococcus* species (but it groups with a hypothetical protein found in *Streptococcus ictaluri*). In general, the patterns are not uniform: whereas within-species or within-genus similarity of the EndoS-like proteins is high for some taxonomic groups (e.g. *Bifidobacterium longum*), there is also considerable within-genus variation in *Corynebacterium* (Figure 3). Strikingly, EndoS and $EndoS_2$ from *Streptococcus* are more closely related to EndoS-like proteins of the fungi *Cordyceps militaris* and *Beauveria bassiana* than to EndoS-like proteins of bacteria such as *Melissococcus*, *Corynebacterium* and *Lactobacillus* (Figure 3), in sharp contrast with the taxonomic relationships (Supplementary Figure S1). Notably, some taxa are paraphyletic in the EndoS-like protein phylogeny, to which could possibly be ascribed the inclusion of non-verified hypothetical proteins. However, the biologically verified EndoS-like proteins of *Enterococcus gallinarum* and *E. faecalis* do not form a monophyletic clade (Figure 3). In all, this picture indicates the occurrence of horizontal gene transfer of *ndoS*-like genes. Even though no known proteins were found to be closely related to $EndoS_2$, the differentiation from *S. pyogenes* EndoS and the high degree of similarity between serotype M49 and other *S. pyogenes* strains combined with the conserved genetic context points towards horizontal gene transfer of *ndoS2* into serotype M49. The strain NZ131 also has an unusually high frequency of transformation, and horizontal gene transfer has been described on several places in the genome [24]. The alternative interpretation, that a particularly strong directional selection on the ancestral *ndoS* gene in serotype M49 resulted in *ndoS2*, seems less plausible.

The active site and tryptophan residues important for activity in EndoS were found to be conserved in $EndoS_2$ even though the proteins are only 37 % identical [13]. Despite this substantial difference in amino acid sequences, $EndoS_2$ hydrolysed the glycan on IgG in a similar fashion to EndoS. In the chromatograms comparing the glycan profile of IgG generated by $EndoS_2$ and PNGase F (Figure 5), a shift was observed that could be explained by the site of action. PNGase F is an amidase that cleaves between the asparagine residue and the first GlcNAc residue of the glycan, whereas $EndoS_2$ cleaves after the first GlcNAc and thus leaves one GlcNAc with or without fucose attached to the protein backbone. Owing to lack of one reducing end GlcNAc in the $EndoS_2$ glycan profile, there is a loss in resolution, which explains why the A2G1 peak could not be separated in the $EndoS_2$ chromatogram, but can be seen as two separate peaks in the PNGase F profile. It has been argued previously that EndoS does not cleave bisecting glycans [41,42]. From the LC–MS data of the present study, we argue that $EndoS_2$ cleaves all glycoforms present of human serum IgG, including bisecting glycans, since all peaks present in the PNGase F glycan profile could be found in the $EndoS_2$ profile (Figure 5).

A striking difference between $EndoS_2$ and EndoS was found when incubated with the human acute-phase protein AGP. The observed activity of $EndoS_2$ was confirmed with LC–MS and revealed that $EndoS_2$ specifically releases biantennary and sialylated structures of AGP (Figure 6). Again, the peaks annotated in the $EndoS_2$ profile could be found in the PNGase F release with the difference of one GlcNAc residue. It is clear that $EndoS_2$ only releases a fraction of the glycans present on AGP. $EndoS_2$ does not cleave tri- and tetra-antennary glycans, with or without outer arm fucosylation, although they are present in great numbers on AGP.

The activity on IgG and AGP raised several questions regarding the specificity of $EndoS_2$. To answer these, we tested the activity of $EndoS_2$ on heat-denatured IgG and AGP, on other glycoproteins, in a chitinase assay and on a pool of free N-glycans. $EndoS_2$ was only active on native IgG and AGP and we draw the conclusion that $EndoS_2$ requires a protein–protein interaction with its substrates for glycan hydrolysis to occur. The activity of $EndoS_2$ on AGP may be the result of reduced protein recognition, since early studies indicate sequence homology between IgG and AGP [43]. On glycoproteins with a completely different fold, we detected no activity with similar assays to the activity on IgG and AGP detected. It was therefore not surprising to find that $EndoS_2$ had no general chitinase activity compared with a chitinase from *S. griseus*. Taken together, these data indicate that $EndoS_2$ specifically interacts with protein folds including IgG and AGP. Furthermore, we dissected the glycoform specificity of $EndoS_2$ by incubating the enzyme with the N-glycan pool from fetuin released by PNGase F and showed that $EndoS_2$ hydrolysed only free biantennary structures with or without terminal sialylation. No bisecting glycans are present on fetuin, which explains why such structures are not present in the chromatograms. On the basis of our findings, we believe that $EndoS_2$ is both site- and glycoform-specific which is a unique property of an endoglycosidase.

The hydrolysis of the glycan of IgG has been shown to have major consequences on the effector functions of the antibody by modulating the binding to FcγR [12]. Since EndoS and $EndoS_2$ have similar hydrolysing activity on the glycan of IgG, both enzymes are expected to affect the functionality of this antibody. The functional consequence for AGP when biantennary sialylated glycans are cleaved off is unknown and lies beyond the scope of the present study.

The expression of $EndoS_2$ was found to depend on the availability of carbohydrates in the bacterial culture medium. C-medium is a poor medium for GAS and expression of $EndoS_2$ could only be detected when GAS was grown in 50 % diluted C-medium. Incubating the supernatants with IgG confirmed the previous work carried out with recombinant $EndoS_2$ and a clear correlation between expression of $EndoS_2$ and hydrolysis of the Fc-glycan on IgG confirmed this. The genes *scrb*, *scra* and *scrk*, surrounding *ndoS2*, are part of a sucrose utilization operon and this could explain the increase of $EndoS_2$ expression when sucrose was added to the culture medium. The presence of glucose, galactose, GlcNAc or mannose completely inhibited expression of $EndoS_2$ indicating that this enzyme is tightly regulated by a mechanism

sensitive to the presence of carbohydrates. Research has indicated that the virulence of GAS is linked to the utilization of available carbohydrates via CcpA (catabolite control protein A), but in the present study of EndoS$_2$, we can only hypothesize that CcpA is involved in the regulation mechanism [32]. This indicates that in the infection scenario, EndoS$_2$ is strictly regulated and that the enzyme is used in an environment where nutrition is scarce, e.g. the human skin. This indicates further that the virulence of GAS is linked to the utilization of complex carbohydrates [44].

The present study shows that the endoglycosidase EndoS$_2$ is conserved and uniquely present in GAS serotype M49. We show that EndoS$_2$ hydrolysed all glycoforms on human serum IgG and biantennary and sialylated glycans on AGP. EndoS$_2$ is secreted by GAS during starvation and the expression is linked to the carbohydrate composition of the culture medium. The enzymatic activity on two key players of the immune system argues that EndoS$_2$ has a role in immunomodulation of the host that could potentially be linked to the pathogenesis of GAS serotype M49 infections.

AUTHOR CONTRIBUTION

Jonathan Sjögren and Mattias Collin conceived the study. Jonathan Sjögren performed experiments and drafted the paper. Weston Struwe, Eoin Cosgrave and Pauline Rudd performed glycan analysis and contributed to the paper. Martin Stervander performed phylogenetic analyses and contributed to the paper. Victor Nizet, Andrew Hollands and Maria Allhorn provided material and valuable input on the text. All authors read and approved the final paper.

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online data

SUPPLEMENTARY ONLINE DATA

$EndoS_2$ is a unique and conserved enzyme of serotype M49 group A *Streptococcus* that hydrolyses N-linked glycans on IgG and α_1-acid glycoprotein

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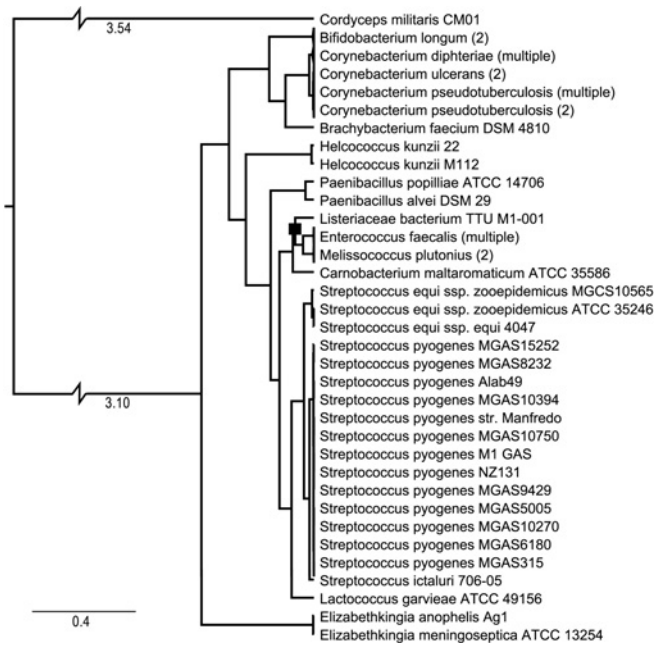

Figure S1 Phylogenetic reconstruction of 16S rRNA sequences, for inference of taxonomic relationship between taxa of the EndoS-like protein phylogeny, based on 1576 nucleotides

All internal nodes were supported by a Bayesian posterior probability (PP) of 0.99–1.0 except for the grouping of the Listeriaceae family bacterium, highlighted with a black squares signifying PP$<$0.80. The scale bar indicates genetic distance. Note that the branches connecting the ingroup and outgroup are truncated owing to the large taxonomic difference (genetic distances stated below branches). For accession numbers, see Table S3.

