BJ Biomolecules

View this PDF in Utopia Documents for more interactive functionality
Download FREE software now

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

Jonathan SJÖGREN*[1,2], Weston B. STRUWE†[3], Eoin F. J. COSGRAVE†[4], Pauline M. RUDD†, Martin STERVANDER‡, Maria ALLHORN*[2], Andrew HOLLANDS§‖[2], Victor NIZET*‖¶[2] and Mattias COLLIN*[2]

*Department of Clinical Sciences, Division of Infection Medicine, Lund University, Biomedical Center B14, SE-22184 Lund, Sweden, †The National Institute for Bioprocess Research and Training, NIBRT, University College Dublin, Fosters Avenue, Mount Merrion, Blackrock, Co. Dublin, Ireland, ‡Molecular Ecology and Evolution Laboratory, Department of Biology, Lund University, Ecology Building, SE-223 62 Lund, Sweden, §School of Biological Sciences, University of Wollongong, Wollongong, NSW 2522, Australia, ‖Department of Pediatrics, University of California San Diego, 9500 Gilman Drive, Mail Code 0687, La Jolla, CA 92093, U.S.A., and ¶Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, 9500 Gilman Drive, Mail Code 0687, La Jolla, CA 92093, U.S.A.

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.

[1] To whom correspondence should be addressed (email jonathan.sjogren@med.lu.se).

[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.

[3] Present address: Chemistry Research Laboratory, Department of Chemistry, University of Oxford, Oxford OX1 3TA, U.K.
[4] Present address: Waters Corporation, 34 Maple Street, Milford, MA 01757, U.S.A.

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).

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

Biochemical Journal www.biochemj.org

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

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

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

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

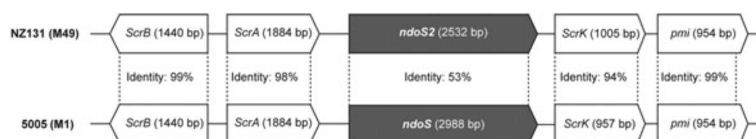

Figure 1 Genetic context analysis of *ndoS* and *ndoS2*

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

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

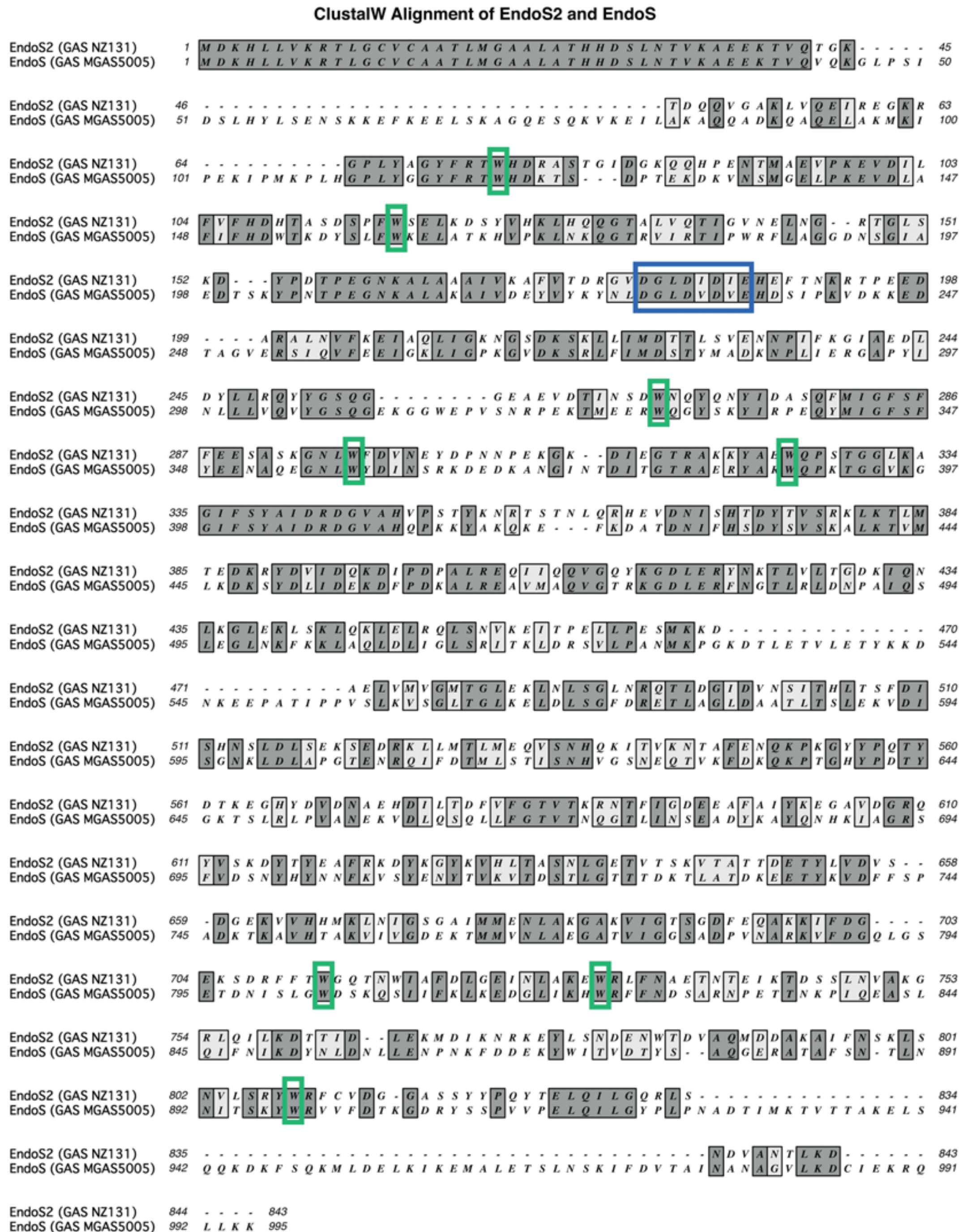

Figure 2 ClustalW alignment of EndoS$_2$ and EndoS

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

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

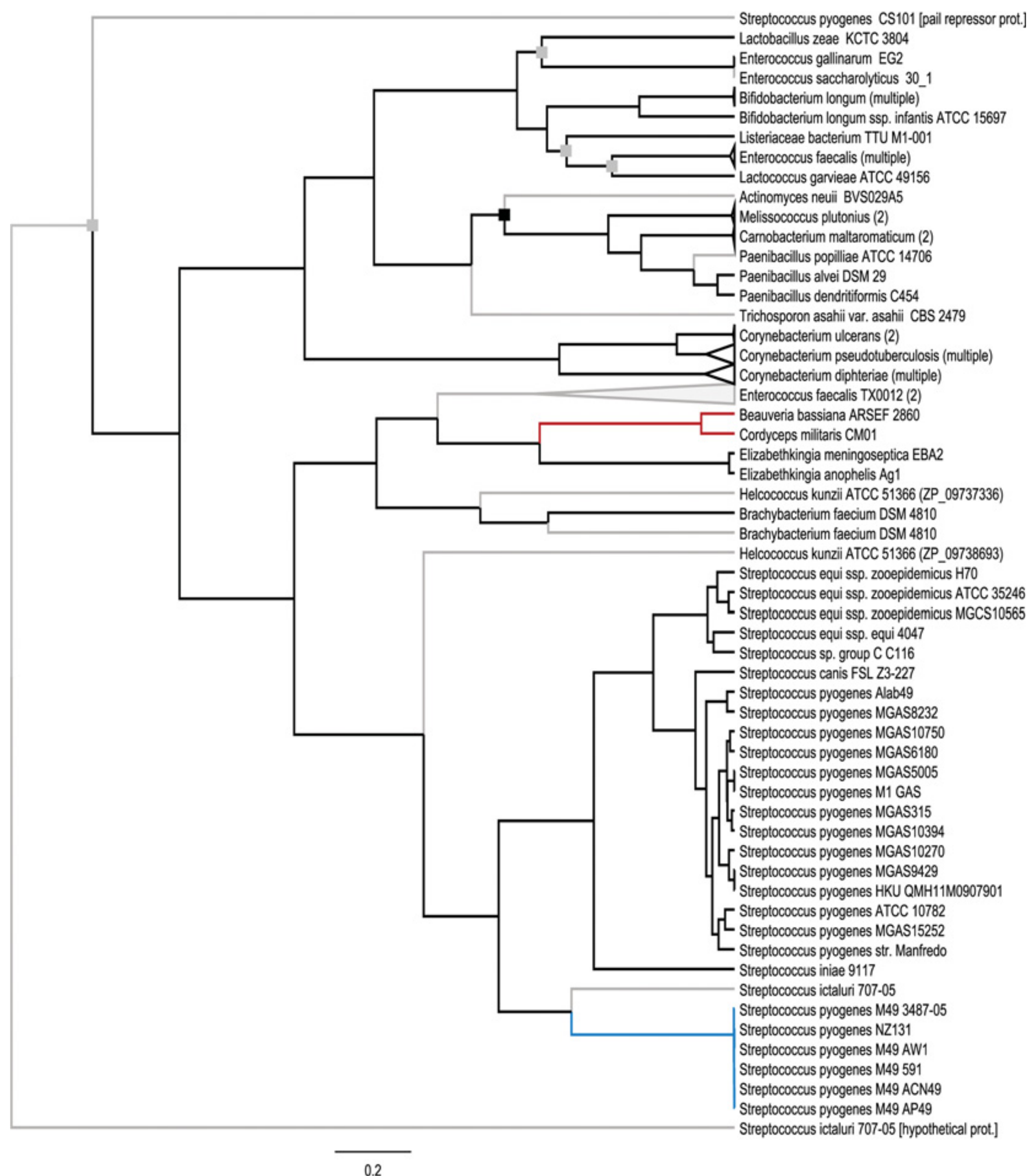

Figure 3 Phylogenetic reconstruction of the protein-specific tree for EndoS-like proteins, based on 1099 amino acids