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[2] Patents for the use of $EndoS_2$ have been applied for by Genovis AB. Jonathan Sjögren, Andrew Hollands, Victor Nizet, Maria Allhorn and Mattias Collin are listed as inventors on the application that is pending.

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The sequences of *Streptococcus pyogenes ndoS2* will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession numbers KC155346 (strain 3487-05), KC155348 (strain AP49), KC155347 (strain ACN49), KC155349 (strain AW1) and KC155350 (strain AW2).

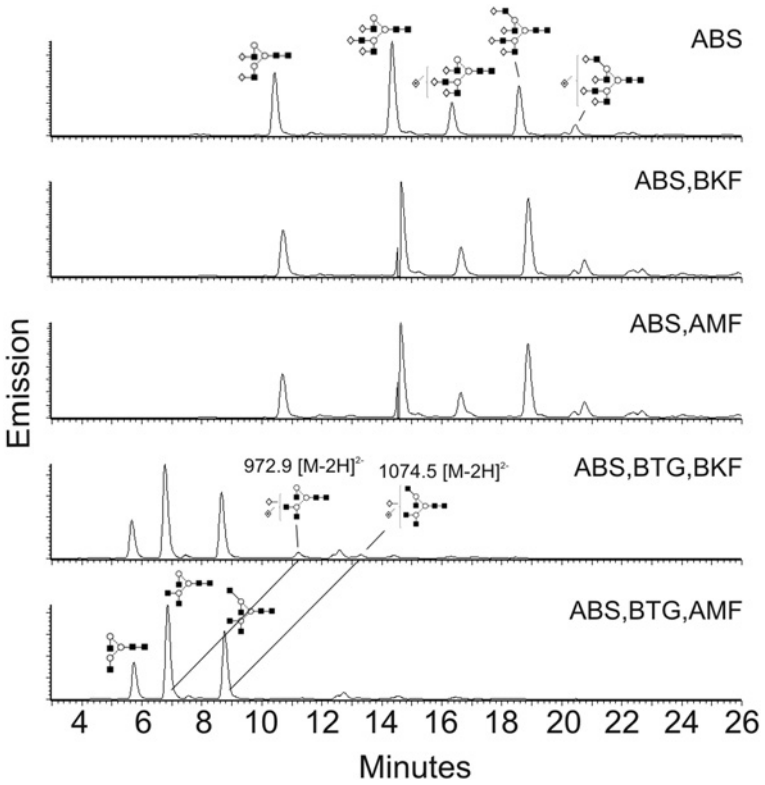

Figure S2 HILIC–FLD–MS and exoglycosidase sequencing of AGP N-glycans

The fucose linkage position was confirmed as α(1,3) to galactose residues on tri- and tetra-antennary glycans and were detected as $[M-2H]^{2-}$ ions 972.9 and 1074.5.

Table S1 *E. coli* and group A streptococcal strains used in the present study

Strains of *E. coli* and *S. pyogenes* with accession numbers. Also indicated is the GenBank® accession number for *ndoS2* sequences. N/A, not applicable.

Strain	Source/accession number	*ndoS2* accession number
E. coli		
Top10	Invitrogen	N/A
BL21 pLysS	Invitrogen	N/A
GAS (serotype)		
NZ131 (M49)	ASM1812v1	ACI61688
3487-05 (M49)	*S. pyogenes*, serotype M49	KC155346
ACN49 (M49)	Strain 3274-98 from Center for Disease Control and prevention. A gift from Actinova	KC155347
AP49 (M49)	*S. pyogenes* strain collection, Institute of Hygiene and Epidemiology, Prague, Czech Republic	KC155348
AW1 (M49)	Nephritis strain H 9449 B from the collection of L. Wannamaker	KC155349
AW2 (M49)	Strain H 5424 from the collection of L. Wannamaker	KC155350

Table S2 Plasmids and primers used in the present study

Plasmids and primers used for recombinant expression and sequencing.

(a) Plasmids

Name	Source
pGEX-5X-3	GE Healthcare
pGEX-ndoS2	The present study
pGEX-ndoS2(E-L)	The present study
pGEX-ndoS	[1]
pCR2.1	Invitrogen

(b) Primers

Name	Sequence (5′→3′)
ndoS2-out-R	GCGCCACTTTCTGGTAGTCTAAC
Seq38-R	TTTGGGACCTCAGCCATAG
Seq42-R	GGATTGTTAGGGTCGTATTCG
Seq54-R	TTTAGCCCCTTTTGCCAG
Seq15-F	CCTGAAGAAGATGCTCGTG
Seq17-F	TGATGACCGAAGACAAACG
Seq24-F	GAGACGAAGAAGCATTTGC
Seq28-F	TCACTTGGGGACAAACTAAC
ndoS2-F-BamHI	CTGTAAGGATCCAGGAGAAGACTG
ndoS2-R-XhoI	GAAACCTCGAGTCTTTGTAATCGTAGGACTT
ndoS2(E-L)-F-BamHI	CTAGATATTGATATTCTTCACGAATTTACGAAC
ndoS2(E-L)-R-XhoI	GTTCGTAAATTCGTGAAGAATATCAATATCTAG

Table S3 Accession numbers for EndoS-like protein and 16S rRNA sequences

For the EndoS-like protein dataset, outgroup sequences are indicated with an asterisk. For the $EndoS_2$ sequences of the present study, see Table S1.

Taxa	Strain	GenBank® accession number	
		EndoS-like protein	16S rRNA
Actinomyces neuii	BVS029A5	ZP_11040533	
Beauveria bassiana	ARSEF 2860	EJP67240	
Bifidobacterium longum subsp. *infantis*	157F	YP_004209228	AP010890
Bifidobacterium longum subsp. *infantis*	ATCC 15697	YP_002323900	AP010889
Bifidobacterium longum subsp. *longum*	44B	EIJ32132	
Bifidobacterium longum subsp. *longum*	1-6B	EIJ25186	
Brachybacterium faecium	DSM 4810	YP_003153977; YP_003155571	X91032
Carnobacterium maltaromaticum	ATCC 35586	ZP_10279268	JF749289
Carnobacterium maltaromaticum	LMA28	YP_006993899	
Chryseobacterium meningosepticum	ATCC 13254	AJ704541	
Cordyceps militaris	CM01	EGX89767	HQ585075
Corynebacterium diphtheriae	241	YP_005126330	CP003207
Corynebacterium diphtheriae	BH8	YP_005161243	CP003209
Corynebacterium diphtheriae	C7 (β)	YP_005163607	CP003210
Corynebacterium diphtheriae	CDCE 8392	YP_005134619	CP003211
Corynebacterium diphtheriae	INCA 402	YP_005128539	CP003208
Corynebacterium diphtheriae	PW8	YP_005143699	CP003216
Corynebacterium diphtheriae	31A	YP_005158899	
Corynebacterium diphtheriae bv. *intermedius* str.	NCTC 5011	EIK55328	
Corynebacterium pseudotuberculosis	31	YP_006214375	CP003421
Corynebacterium pseudotuberculosis	258	YP_006353466	CP003540
Corynebacterium pseudotuberculosis	Cp162	YP_006437976	CP003652
Corynebacterium pseudotuberculosis	FRC41	YP_003784295	CP002097
Corynebacterium pseudotuberculosis	PAT10	YP_005691331	CP002924
Corynebacterium pseudotuberculosis	X81907		
Corynebacterium pseudotuberculosis	316	YP_005304514	CP003077
Corynebacterium pseudotuberculosis	CIP 52.97	YP_005695446	
Corynebacterium ulcerans	809	YP_005711595	CP002790
Corynebacterium ulcerans	BR-AD22	YP_004630749	CP002791
Elizabethkingia anophelis	Ag1	ZP_09415888	EF426425
Elizabethkingia meningoseptica	EBA2	FLAME	
Enterococcus faecalis	ATCC 29200	ZP_04439322	ACHK01000081
Enterococcus faecalis	OG1RF	CP002621	
Enterococcus faecalis	V583	NP_813917	
Enterococcus faecalis	HER1044	AAR20477	
Enterococcus faecalis	DS5	ZP_05561082	
Enterococcus faecalis	ERV85	EJV36587	
Enterococcus faecalis	PC1.1	ZP_06746878	
Enterococcus faecalis	R508	EJV39420	
Enterococcus faecalis	R712	ZP_06630726	
Enterococcus faecalis	T1	ZP_05422239	
Enterococcus faecalis	T2	ZP_05425204	
Enterococcus faecalis	T3	ZP_05502539	
Enterococcus faecalis	T8	ZP_05560447	
Enterococcus faecalis	TX0012	EFT95260; EFT95516; EFT95520	
Enterococcus faecalis	TX0104	ZP_03950001	
Enterococcus faecalis	TX0109	ZP_07567140	
Enterococcus faecalis	TX0309B	EFU85828	
Enterococcus faecalis	TX0470	ZP_07760464	
Enterococcus faecalis	TX0855	ZP_07555326	
Enterococcus faecalis	TX0860	ZP_07559314	
Enterococcus faecalis	TX1322	ZP_04435748	
Enterococcus faecalis	TX1341	EFU12187	
Enterococcus faecalis	TX1342	EFU15396	
Enterococcus faecalis	TX1346	EFU16620	
Enterococcus faecalis	TX1467	EGG58918	
Enterococcus faecalis	TX2137	EFT38634	
Enterococcus faecalis	TX4244	EFT91060	
Enterococcus gallinarum	EG2	ZP_05648160	
Enterococcus saccharolyticus	30_1	ZP_09110633	
Helcococcus kunzii	ATCC 51366	ZP_09737336; ZP_09738693	
Helcococcus kunzii	22	NR_029237	
Helcococcus kunzii	M112	JN861738	
Lactobacillus zeae	KCTC 3804	ZP_09453558	
Lactococcus garvieae	ATCC 49156	YP_004779109	AP009332
Listeriaceae family bacterium	TTU M1-001	ZP_09890591	JQ287762
Melissococcus plutonius	ATCC 35311	YP_004455741	AP012200

Table S3 Continued

Taxa	Strain	GenBank® accession number	
		EndoS-like protein	16S rRNA
Melissococcus plutonius	DAT561	YP_005320150	AP012282
Paenibacillus alvei	DSM 29	ZP_10866627	AJ320491
Paenibacillus dendritiformis	C454	ZP_09678120	
Paenibacillus popilliae	ATCC 14706	GAC41484	AB073198
Streptococcus canis	FSL Z3-227	ZP_10274531	
Streptococcus equi subsp. *equi*	4047	YP_002745815	FM204883
Streptococcus equi subsp. *zooepidemicus*	MGCS10565	YP_002122753	CP001129
Streptococcus equi subsp. *zooepidemicus*	ATCC 35246	AEJ24585	CP002904
Streptococcus equi subsp. *zooepidemicus*	H70	YP_002745127	
Streptococcus ictaluri	707-05	ZP_09127116; ZP_09126970*	
Streptococcus ictaluri	706-05	DQ462420	
Streptococcus iniae	9117	ZP_11067943	
Streptococcus pyogenes	Alab49	YP_006072662	CP003068
Streptococcus pyogenes	M1 GAS	NP_269818	AE006615
Streptococcus pyogenes	MGAS315	NP_665372	AE014074
Streptococcus pyogenes	MGAS5005	YP_282903	CP000017
Streptococcus pyogenes	MGAS6180	YP_280992	CP000056
Streptococcus pyogenes	MGAS8232	NP_607886	AE009954
Streptococcus pyogenes	MGAS9429	YP_597275	CP000259
Streptococcus pyogenes	MGAS10270	YP_599216	CP000260
Streptococcus pyogenes	MGAS10394	YP_060848	CP000003
Streptococcus pyogenes	MGAS10750	YP_603093	CP000262
Streptococcus pyogenes	MGAS15252	YP_005389405	CP003116
Streptococcus pyogenes	str. Manfredo	YP_001127900	AM295007
Streptococcus pyogenes	NZ131	YP_002286383	CP000829
Streptococcus pyogenes	ATCC 10782	ZP_07460044	
Streptococcus pyogenes	HKU QMH11M0907901	EIK41346	
Streptococcus pyogenes	M49 591	ZP_00365754	
Streptococcus pyogenes	CS101	U78969_1*	
Streptococcus sp. group C	C116	ADC53484	
Trichosporon asahii var. *asahii*	CBS 2479	EJT46459	

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PAPER III

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PLOS ONE

EndoE from *Enterococcus faecalis* Hydrolyzes the Glycans of the Biofilm Inhibiting Protein Lactoferrin and Mediates Growth

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Abstract

Glycosidases are widespread among bacteria. The opportunistic human pathogen *Enterococcus faecalis* encodes several putative glycosidases but little is known about their functions. The identified endo-β-*N*-acetylglucosaminidase EndoE has activity on the N-linked glycans of the human immunoglobulin G (IgG). In this report we identified the human glycoprotein lactoferrin (hLF) as a new substrate for EndoE. Hydrolysis of the N-glycans from hLF was investigated using lectin blot, UHPLC and mass spectrometry, showing that EndoE releases major glycoforms from this protein. hLF was shown to inhibit biofilm formation of *E. faecalis in vitro*. Glycans of hLF influence the binding to *E. faecalis,* and EndoE-hydrolyzed hLF inhibits biofilm formation to lesser extent than intact hLF indicating that EndoE prevents the inhibition of biofilm. In addition, hLF binds to a surface-associated enolase of *E. faecalis*. Culture experiments showed that the activity of EndoE enables *E. faecalis* to use the glycans derived from lactoferrin as a carbon source indicating that they could be used as nutrients *in vivo* when no other preferred carbon source is available. This report adds important information about the enzymatic activity of EndoE from the commensal and opportunist *E. faecalis*. The activity on the human glycoprotein hLF, and the functional consequences with reduced inhibition of biofilm formation highlights both innate immunity functions of hLF and a bacterial mechanism to evade this innate immunity function. Taken together, our results underline the importance of glycans in the interplay between bacteria and the human host, with possible implications for both commensalism and opportunism.

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Introduction

Enterococcus faecalis is a well-known member of the microbial consortium in the human gastrointestinal tract and the oral cavity [1,2], but it is also known to cause infectious endocarditis [3], urinary tract infections [4], bacteremia as well as sepsis [5]. The treatment of enterococcal infections is difficult due to the increasing antibiotic resistance which can be transferred among *E. faecalis* strains via mobile genetic elements [1,6]. It has also been shown that *E. faecalis* is able to adhere to and form biofilms on different biomaterials and medical devices suggesting an involvement of biofilms in the process of infection [7–11].

Many human pathogens have evolved intricate mechanisms to survive in the human body. Escaping the immune system and gaining nutrients are challenging obstacles for pathogenic bacteria. Secreted proteins with glycosidase activity have been identified in different bacteria such as *Streptococcus pyogenes* [12,13], *Streptococcus oralis* [14] and *Capnocytophaga canimorsus* [15]. Glycosidases from pathogens can target different glycoproteins of the host, for example, proteins of the immune system, thereby changing the function of the protein and help the intruding bacteria to escape the immune response [16–19]. Prominent examples of pathogenic glycosidases are EndoS from *S. pyogenes* and the pneumococcal enzymes NanA, BgaA and StrH. EndoS is able to cleave the N-linked glycans from IgG and thereby inhibit the immunoglobulin-mediated opsonophagocytosis, which increases the survival of *S. pyogenes* in blood [20]. The three exoglycosidases NanA, BgaA and StrH from *Streptococcus pneumoniae* are responsible for the sequential

deglycosylation of human glycoproteins and play a major role in evasion of opsonophagocytosis, adherence to epithelial surfaces and nutrient acquisition [17]. Bacterial hydrolysis of host glycoproteins can have direct modulating activity immune functions as exemplified by endoglycosidase with activity on human antibodies. In addition to this, it is clear that several bacteria, such as oral streptococci, liberate carbohydrates that can be used as nutrients during colonization and infection [14,21].

The endoglycosidase EndoE from *E. faecalis* was identified due to similarities to the well investigated endoglycosidase EndoS from *S. pyogenes* [22]. EndoE has a unique feature, namely, it combines two enzymatic domains with different glycosyl hydrolase activities. The α domain of EndoE contains a family 18 glycosyl hydrolase (GH18) motif while the β domain contains a family 20 glycosyl hydrolase (GH20) motif and is able to release the glycans from the immunoglobulin IgG [22].

Many of the proteins involved in adaptive and innate immunity are glycosylated. The carbohydrates are important for the stability and also for recognition [23]. The non-heme iron binding protein lactotransferrin (lactoferrin, hLf) belongs to the family of transferrins that are involved in the regulation of iron homeostasis [24]. This protein has a mass of 80 kDa, is present at concentrations of up to 7 mg/ml in milk but can also be found on mucosal surfaces, for example in the gastrointestinal tract or the respiratory tract, at lower concentrations around 2 mg/ml [25]. More recently, hLF has been described to have multiple biological functions in blood and mucosal surfaces and is considered as a component of the innate immune system with antimicrobial activity [26–29]. The antimicrobial activity of this protein has been attributed to deprivation of essential iron [30], outer membrane damage in Gram-negative bacteria [31], and recently, the inhibition of bacterial H^+-ATPase [26]. Moreover, it has been described that hLF is able to inhibit biofilm formation of bacteria like *Pseudomonas aeruginosa*, *Streptococcus mutans*, *Porphyromonas gingivalis* and *Prevotella intermedia* [32–34]. Human hLF from milk contains three putative N-glycosylation sites (Asn138, Asn479 and Asn624), of which only two sites (Asn138 and Asn479) are linked to highly branched, highly sialylated and highly fucosylated complex type N-glycans [35]. Not much is known about the role of the glycans, but it has been described that de-glycosylated hLF has identical affinity to iron and bacterial lipopolysaccharide [36].

In this study, we hypothesized that the endoglycosidase EndoE from *E. faecalis* has activity on human glycoproteins and that this activity might help *E. faecalis* to persist and/or survive in the human body. We showed that EndoE cleaves complex type N-linked glycans from hLF, which leads to a reduction of the biofilm inhibiting properties of this protein. Removal of the glycans by EndoE restored biofilm formation of *E. faecalis*, showing a new mechanism for *E. faecalis* to interfere with a possible defense mechanism of the human body. In addition, the released glycans can be metabolized by *E. faecalis* and could support nutrient acquisition.

Results

EndoE Binds to and has Glycosidase Activity on Human Lactoferrin from Milk

It has been described previously that the β-*N*-acetylglucosaminidase EndoE has activity on the glycans of human IgG and on the glycans of the model glycoprotein RNaseB [22]. We identified a new substrate for EndoE, the human glycoprotein lactoferrin (hLF), that contains two complex type N-glycans [35].