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

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

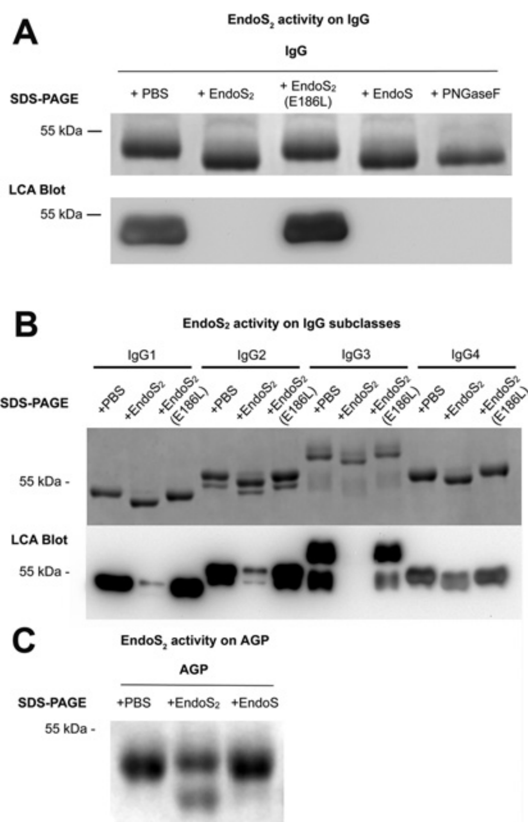

Figure 4 Activity of EndoS$_2$ on IgG, IgG subclasses and AGP

(**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).

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

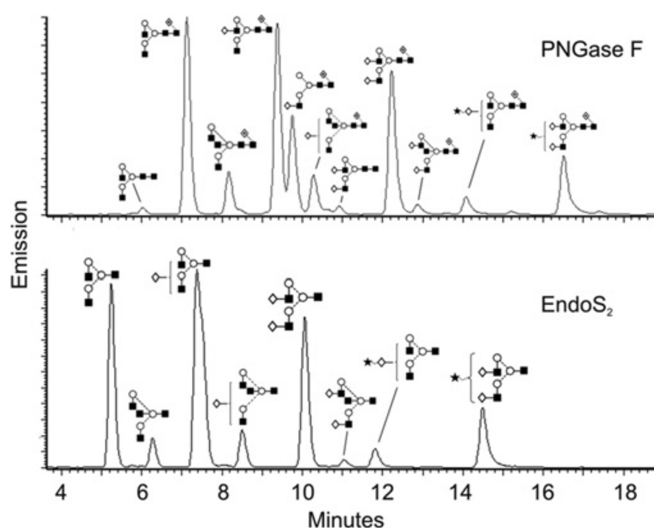

Figure 5 Glycan fluorescent profiles from human IgG released by EndoS$_2$ and PNGase F

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].