To investigate the glycan hydrolyzing activity of EndoE, recombinant enzyme or PBS as control was incubated with hLF from milk and analyzed with SDS-PAGE (Fig. 1 A, upper panel) and Concanavalin A (ConA) lectin blot (Fig. 1 A, lower panel), detecting α-mannosidic structures of the N-linked glycan. EndoE was found to have glycosidase activity on hLF as indicated by a shift of the native glycoprotein into three bands compared to the control seen as one band at 80 kDa on the SDS-PAGE gel. The lectin blot analysis showed that the lowest band in the SDS gel corresponds to de-glycosylated hLF since no signal was obtained with the ConA lectin, indicated by the lack of the last band in the lectin blot. Since hLF contains two N-linked glycans, the presented results indicate that lactoferrin occurs in three protein forms after EndoE treatment: fully glycosylated hLF (hLF with the highest molecular weight, similar to the control), hLF with partially cleaved N-linked glycans and de-glycosylated hLF. However, the possible presence of one or two remaining *N*-acetylglucosamines (GlcNAc) and a α1–6-linked fucose on the protein backbone as well as the nature of the partially cleaved N-linked glycans cannot be verified with this method but will be further described below. It has been described previously that EndoE has two domains with different enzymatic activity [22]. To investigate which domain is responsible for the glycan hydrolysis on hLF, we incubated the glycoprotein with site-directed mutagenized EndoE. As described previously, two glutamic acid residues (Glu186 and Glu662), which are important for the activity of the α domain and β domain, respectively, were exchanged to glutamine, leading to an EndoE with active α domain but inactive β domain (EndoE(E662Q)) and an EndoE with active β domain but inactive α domain (EndoE(E186Q)) [22]. Fig. 1A shows that the α domain of EndoE with family 18 glycosyl hydrolase activity is responsible for the hydrolysis of hLF since the mutated EndoE(E186Q) lost its activity on hLF.

To verify the physical interaction between EndoE and lactoferrin, surface plasmon resonance analysis with EndoE(E186Q) and EndoE(E662Q) was performed (Fig. 1B). This revealed that both, EndoE(E186Q) with inactive α domain and EndoE(E662Q) with inactive β domain, interact with hLF. The

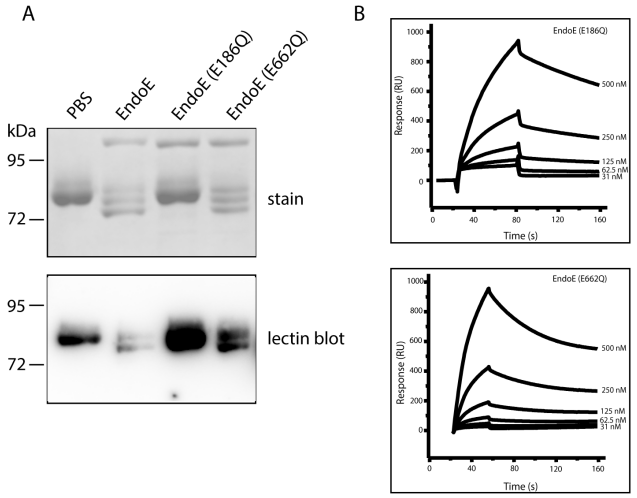


Figure 1. Activity on and binding of EndoE to human lactoferrin. A. Human lactoferrin (hLF) was incubated with EndoE, EndoE(E186Q) and EndoE(E662Q), separated on 10% SDS-PAGE and stained with Coomassie (upper panel), or electro-blotted onto PVDF membranes and was analyzed with ConA lectin (lower panel). Incubation of hLF with PBS was used as a negative control. B. Plasmon surface resonance assay to analyze binding of EndoE to hLF. The plots show binding of EndoE(E186Q) and EndoE(E662Q) to hLF.
doi:10.1371/journal.pone.0091035.g001

K_D value for EndoE(E662Q) was calculated to be 3.7 nM whereas the K_D value for EndoE(E186Q) was calculated to be 44 nM, indicating a stronger interaction of hLF with EndoE(E662Q) than EndoE(E186Q) of which the latter variant shows no activity on hLF in the lectin blot analysis (see above). No interaction between wild type EndoE and hLF could be detected using this method (data not shown). This is most likely due to a transient interaction between the active endoglycosidase and the substrate glycoprotein as has been shown for the related enzyme EndoS [16].

EndoE Releases Major Glycoforms from Human Lactoferrin

As described above, EndoE is able to hydrolyze glycans from hLF (Fig. 1A). Although we detected de-glycosylated hLF, we also detected fully glycosylated hLF and hLF with partially cleaved glycans according to the SDS-PAGE gel and ConA lectin blot. Longer incubation did not change the shifts to only de-glycosylated hLF (data not shown), indicating that EndoE might not have activity on all occurring N-glycan structures of hLF. The glycosylation profile of hLF from milk was recently investigated, indicating the presence of 17 different, complex-type N-glycan structures attached to hLF on two sites: Asn138 and Asn479 [35]. Moreover, it has also been described that the glycans at Asn138 were more complex, more branched and more fucosylated [35]. To investigate what glycans were hydrolyzed by EndoE, the N-glycans from lactoferrin were released with either PNGaseF or EndoE and comparatively analyzed using UHPLC (Ultra high performance liquid chromatography) and LC-MS (liquid chromatography-mass spectrometry) (Fig. 2). PNGaseF (Peptide-N-Glycosidase F from *Elizabethkingia meningoseptica*) is an amidase that is widely used in glycan analysis of N-linked glycans as a control enzyme that hydrolyzes the bond between the protein backbone and the first GlcNAc of the glycan.

It has previously been shown that EndoE cleaves between the two GlcNAc residues in the chitobiose core structure of IgG N-glycan [22], and this activity was also confirmed on hLF (Figure 3). Most glycoforms of hLF that were identified in the analysis released by PNGaseF could also be detected in the glycan profile of hLF incubated with EndoE, as indicated by the peaks in the chromatogram (Peaks 3, 6, 7, 8, 9, 10). The peaks corresponding to the glycans released by PNGaseF are shifted by roughly 0.5 GU (glucose unit) since it releases both GlcNAcs from the protein whereas EndoE cleaves between the two core GlcNAcs. The structures from the identified released glycans are shown in Figure 3 and 4. Interestingly, peaks 2, 4 and 5 from the chromatogram correspond to glycans with single antennary structures that were released by EndoE but were not found among the glycans released by PNGaseF. However, these glycans could be the products of the glycans 14/15, 21 and 20 respectively, but further processed by glycosidase activity between the mannose and the GlcNAc. Also, glycans containing sialic acid α1–6-linked to galactose released by PNGaseF (19, 22, 23 and 24) were not found in the glycan profile of EndoE. This indicates that EndoE might not be able to cleave the glycans containing sialic acids, which could be an explanation for the detection of hLF with partial deglycosylation in the lectin blot analysis.

Lactoferrin Inhibition of Biofilm Formation of *E. faecalis* is Glycan Dependent

The multifunctional protein hLF has been described to inhibit biofilm formation by several bacterial species. We investigated whether hLF affects the biofilm formation of *E. faecalis*. *E. faecalis* was grown in the presence of 0.025 mg/ml hLF in 96 well plates and attached cells were stained with crystal violet to examine biofilm formation (Fig. 5). In the presence of hLF, biofilm formation was inhibited by 80% compared to the control without hLF. Moreover, it has also been described that hLF has bactericidal effects on bacteria. To rule out any potential killing effect of hLF on biofilm formation, we measured the colony forming units (CFU) before staining with crystal violet and determined the amount of viable cells. No bactericidal effects for the used hLF concentration were observed in the experiment (data not shown). To rule out that the growth rate of *E. faecalis* is not affected by lactoferrin, which could cause decreased biofilm formation, we performed growth experiments in the presence of 0.1 mg/ml hLF but could not detect any effect on the growth rate of *E. faecalis* (data not shown). Next, we examined if the glycans of hLF could play a role in the inhibition of biofilm formation. Therefore, hLF was first treated with recombinant EndoE and purified. The purified deglycosylated hLF (de-hLF) was subsequently used for the biofilm assay as described above. Fig. 5 shows that the removal of glycans from lactoferrin restored biofilm formation of *E. faecalis*. However, the restoration of biofilm formation is not complete, which is probably due to the fact that EndoE cannot completely deglycosylate hLF (Fig. 1A).

Lactoferrin Binding to the Surface Associated Enolase is Glycan Dependent

Glycans are often important for the interaction or binding to other proteins or sugars. To identify the function of the biofilm inhibitory effect of hLF we investigated if hLF can bind to *E. faecalis* and whether it binds to a specific surface protein. The binding of hLF to the surface of *E. faecalis* could lead to a reduced adherence of *E. faecalis* to different surfaces and could explain the biofilm inhibitory effect described above. First, we incubated *E. faecalis* with hLF in different concentrations, washed the cells and detected cell bound hLF with Western blot analysis using anti-hLF antibodies (Fig. 6 A). We observed a concentration dependent binding of hLF to *E. faecalis* and investigated next if the binding is dependent on the glycans of hLF. Fig. 6 A shows that the binding of de-hLF to *E. faecalis* was strongly reduced compared to the fully glycosylated hLF. These results indicate that the binding to *E. faecalis* is glycan dependent and might be mediated by a specific surface protein or component. To identify a possible hLF binding surface protein from *E. faecalis*, we coupled hLF to CnBr activated sepharose and incubated the sepharose with an *E. faecalis* cell extract. The proteins that were bound to hLF were eluted and analyzed via SDS-PAGE. We observed only one distinct band with a size of around 50 kDa and identified the protein via mass spectrometry as a surface associated enolase (EF1961) (Protein identification report: File S1). To verify the interaction between hLF and the enolase we immobilized recombinant enolase to a microtiter plate in an ELISA experiment to show binding of hLF to the enolase *in vitro* (Fig. 6 B).