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

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

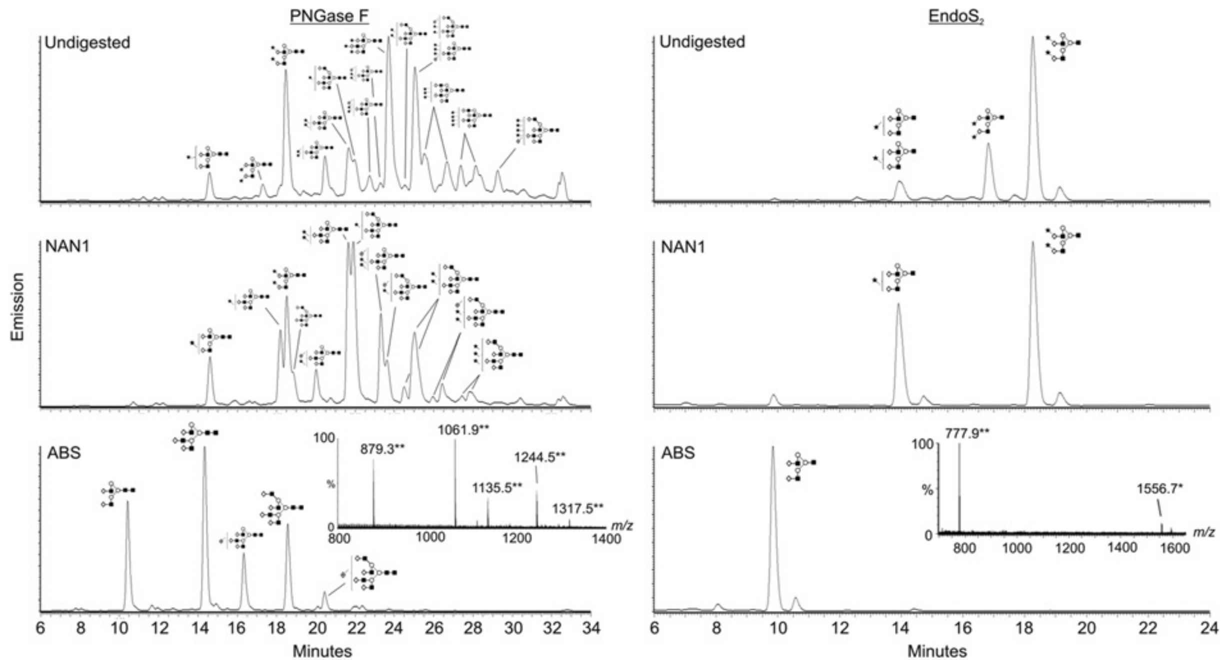

Figure 6 Glycan fluorescent profiles from human AGP released by PNGase F and EndoS$_2$

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.

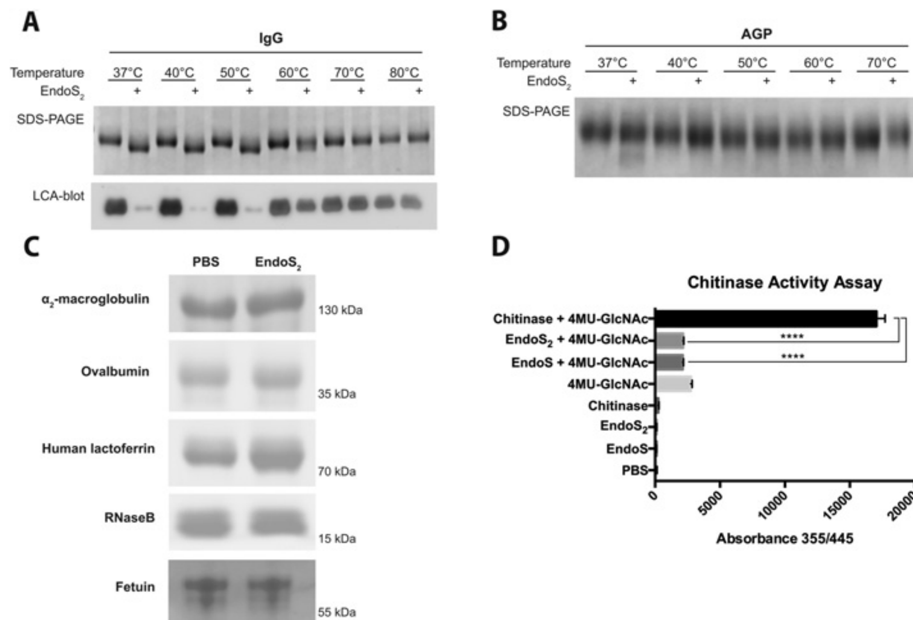

Figure 7 Activity of EndoS$_2$ on native and denatured IgG and AGP, other glycoproteins and chitinase assay

(**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 $P<0.05$. ****$P<0.001$.