Surface displayed enolase is in streptococci known to bind host proteins like plasminogen, fibrinogen and mucin [37–39], but to our knowledge, nothing is known about its interaction with hLF. Our results indicate that hLF inhibits biofilm formation by binding to the surface of *E. faecalis*, that this process is glycan dependent, and that the binding might be, at least in part, mediated through binding to the surface associated enolase. In addition, restoration of the biofilm formation by EndoE indicates that *E. faecalis* potentially uses this endoglycosidase to reverse the effect of biofilm inhibition by hLF.

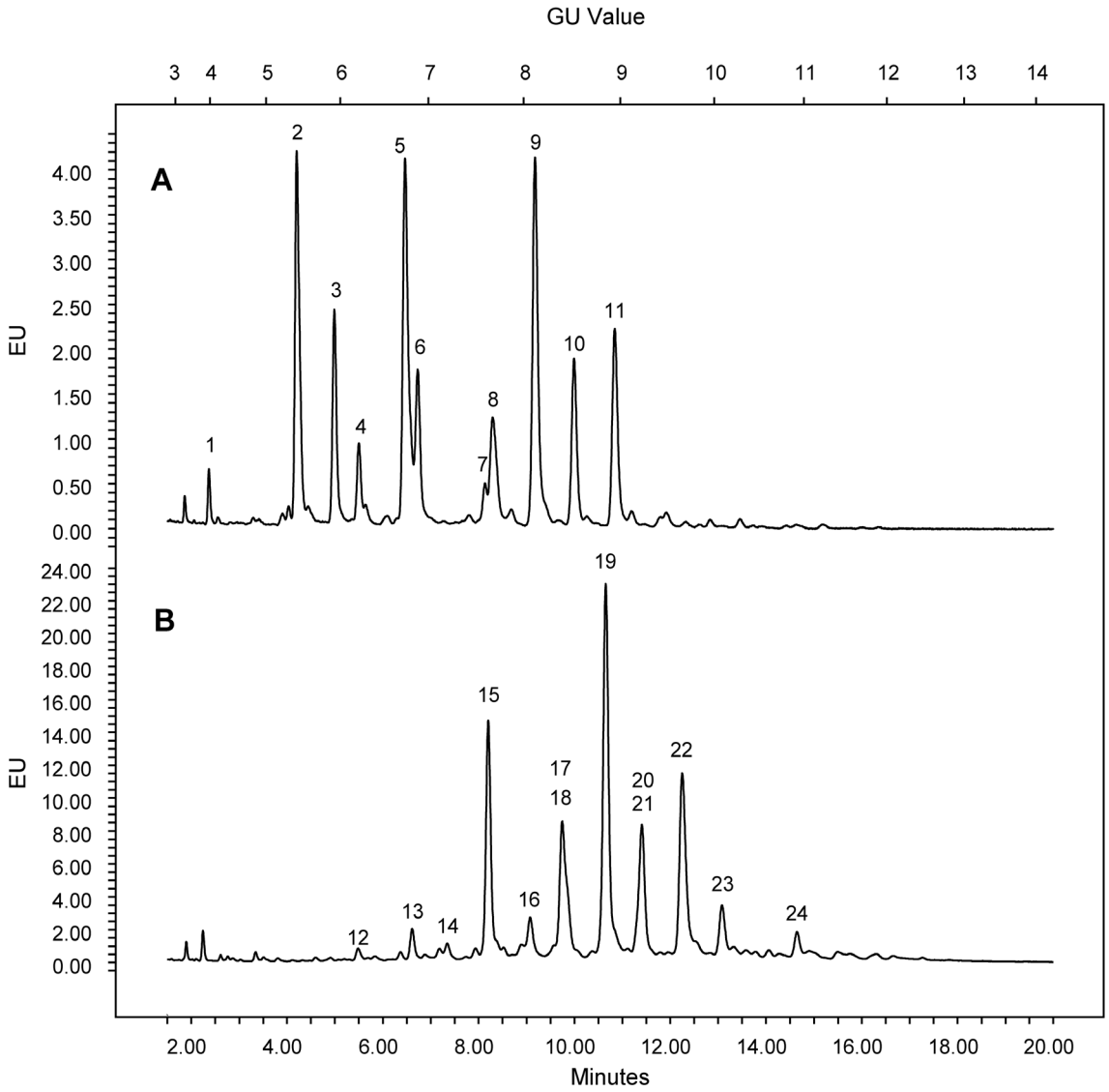


Figure 2. Glycan analysis of lactoferrin. Hydrophilic interaction liquid chromatography (HILIC)-fluorescence chromatogram of 2-AB labeled glycans released from human lactoferrin by the endoglycosidase EndoE (A) and the endoglycosidase PNGaseF, respectively (B). Identified glycans are separated into peaks. The numbers correspond to the glycan structures depicted in Figure 3 and 4.
doi:10.1371/journal.pone.0091035.g002

E. faecalis is able to Utilize the Glycans from Lactoferrin as Nutrients

It has been described that *E. faecalis* is able to grow with RNaseB as a single carbon source and it was assumed that deglycosylation of glycoproteins could be important for nutrient acquisition during growth *in vivo* [40]. Especially in nutrient poor environments, the glycans released from glycoproteins could be essential for the bacteria to persist and survive in a certain environment. Therefore, we were interested in whether *E. faecalis* is able to use the complex glycans from hLF to promote its growth. Culture experiments were performed in diluted THB medium with and without hLF as well as with the N-linked glycans isolated from hLF. This showed that *E. faecalis* is able to reach a higher optical density in the presence of hLF and in the presence of the isolated glycans than in diluted THB medium, indicating that the glycans can indeed promote the growth of *E. faecalis* (Fig. 7).

Discussion

Glycosidases are widespread among bacterial pathogens and non-pathogens and play key roles in modulating host proteins, nutrient acquisition and escaping the immune system [14,17,18,41]. There is limited information about the biological function of the endoglycosidase EndoE from the opportunistic pathogen *E. faecalis*. It cleaves the glycans from human IgG [22] and might be important to gain nutrients [40]. In this study we identified a new substrate for EndoE, the human glycoprotein lactoferrin (hLF). hLF is a 80 kDa non-heme iron binding protein considered to be a component of innate immunity [27,42,43]. Using lectin blot, UHPLC and LC-MS analysis, we showed that EndoE hydrolyzes glycans from hLF and that the activity of EndoE on hLF is dependent on the α-domain with GH18 activity. Moreover, we were able to show that most glycans released from hLF by EndoE correspond to the glycans released by PNGaseF. Due to the difference in site of cleavage of EndoE and PNGaseF,

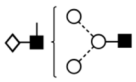
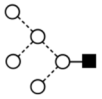
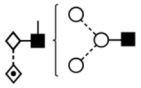
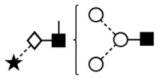
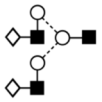
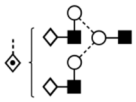
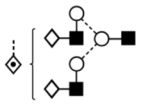
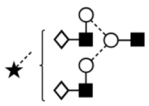
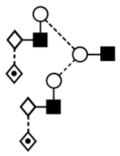
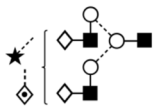
Peak	Name	Structure	GU Value	Peak Area (%)	m/z Value
1	$M3_E$		3.86	1.21	826.36*
2	$A1_EG(4)1$		5.49	15.69	1191.52*
3	$M5_E$		6.04	8.28	1150.48*
4	$A1_EG1F(2)1$		6.34	3.08	1337.59*
5	$A1_EG(4)1S(3)1$		6.84	18.98	1482.64* 740.81
6	$A2_EG(4)2$		6.97	7.32	777.83
7	$A2_EG(4)2F(2)1$ iso		7.58	1.58	850.86
8	$A2_EG(4)2F(2)1$ iso		7.65	7.03	850.86
9	$A2_EG(4)2S(3)1$		8.03	18.91	923.38
10	$A2_EG(4)2F(2)2$		8.41	7.89	923.89
11	$A2_EG(4)2F(2)1S(3)1$		8.83	10.02	996.42

Figure 3. EndoE released N-glycans identified from human lactoferrin. The depicted glycan structure is based on the Oxford glycan nomenclature [56]. Glycans were detected as [M-H] and $[M-2H]^{2-}$ ions. * denotes single charged ions. Glycan names denoted with a subscript E refer to glycans released using EndoE. GU values were generated as previously described [54]. In situations where chromatographic peaks containing multiple structures, the associated peak area was divided equally among the structures for simplicity.
doi:10.1371/journal.pone.0091035.g003

the glycoforms show up with a shift of roughly 0.5 GU (e.g. peak 6 and 15, as well as 10 and 21). Nevertheless, in the chromatogram with glycans released by EndoE, peaks 2, 4 and 5 represents glycans with single antennary structures ($A1_EG(4)1$, $A1_EG1F(2)1$, $A1_EG(4)1S(3)1$) not present in the PNGaseF glycan profile. This phenomenon can be explained in two ways; first, PNGaseF does not have activity on these glycans and they are therefore not released from the glycoprotein. Or secondly, EndoE harbors a glycan hydrolyzing activity that further trims the glycans and gives rise to the observed peaks. Due to the broad activity of PNGaseF and the two active sites of EndoE we favor the second explanation. It can be speculated that EndoE holds two functions; one to modulate the function of the glycoprotein hLF by releasing the N-linked glycan and the other function to further process the released complex glycans. As judged from the lectin blot, it also seems like that the α-domain is responsible for the glycan release based on the glycosidase activity between the two GlcNAcs. The β-domain on the other hand could be responsible for the further trimming of the glycans by glycosidase activity between mannose and GlcNAc, which could not been seen with lectin blot analysis since the lectin ConA binds to mannose within the glycan. This is also supported by the similarity between EndoE and the exoglycosidase StrH

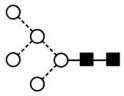
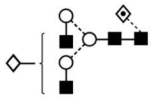
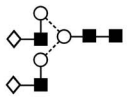
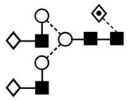
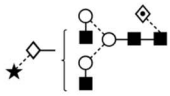
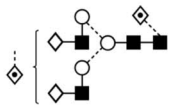
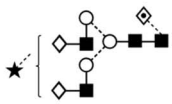
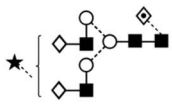
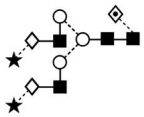
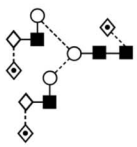
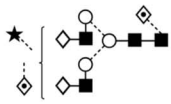
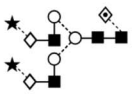
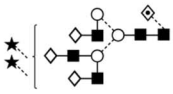
Peak	Name	Structure	GU Value	Peak Area (%)	m/z Value
12	M5		6.23	0.58	ND
13	F(6)A2G(4)1		6.81	1.54	871.35
14	A2G(4)2		7.14	0.93	ND
15	F(6)A2G(4)2		7.50	14.52	952.39
16	F(6)A2G(4)1S(3)1		7.86	3.01	1016.90
17	F(6)A2G(4)2F(2)1		8.16	6.65	1025.42
18	F(6)A2G(4)2S(3)1		8.16	6.66	1097.94
19	F(6)A2G(4)2S(6)1		8.58	28.08	1097.94
20	F(6)A2G(4)2S(3)2		8.97	6.05	1243.50
21	F(6)A2G(4)2F(2)2		8.97	6.06	1098.46
22	F(6)A2G(4)2F(2)1S(6)1		9.42	17.85	1170.97
23	F(6)A2G(4)2S(6)2		9.89	5.25	1243.50
24	F(6)A3G(4)3S(6)2		10.82	2.81	1426.06

Figure 4. PNGaseF released N-glycans identified from human lactoferrin. The depicted glycan structure is based on the Oxford glycan nomenclature [56]. Glycans were detected as $[M\text{-}2H]^{2-}$ ions. GU values were generated as previously described [54]. In situations where chromatographic peaks containing multiple structures, the associated peak area was divided equally among the structures for simplicity.
doi:10.1371/journal.pone.0091035.g004

from *Streptococcus pneumoniae* which also cleaves between mannose and GlcNAc [22,44]. The absence of glycans that contain sialic acids α1–6 attached to galactose in the chromatogram obtained from EndoE could have two reasons: either EndoE has no activity on this type of glycans due to for example steric hindrance, or EndoE has a strong preference for non-sialylated glycans. In both cases the glycans would not appear in the chromatogram. However, independently of the incubation time of hLF with EndoE, fully glycosylated hLF was detected in the lectin blot analysis indicating that EndoE might not have activity on all hLF

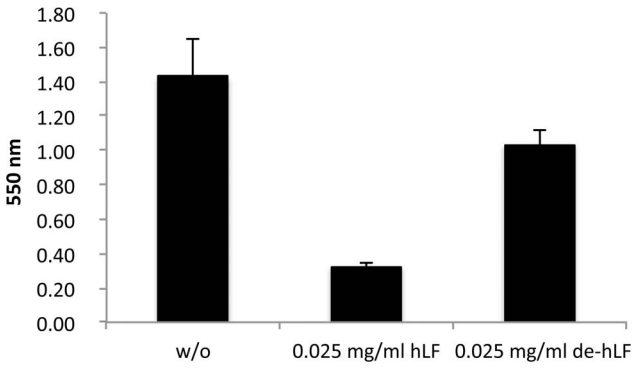


Figure 5. Influence of human lactoferrin on biofilm formation of *Enterococcus faecalis*. Biofilm formation of *E. faecalis* was measured using the crystal violet assay and is expressed as OD_{550}. Human lactoferrin (hLF) was added either fully glycosylated (hLF) or deglycosylated (de-hLF) due to the treatment with EndoE. Error bars indicate the standard deviation from the mean of three independent experiments with three replicates. w/o: no hLF added.
doi:10.1371/journal.pone.0091035.g005

glycans. Taken together, EndoE hydrolyzes most glycoforms of hLF, and might even harbor two different glycosidase activities on hLF. Further investigation of the released glycans by the different domains of EndoE will help to explain the appearance of the glycans with single antennary structure and whether EndoE is able to cleave glycans with α1–6 linked sialic acids or not.

As an inhabitant of the gastrointestinal tract, but also as an opportunistic pathogen, *E. faecalis* encounters different niches where it is exposed to hLF. However, as described recently, hLF contains 17 structurally different glycans [35] and it is not known to what extent the glycans differ in the diverse niches within the human body. To our knowledge, the role of the N-linked glycans from hLF is poorly investigated with regards to the interaction with bacteria. hLF has been described to have antimicrobial activity, both bacteriostatic and bactericidal, and it has been shown to interact with bacteria in different ways. Bactericidal effects are explained by the inhibition of the bacterial H+-ATPase and with the direct interaction of hLF to bacterial surfaces that results in the damage of the outer membrane in Gram-negative bacteria, or the cell wall in Gram-positive bacteria [26,31,45,46]. The bacteriostatic activity of hLF can be explained by iron deprivation of the bacteria [29,47]. This iron sequestration was also suggested to be important for the biofilm inhibition of the Gram-negative bacterium *Pseudomonas aeruginosa* [32]. Biofilm inhibition by hLF has also been observed for other bacteria such as *Streptococcus mutans*, *Porphyromonas gingivalis* and *Prevotella intermedia* [33,34]. However, the exact mechanism of biofilm inhibition is not clear yet and it is unclear whether the inhibition of biofilms by hLF is a universal effect or specific to certain bacteria. Biofilm-related infections are estimated to be responsible for up to 60% of nosocomial infections [48] and therefore play a critical role in the treatment of infections, since growth in a biofilm is often associated with a higher antibiotic resistance, which has also been described for enterococci [49]. Here we report a novel mechanism for the biofilm inhibition of *E. faecalis* by hLF. We showed in a crystal violet assay that hLF strongly inhibits biofilm formation. Deglycosylation of hLF by the endoglycosidase EndoE on the other hand partially restored the biofilm forming ability of *E. faecalis*, strongly suggesting that the inhibition is dependent on the glycans of hLF. Removal of glycans does not change the affinity to iron as reported previously [36] and a biofilm inhibitory effect due to iron sequestration can therefore be excluded. Moreover, we showed that hLF binds to the surface of *E. faecalis* and that this binding is also glycan dependent. A possible reason for the biofilm inhibition could be that the attachment or adhesion to surfaces is decreased due to binding of hLF to *E. faecalis*. It has also been described for *Actinobacillus actinomycetemcomitans* and *Prevotella intermedia* that hLF inhibits the adhesion to fibroblasts and epithelial cells suggesting that hLF prevents the adhesion of bacteria to periodontal tissues [50]. The activity of EndoE on the complex type glycans of hLF could be a mechanism for *E. faecalis* to reverse the biofilm inhibiting effect.

We here identified a surface associated hLF binding protein of *E. faecalis*, the α-enolase. This protein has until now not been known to interact with hLF. The enolase belongs to the so-called moonlighting enzymes with multifunctional properties. These proteins are often involved in metabolic processes, for example, the enolase converts 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway [51,52]. On the other hand, as mentioned above, it has been shown that this protein is not only present intracellularly but interacts as a surface protein with other human proteins like plasminogen, fibrinogen and mucin [37–39]. It has been speculated that these additional biological functions are involved in bacterial virulence [51,52]. Moreover, it is not known if this protein is important for the biofilm formation of *E. faecalis* or other bacteria. Future studies concerning the interaction of the enolase with hLF and its role in the biofilm formation of *E. faecalis* will most likely reveal additional details of the mechanism proposed above.

It has previously been described that the glycans of glycoproteins can serve as nutrients for bacteria and help to survive or persist *in vivo* [14,21,40]. We showed that the glycans of hLF can promote growth of *E. faecalis* in a nutrient limited medium. It seems likely that these glycans could also promote growth of cells within the nutrient limited environment of a biofilm. Similar to the enolase that is involved in metabolism and virulence, EndoE might also be important for metabolic purposes and virulence in *E. faecalis*.

We here provide novel information about the biofilm inhibiting potential of hLF and a possible function of its glycans when interacting with bacteria like *E. faecalis*. EndoE could have two roles: nutrient acquisition and to reverse the biofilm inhibiting effect of hLF. Taken together, the results of this study underline the importance of glycans in the interplay between bacteria and the human host, with possible implications for both commensalism and opportunism.

Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was routinely propagated in LB broth (Difco, Detroit, MI) at 37°C with aeration. *E. faecalis* strains were cultured in Todd-Hewitt broth (THB; Difco, Detroit, MI) at 37°C without aeration. 1.5% (w/v) agar (Difco, Detroit, MI) was used to solidify the medium when needed. Antibiotics were used at the following concentrations: carbenicillin 100 μg/ml (*E. coli*), chloramphenicol 34 μg/ml (*E. coli*).