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

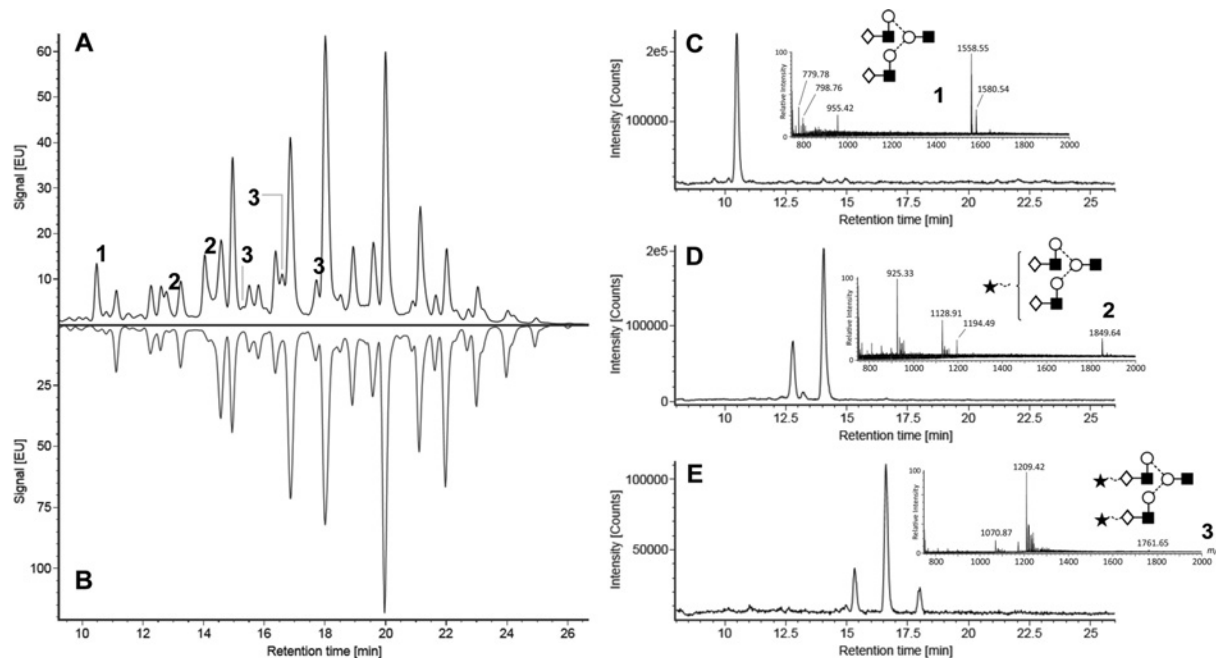

Figure 8 $EndoS_2$ hydrolyses biantennary free glycans

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 $[M+\mathrm{H}]^+$, 925.33 $[M+2\mathrm{H}]^{2+}$ and 1070.87 $[M+2\mathrm{H}]^{2+}$ 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 $[M+\mathrm{H}]^+$ 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

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

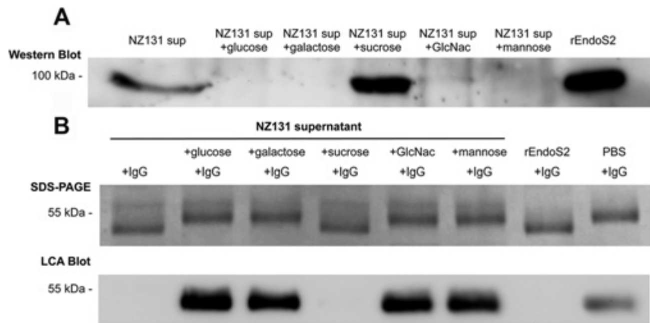

Figure 9 EndoS$_2$ expression and activity in GAS strain NZ131

(**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

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

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.

FUNDING

This work was supported by the Swedish Research Council [grant number 2010-57X-20240], the Åke Wiberg Foundation, the Alfred Österlund Foundation, the Petrus and Augusta Hedlund Foundation, the Clas Groschinsky Foundation, the Torsten och Ragnar Söderberg Foundation, the Swedish Society for Medicine, the Royal Physiografic Society in Lund, King Gustaf V's 80 Years Fund, the Medical Faculty at Lund University, Swedish governmental funding for clinical research (ALF), and Hansa Medical AB. The funders had no role in the preparation of the paper or the decision to publish.

REFERENCES

1 Rudd, P., Elliott, T., Cresswell, P., Wilson, I. and Dwek, R. (2001) Glycosylation and the immune system. Science **291**, 2370–2376

2 Raju, T. S. (2008) Terminal sugars of Fc glycans influence antibody effector functions of IgGs. Curr. Opin. Immunol. **20**, 471–478

3 Lux, A. and Nimmerjahn, F. (2012) Impact of differential glycosylation on IgG activity. Adv. Exp. Med. Biol. **780**, 113–124

4 Lux, A., Aschermann, S., Biburger, M. and Nimmerjahn, F. (2009) The pro and anti-inflammatory activities of immunoglobulin G. Ann. Rheum. Dis. **69**, i92–i96

5 Barb, A. W. and Prestegard, J. H. (2011) NMR analysis demonstrates immunoglobulin G *N*-glycans are accessible and dynamic. Nat. Chem. Biol. **7**, 147–153

6 Arnold, J. N., Wormald, M. R., Sim, R. B., Rudd, P. M. and Dwek, R. A. (2007) The impact of glycosylation on the biological function and structure of human immunoglobulins. Annu. Rev. Immunol. **25**, 21–50

7 Nizet, V. (2007) Understanding how leading bacterial pathogens subvert innate immunity to reveal novel therapeutic targets. J. Allergy Clin. Immunol. **120**, 13–22

8 Carapetis, J., Steer, A., Mulholland, E. and Weber, M. (2005) The global burden of group A streptococcal diseases. Lancet Infect. Dis. **5**, 685–694

9 Cunningham, M. W. (2000) Pathogenesis of group A streptococcal infections. Clin. Microbiol. Rev. **13**, 470–511

10 Collin, M. and Olsén, A. (2001) EndoS, a novel secreted protein from *Streptococcus pyogenes* with endoglycosidase activity on human IgG. EMBO J. **20**, 3046–3055

11 Collin, M., Svensson, M., Sjöholm, A., Jensenius, J., Sjöbring, U. and Olsén, A. (2002) EndoS and SpeB from *Streptococcus pyogenes* inhibit immunoglobulin-mediated opsonophagocytosis. Infect. Immun. **70**, 6646

12 Allhorn, M., Olin, A. I., Nimmerjahn, F. and Collin, M. (2008) Human IgG/FcγR interactions are modulated by streptococcal IgG glycan hydrolysis. PLoS ONE **3**, e1413