Growth Experiments with Lactoferrin

50 μg human lactoferrin from milk (hLF) (Sigma-Aldrich) was incubated with 50 μg recombinant EndoE in PBS at 37°C overnight and released glycans were separated from the mixture using 10 MWCO centrifugal filters. *E. faecalis* was cultured overnight in THB and then diluted 1:20 in 20× diluted THB medium with addition of 2 mg/mL hLF or released glycans from

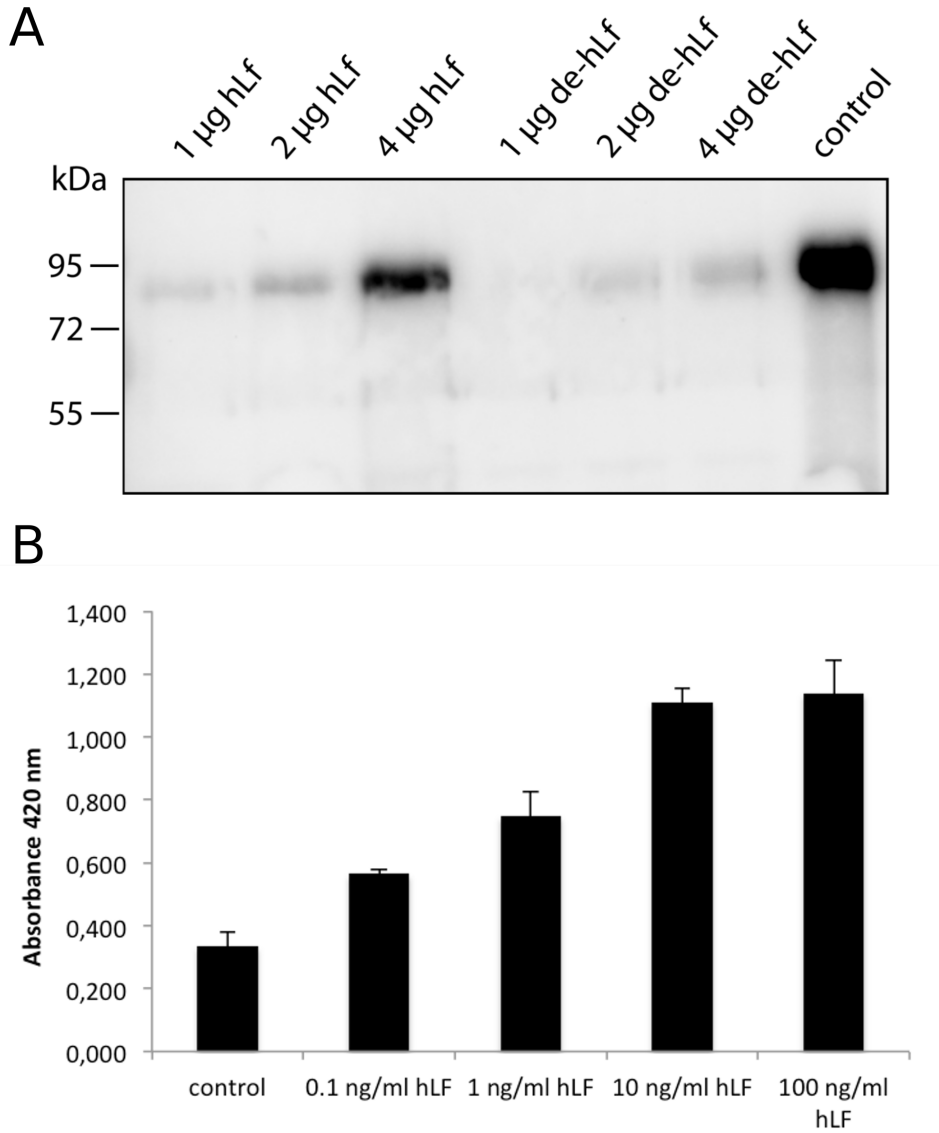


Figure 6. Binding of human lactoferrin to the surface of *Enterococcus faecalis* and recombinant enolase. A. Western blot analysis, using anti-lactoferrin antibodies, of human lactoferrin (hLF) bound to *E. faecalis* OG1. *E. faecalis* was incubated with indicated concentrations of hLF or EndoE treated hLF (de-hLF). *E. faecalis* cell extract was separated on 10% SDS-PAGE and electro-blotted onto PVDF membranes. Control: 1 µg hLF. B. Binding of different hLF concentrations to recombinant enolase immobilized to a microtiter plate. Anti-hLF antibodies were used to detect the binding of hLF to the enolase. Error bars indicate the standard deviation from the mean of three independent experiments.
doi:10.1371/journal.pone.0091035.g006

approximately 50 µg hLF. The growth was followed by measuring OD at 620 nm over time.

PCR Analysis and Sequencing

To amplify DNA from *E. faecalis* strains, genomic DNA was isolated using the DNA extraction kit Gentra Puregene Yeast/Bact (Qiagen AB, Sweden) according to the manufacturer's instructions. 30 ng genomic DNA was used as a template in a standard PCR using TrueStart *Taq* polymerase (Fermentas AB, Sweden). PCR products were separated on 1% TAE agarose and stained with SYBR Safe (Invitrogen). PCR fragments or plasmids were sequenced using the sequencing service of GATC Biotech (Konstanz, Germany).

Recombinant Expression of EndoE

EndoE, EndoE(E186Q) and EndoE(E662Q) were recombinantly expressed in *E. coli* as previously described using the GST gene fusion system from GE Healthcare [22]. Recombinant expression of the enolase (EF1961) was performed using the same protocol. Briefly, a 1299 bp fragment of EF1961 was amplified using the following primers: 5′-CGGGATCCACATGTCAATTATTACTGATA-3′ with a *BamH*I site (underlined) and 5′-CCCTCGAGTTATTTGTTTTTTAAGTTG-3′ with an *Xho*I site (underlined). The PCR fragment was cloned into the pGEX-5x-3 vector (GE Healthcare) and transformed into the *E. coli* BL21 (DE3) pLysE (Invitrogen) strain for expression of EF1961.

Lectin Blot Analysis

Activity of EndoE on human lactoferrin from milk (hLF) (Sigma-Aldrich) was tested as described with other glycoproteins

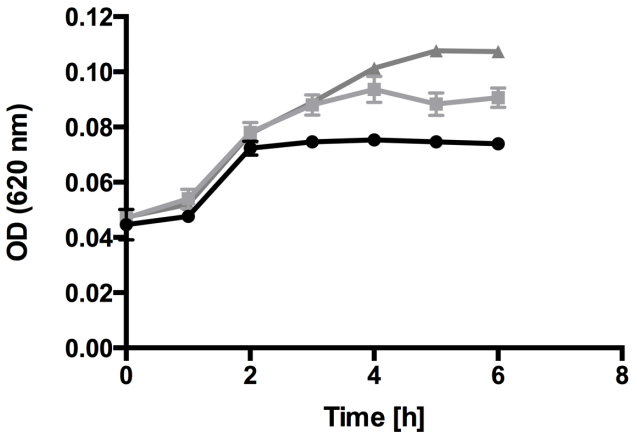


Figure 7. Growth of *Enterococcus faecalis* in the presence of lactoferrin. Growth curve of *E. faecalis* in diluted THB medium (●) and in diluted THB medium supplemented with 2 mg/ml hLF (■) or isolated N-linked glycans from hLF (▲). Optical density (OD) at 620 nm was determined at indicated time points. Error bars indicate the standard deviation from the mean of three independent experiments.
doi:10.1371/journal.pone.0091035.g007

previously [22]. Briefly, 2 μg of hLF was incubated with 2 μg recombinant EndoE, EndoE(E186Q) or EndoE(E662Q) in a total volume of 20 μl of PBS buffer for 16 h at 37°C. Activity was analyzed with 10% SDS PAGE (stained with Coomassie Brilliant Blue G-250) and lectin blot analysis was performed using 0.5 μg/ml biotinylated ConA lectin (Vector Laboratories, Burlingame, CA) [22,53].

UHPLC Analysis

Glycans from 1 μg hLF were released with EndoE or N-glycosidase F (PNGaseF; ProZyme) in PBS at 37°C overnight. The released glycans were isolated with 10 MWCO spin columns (Pall), desalted using porous graphitic carbon and labeled with 2-AB using the LudgerTag Glycan Labeling Kit (Ludger Ltd) according to the manufacturer's instructions. Glycans labeled with 2-AB were analyzed using a Waters 1.7 μm BEH Glycan (2.1 mm×150 mm) column coupled to a Waters H-class UHPLC instrument. Separations were carried out using a 30–58% gradient of 50 mM ammonium formate pH 4.4 against acetonitrile with a column temperature of 40°C over a 30 min duration. Labeled glycans were visualized using fluorescence detection with excitation and emission wavelengths of 330 nm and 420 nm, respectively. Individual peaks appearing within chromatograms were converted to glucose unit (GU) values for comparative purposes. GU values were calculated through use of a 2-AB labeled dextran hydrolysate as a glycan ladder, which provided a means of converting peak retention time to a standardized GU value [54]. Retention times for peaks of interest in each glycan sample were integrated and converted to GU values using Empower 3 software (Waters, MA, USA).

LC-FLD-MS

Online coupled fluorescence (FLD)-mass spectrometry detection was performed using a Waters Xevo G2 QTof with Acquity UPLC and BEH Glycan column (1.0×150 mm, 1.7 μm particle size). MS data was acquired in negative mode with the following conditions: 2500 V capillary voltage, 50 V cone voltage, 280°C desolvation temperature, 600 L/hour desolvation gas and 100°C source temperature. The analyzer was set to sensitivity mode. The fluorescence data rate was 1 pts/second and a PMT gain = 10 with altering excitation and emission wavelengths that varied based on the experiment and glycan label used. Sample injection volumes were 10 μl and the flow rate was 0.150 μl/min. Solvent A was 50 mM ammonium formate pH 4.4 and solvent B was acetonitrile. A 40 minute linear gradient was used and was as follows: 28–43% A for 32 minutes, 70% A for 4 minutes and 28% solvent A for 4 minutes. Samples were diluted in 65% acetonitrile prior to analysis.

BIAcore Surface Plasmon Resonance (SPR) Interaction Analysis

Lactoferrin was diluted in 10 mM sodium acetate, pH 4, and immobilized via amine coupling to a flow cell of Sensor Chip CM3 (GE Healthcare, Uppsala, Sweden). Immobilization levels were optimized to around 1000 response units. A flow cell subjected to the immobilization protocol but without any addition of protein was used as control for buffer bulk changes and nonspecificity.

For affinity measurements, the binding and dissociation phases were monitored with a BIAcore 2000 instrument (GE Healthcare). In control experiments for possible mass transfer limitations,

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics	Reference or source
E. faecalis		
OG1RF	Derivative of *E. faecalis* OG1, resistant to rifampicin	[57]
E. coli		
TOP10	Cloning strain	Invitrogen
BL21(DE3)pLys	Cloning/expression strain	Invitrogen
Plasmids		
pCR2.1	PCR cloning vector	Invitrogen
pGEX-5X-3	GST fusion vector	GE Healthcare
pGEX*ndoE*	Expression vector for full length EndoE	[22]
pGEX*ndoE*(E186Q)	E186Q mutation in EndoE	[22]
pGEX*ndoE*(E662Q)	E662Q mutation in EndoE	[22]
pGEX EF1961	Expression vector for full length EF1961	This study

doi:10.1371/journal.pone.0091035.t001

EndoE variants were injected over the surfaces at different flow rates. No differences in initial binding were observed at 5 µl/min or above indicating no limitations to any combinations of ligands. EndoE(E186Q) and EndoE(E662Q) were injected at different concentrations (typically 31–500 nM) at 15 µl/min and 25°C over the lactoferrin flow cell in running buffer (10 mM Hepes, pH 7.5, 150 mM NaCl, and 0.005% surfactant P20).

Between experiments, the surfaces were strictly regenerated with micro-pulses of 0.1 M $NaHCO_3$, pH 12 and with running buffer containing 2 M NaCl followed by an extensive wash procedure after reaching flow cell baseline. After X and Y normalization of data (injection starting points set to zero), the blank curves from control flow cells of each injected concentration were subtracted.

The association (k_a) and dissociation (k_d) rate constants were determined simultaneously using the equation for 1:1 Langmuir binding in the BIA Evaluation 4.1 software (GE Healthcare). The binding curves were fitted locally and the equilibration dissociation constants (K_D) were calculated from mean values of the obtained rate constants.

Biofilm Assay

Quantification of biofilm formation was performed using a modified crystal violet microtiter plate assay as described previously [55]. A bacterial overnight culture was diluted 1:10 in THB medium supplemented with 0.4% glucose, 200 µl of bacterial suspension was filled in each flat bottomed 96-well microtiter plate (Nunc) and incubated for 24 h at 37°C, 5% CO_2. The content of each well was discarded and the wells were washed three times with 190 µl sterile PBS buffer. The attached bacteria were fixed with 200 µl methanol for 10 min. The methanol was discarded and the wells air-dried at room temperature for 15 min. Bacteria were stained for 5 min with 160 µl 1% (w/v in H_2O) crystal violet solution. After staining, the wells were washed three times with 190 µl PBS and the bound dye was finally re-solubilized with 200 µl ethanol/acetone plate and the OD was immediately measured at a wavelength of 550 nm. To study the biofilm formation in the presence of lactoferrin the protein was directly added to the diluted bacteria at indicated concentrations. For deglycosylation, hLF was first incubated with recombinant EndoE-GST fusion protein in equal amounts for 16 h at 37°C. EndoE was removed using glutathione sepharose (GE Healthcare) and the EndoE treated hLF (de-hLF) was sterile filtered. Each experiment is displayed as the mean of three independent experiments with three replicates.

To study the amount of viable cells in the biofilm assay, control cultures were vigorously mixed after 24 h to detach the cells from the bottom of the well, diluted in PBS-buffer and the colony forming unit (CFU) was determined. Each experiment was performed in three independent experiments.

Binding of Lactoferrin to *Enterococcus faecalis*

5 ml stationary phase culture of *E. faecalis* was washed 3 times with PBS buffer and resuspended in PBS buffer. 50 µl cells (2.5×10^7 cells) were incubated with indicated concentrations of human lactoferrin (hLF) or EndoE treated hLF (de-hLF, see above) for 2 h at 37°C. The bacteria were washed 3 times with PBS to remove non-bound hLF, pelleted and dissolved in SDS-buffer. Cell extract was separated on 10% SDS-PAGE and electro-blotted onto PVDF membranes. Membranes were processed as described [22] and hLF was detected using anti-lactoferrin antibodies.

Identification of a Lactoferrin Binding Protein from *Enterococcus faecalis*

In order to identify a protein on the enterococcal surface that binds lactoferrin, lactoferrin was coupled to CnBr-activated sepharose (Pharmacia Biotech) according to the manufacturer's instructions. 30 ml of enterococcal over night culture was washed twice with PBS buffer, resuspended in 10 ml of 0.01 M KH_2PO_4 buffer, pH 6.2 and treated with 250 U mutanolysin at 37°C until lysis occurred. The lysate was centrifuged to remove cell debris and the supernatant was incubated with the CnBr activated sepharose with coupled lactoferrin. The lactoferrin bound protein from the enterococcal cell extract was eluted with 0.1 M glycine, pH 2 and concentrated using trichloroacetic acid at a final concentration of 5%. Proteins were separated on 10% SDS-PAGE, stained with Coomassie Brilliant Blue R-250 and identified using the protein identification service from Alphalyse (Odense, Denmark).

Enzyme Linked Immunosorbent Assay (ELISA)

Microtiter plates (MaxiSorp, NUNC, Roskilde, Denmark) were coated with 5 µg/ml hLF in coatingbuffer (16 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) at 4°C overnight. The plates were washed three times with PBS +0.05% Tween (PBST). Non-specific binding was blocked with 2% (w/v) BSA in PBST for 30 min at room temperature followed by washing with PBST. hLF was diluted in PBST +2% (w/v) BSA (concentrations indicated), added to the wells and incubated for 1 h at 37°C. Wells were washed three times with PBST, anti-hLF antibody was added (1:1000 in PBST +2% (w/v) BSA) and incubation proceeded for another hour at 37°C. Secondary HRP conjugated antibody (1:3000 in PBST +2% (w/v) BSA) was added after washing three times with PBST. Following three more washes, the color reaction was developed with 0.1 M citric acid monohydrate, 0.1 M $Na_2HPO_4\times2H_2O$ buffer pH 4.5 containing 0.012% (v/v) H_2O_2 and 1.7 mM 2,29-azino-bis(3- ethylbenzthiazoline-6-sulphonic acid) (ABTS). The absorbance was read on a model 550 micro plate reader (BIO-RAD, Hercules, CA, USA) at 420 nm. The experiments were made in triplicates.

Supporting Information

File S1 Protein identification report and MS spectrum.
(PDF)

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Author Contributions

Conceived and designed the experiments: JG MC. Performed the experiments: JG JS MB AIO. Analyzed the data: JG JS EC WBS MB AIO. Contributed reagents/materials/analysis tools: PMR MC. Wrote the paper: JG JS.

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PAPER IV

PAPER V

Bacterial modulation of host glycosylation

Jonathan Sjögren

A majority of the proteins of the immune system are glycosylated and the glycans of IgG are essential for its functionality. Bacteria display enzymes that modulate the glycans of the immune system to weaken the host defense and favor bacterial survival. In this thesis we aimed at exploring bacterial modulation of host glycosylation in infection and to evaluate the usefulness of bacterial enzymes in biotechnology and for therapeutic use.

The role of IgG endoglycosidase EndoS in streptococcal virulence was evaluated in a murine model of invasive infection and we found significant contribution when heterologously expressed. We also discovered and characterized EndoS2, a novel enzyme specific and conserved in serotype M49 of streptococci, with enzymatic activity on the glycans of IgG and α_1-acid glycoprotein. Enterococcal pathogenesis was studied, and we found that the endoglycosidase EndoE cleaved glycans of lactoferrin to reduce the antibacterial functions and to support bacterial growth. A glycoform specificity difference between EndoS and EndoS2 was observed, and we suggested a method for quantification of high-mannose glycans on therapeutic antibodies, a key quality attribute. Finally, we explored the importance of Fc glycosylation of IgE and showed that EndoS cleaved glycans of this immunoglobulin causing a reduction of the immune cell activation *in vivo*, a potential new therapeutic strategy for severe IgE mediated allergies.

In this thesis we demonstrate that glycans are an integral part of the immune system, and that the study of bacterial effectors of glycosylation paves the way for a deeper understanding of infections, for novel tools supporting the biotech arena, and for new therapeutic strategies.

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