13 Allhorn, M., Olsén, A. and Collin, M. (2008) EndoS from *Streptococcus pyogenes* is hydrolyzed by the cysteine proteinase SpeB and requires glutamic acid 235 and tryptophans for IgG glycan-hydrolyzing activity. BMC Microbiol. **8**, 3

14 Collin, M. and Olsén, A. (2001) Effect of SpeB and EndoS from *Streptococcus pyogenes* on human immunoglobulins. Infect. Immun. **69**, 7187–7189

15 Sjögren, J., Okumura, C. Y., Collin, M., Nizet, V. and Hollands, A. (2011) Study of the IgG endoglycosidase EndoS in group A streptococcal phagocyte resistance and virulence. BMC Microbiol. **11**, 120

16 Nandakumar, K. S., Collin, M., Olsén, A., Nimmerjahn, F., Blom, A. M., Ravetch, J. V. and Holmdahl, R. (2007) Endoglycosidase treatment abrogates IgG arthritogenicity: importance of IgG glycosylation in arthritis. Eur. J. Immunol. **37**, 2973–2982

17 Allhorn, M., Briceno, J. G., Baudino, L., Lood, C., Olsson, M. L., Izui, S. and Collin, M. (2010) The IgG-specific endoglycosidase EndoS inhibits both cellular and complement-mediated autoimmune hemolysis. Blood **115**, 5080–5088

18 Albert, H., Collin, M., Dudziak, D., Ravetch, J. and Nimmerjahn, F. (2008) *In vivo* enzymatic modulation of IgG glycosylation inhibits autoimmune disease in an IgG subclass-dependent manner. Proc. Natl. Acad. Sci. U.S.A. **105**, 15005–15009

19 van Timmeren, M. M., van der Veen, B. S., Stegeman, C. A., Petersen, A. H., Hellmark, T., Collin, M. and Heeringa, P. (2010) IgG glycan hydrolysis attenuates ANCA-mediated glomerulonephritis. J. Am. Soc. Nephrol. **21**, 1103–1114

20 Collin, M., Shannon, O. and Björck, L. (2008) IgG glycan hydrolysis by a bacterial enzyme as a therapy against autoimmune conditions. Proc. Natl. Acad. Sci. U.S.A. **105**, 4265–4270

21 Benkchoucha, M., Molnarfi, N., Santiago-Raber, M.-L., Weber, M. S., Merkler, D., Collin, M. and Lalive, P. H. (2012) IgG glycan hydrolysis by EndoS inhibits experimental autoimmune encephalomyelitis. J. Neuroinflamm. **9**, 209

22 Huang, W., Giddens, J., Fan, S.-Q., Toonstra, C. and Wang, L.-X. (2012) Chemoenzymatic glycoengineering of intact IgG antibodies for gain of functions. J. Am. Chem. Soc. **134**, 12308–12318

23 Goodfellow, J. J., Baruah, K., Yamamoto, K., Bonomelli, C., Krishna, B., Harvey, D. J., Crispin, M., Scanlan, C. N. and Davis, B. G. (2012) An endoglycosidase with alternative glycan specificity allows broadened glycoprotein remodelling. J. Am. Chem. Soc. **134**, 8030–8033

24 McShan, W. M., Ferretti, J. J., Karasawa, T., Suvorov, A. N., Lin, S., Qin, B., Jia, H., Kenton, S., Najar, F., Wu, H. et al. (2008) Genome sequence of a nephritogenic and highly transformable M49 strain of *Streptococcus pyogenes*. J. Bacteriol. **190**, 7773–7785

25 Chaussee, M. S., Ajdic, D. and Ferretti, J. J. (1999) The *rgg* gene of *Streptococcus pyogenes* NZ131 positively influences extracellular SpeB production. Infect. Immun. **67**, 1715–1722

26 Drummond, A. J., Suchard, M. A., Xie, D. and Rambaut, A. (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. **29**, 1969–1973

27 Henikoff, S. and Henikoff, J. G. (1992) Amino acid substitution matrices from protein blocks. Proc. Natl. Acad. Sci. U.S.A. **89**, 10915–10919

28 Gernhard, T. (2008) The conditioned reconstructed process. J. Theor. Biol. **253**, 769–778

29 Ahn, J., Bones, J., Yu, Y. Q., Rudd, P. M. and Gilar, M. (2010) Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction chromatography columns packed with 1.7 microm sorbent. J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. **878**, 403–408

30 Plummer, Jr, T. H. and Tarentino, A. L. (1991) Purification of the oligosaccharide-cleaving enzymes of *Flavobacterium meningosepticum*. Glycobiology **1**, 257–263

31 Fournier, T., Medjoubi-N, N. and Porquet, D. (2000) α1-Acid glycoprotein. Biochim. Biophys. Acta **1482**, 157–171

32 Shelburne, S. A., Keith, D., Horstmann, N., Sumby, P., Davenport, M. T., Graviss, E. A., Brennan, R. G. and Musser, J. M. (2008) A direct link between carbohydrate utilization and virulence in the major human pathogen group A *Streptococcus*. Proc. Natl. Acad. Sci. U.S.A. **105**, 1698–1703

33 Garbe, J. and Collin, M. (2012) Bacterial hydrolysis of host glycoproteins: powerful protein modification and efficient nutrient acquisition. J. Innate Immun. **4**, 121–131

34 Collin, M. and Fischetti, V. A. (2004) A novel secreted endoglycosidase from *Enterococcus faecalis* with activity on human immunoglobulin G and ribonuclease B. J. Biol. Chem. **279**, 22558–22570

35 Tarentino, A. and Plummer, T. (1994) Enzymatic deglycosylation of asparagine-linked glycans: purification, properties, and specificity of oligosaccharide-cleaving enzymes from *Flavobacterium meningosepticum*. Methods Enzymol. **230**, 44–57

36 Tarentino, A. L., Quinones, G., Schrader, W. P., Changchien, L. M. and Plummer, T. H. (1992) Multiple endoglycosidase (Endo) F activities expressed by *Flavobacterium meningosepticum*. Endo F1: molecular cloning, primary sequence, and structural relationship to Endo H. J. Biol. Chem. **267**, 3868–3872

37 Trimble, R. B. and Tarentino, A. L. (1991) Identification of distinct endoglycosidase (endo) activities in *Flavobacterium meningosepticum*: endo F1, endo F2, and endo F3. Endo F1 and endo H hydrolyze only high mannose and hybrid glycans. J. Biol. Chem. **266**, 1646–1651

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

38 Tarentino, A. L., Quinones, G., Changchien, L. M. and Plummer, Jr, T. H. (1993) Multiple endoglycosidase F activities expressed by *Flavobacterium meningosepticum* endoglycosidases F2 and F3: molecular cloning, primary sequence, and enzyme expression. J. Biol. Chem. **268**, 9702–9708

39 Renzi, F., Manfredi, P., Mally, M., Moes, S., Jenö, P. and Cornelis, G. (2011) The *N*-glycan glycoprotein deglycosylation complex (Gpd) from *Capnocytophaga canimorsus* deglycosylates human IgG. PLoS Pathog. **7**, 17

40 King, S. J., Hippe, K. R. and Weiser, J. N. (2006) Deglycosylation of human glycoconjugates by the sequential activities of exoglycosidases expressed by *Streptococcus pneumoniae*. Mol. Microbiol. **59**, 961–974

41 Anumula, K. R. (2012) Quantitative glycan profiling of normal human plasma derived immunoglobulin and its fragments Fab and Fc. J. Immunol. Methods **382**, 167–176

42 Baruah, K., Bowden, T. A., Krishna, B. A., Dwek, R. A., Crispin, M. and Scanlan, C. N. (2012) Selective deactivation of serum IgG: a general strategy for the enhancement of monoclonal antibody receptor interactions. J. Mol. Biol. **420**, 1–7

43 Schmid, K., Kaufmann, H., Isemura, S., Bauer, F., Emura, J., Motoyama, T., Ishiguro, M. and Nanno, S. (1973) Structure of α1-acid glycoprotein: complete amino acid sequence, multiple amino acid substitutions, and homology with the immunoglobulins. Biochemistry **12**, 2711–2724

44 Shelburne, S. A., Davenport, M. T., Keith, D. B. and Musser, J. M. (2008) The role of complex carbohydrate catabolism in the pathogenesis of invasive streptococci. Trends Microbiol. **16**, 318–325

45 Harvey, D. J., Merry, A. H., Royle, L., P Campbell, M., Dwek, R. A. and Rudd, P. M. (2009) Proposal for a standard system for drawing structural diagrams of N- and O-linked carbohydrates and related compounds. Proteomics **9**, 3796–3801

Received 23 January 2013/10 July 2013; accepted 19 July 2013
Published as BJ Immediate Publication 19 July 2013, doi:10.1042/BJ20130126

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

Biochem. J. (2013) **455**, 107–118 (Printed in Great Britain) doi:10.1042/BJ20130126

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

Jonathan SJÖGREN*[1,2] , Weston B. STRUWE†[3], Eoin F. J. COSGRAVE†[4], Pauline M. RUDD†, Martin STERVANDER‡, Maria ALLHORN*[2], Andrew HOLLANDS§||[2], Victor NIZET*||¶[2] and Mattias COLLIN*[2]

*Department of Clinical Sciences, Division of Infection Medicine, Lund University, Biomedical Center B14, SE-22184 Lund, Sweden, †The National Institute for Bioprocess Research and Training, NIBRT, University College Dublin, Fosters Avenue, Mount Merrion, Blackrock, Co. Dublin, Ireland, ‡Molecular Ecology and Evolution Laboratory, Department of Biology, Lund University, Ecology Building, SE-223 62 Lund, Sweden, §School of Biological Sciences, University of Wollongong, Wollongong, NSW 2522, Australia, ||Department of Pediatrics, University of California San Diego, 9500 Gilman Drive, Mail Code 0687, La Jolla, CA 92093, U.S.A., and ¶Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, 9500 Gilman Drive, Mail Code 0687, La Jolla, CA 92093, U.S.A.

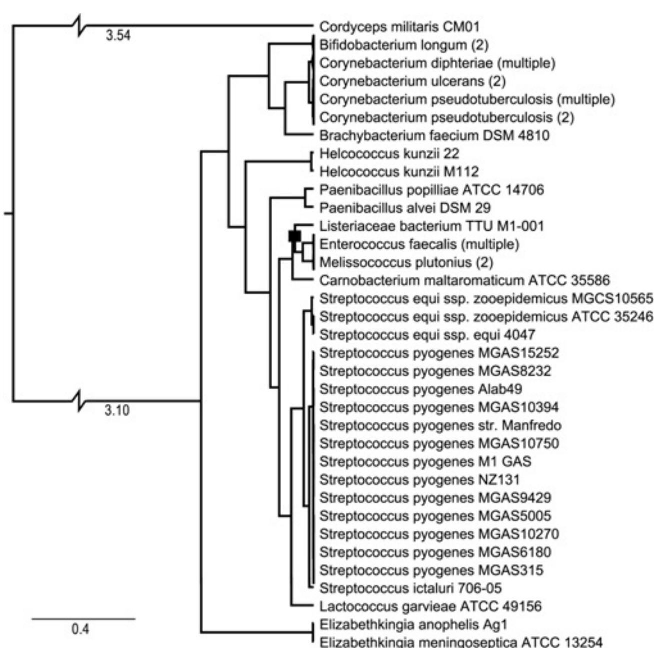

Figure S1 Phylogenetic reconstruction of 16S rRNA sequences, for inference of taxonomic relationship between taxa of the EndoS-like protein phylogeny, based on 1576 nucleotides

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.

[1] To whom correspondence should be addressed (email jonathan.sjogren@med.lu.se).
[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.
[3] Present address: Chemistry Research Laboratory, Department of Chemistry, University of Oxford, Oxford OX1 3TA, U.K.
[4] Present address: Waters Corporation, 34 Maple Street, Milford, MA 01757, U.S.A.

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).

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

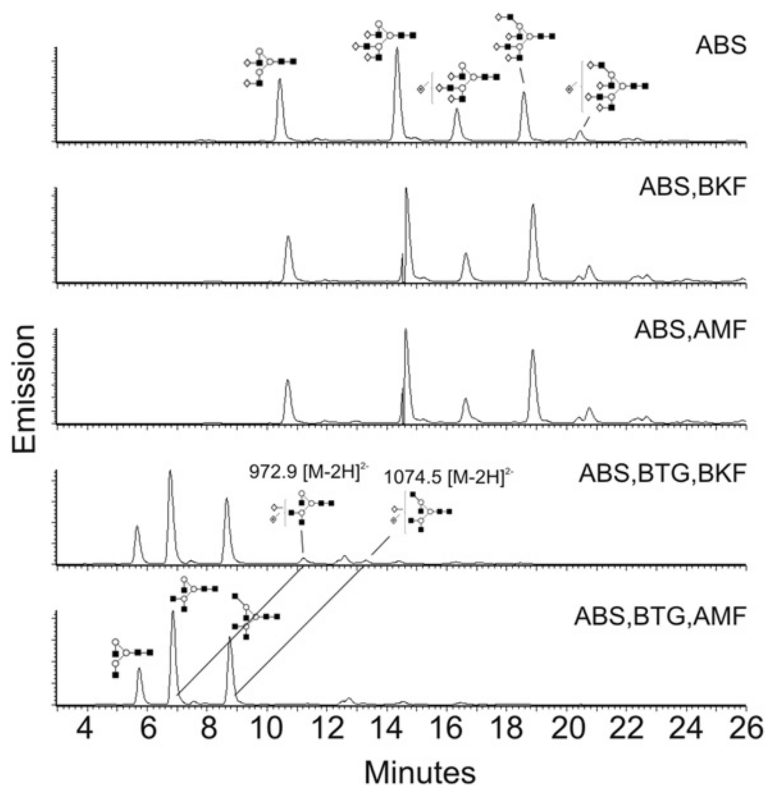

Figure S2 HILIC–FLD–MS and exoglycosidase sequencing of AGP N-glycans

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

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

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

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

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.

		GenBank® accession number	
Taxa	Strain	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

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

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	

REFERENCE

1 Collin, M. and Olsén, A. (2001) EndoS, a novel secreted protein from *Streptococcus pyogenes* with endoglycosidase activity on human IgG. EMBO J. **20**, 3046–3055

Received 23 January 2013/10 July 2013; accepted 19 July 2013
Published as BJ Immediate Publication 19 July 2013, doi:10.1042/BJ20130126

© 2013 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.