

EndoS, a novel secreted protein from *Streptococcus pyogenes* with endoglycosidase activity on human IgG

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***Streptococcus pyogenes* is an important human pathogen that selectively interacts with proteins involved in the humoral defense system, such as immunoglobulins and complement factors. In this report we show that *S. pyogenes* has the ability to hydrolyze the chitobiose core of the asparagine-linked glycan on immunoglobulin G (IgG) when bacteria are grown in the presence of human plasma. This activity is associated with the secretion of a novel 108 kDa protein denoted EndoS. EndoS has endoglycosidase activity on purified soluble IgG as well as IgG bound to the bacterial surface. EndoS is required for the activity on IgG, as an isogenic EndoS mutant could not hydrolyze the glycan on IgG. In addition, we show that the secreted streptococcal cysteine proteinase SpeB cleaves IgG in the hinge region in a papain-like manner. This is the first example of an endoglycosidase produced by a bacterial pathogen that selectively hydrolyzes human IgG, and reveals a novel mechanism which may contribute to *S. pyogenes* pathogenesis.**

Keywords: cysteine proteinase/endo- β -*N*-acetylglucosaminidase/endoglycosidase/IgG/*Streptococcus pyogenes*

Introduction

An enzyme expressed by *Streptococcus pyogenes* capable of releasing the terminal sialic acid residues from glycoproteins such as immunoglobulins was first described by Hayano and Tanaka (1967). Other studies have shown that this release of sialic acid from immunoglobulin G (IgG) is due to a neuraminidase activity produced by *S. pyogenes*, resulting in autoantigenic and nephritogenic properties in patients with glomerulonephritis (Mosquera *et al.*, 1985). Related endoglycosidases found in *Staphylococcus aureus* have been shown to be cluster-dispersing enzymes involved in cell separation (Sugai *et al.*, 1995), and glycosidases in *Streptococcus oralis* that sequentially deglycosylate N-linked glycoproteins promote growth of the bacterium (Byers *et al.*, 1999). It has also been suggested that a β -*N*-acetylglucosaminidase from *Streptococcus pneumoniae* contributes to pathogenicity, since N-linked sugar residues are common features of several cell-surface proteins in host tissue (Clarke *et al.*, 1995).

Oligosaccharide-cleaving enzymes from other species have been studied in detail, and some are standard tools in glycobiology research. For example, endoglycosidase H from *Streptomyces plicatus* (Trumbly *et al.*, 1985), and

endoglycosidases F₁, F₂ and F₃ from *Flavobacterium meningosepticum* (Plummer and Tarentino, 1991; Trimble and Tarentino, 1991) are commonly used. These enzymes all belong to family 18 of chitinases (Henrissat, 1991) and catalyze the hydrolysis of the β -1,4-*N*-acetyl-D-glucosamine linkages in the chitin core of N-linked oligosaccharides in glycoproteins. These enzymes are referred to as true endoglycosidases (Alexander and Elder, 1989; Tarentino and Plummer, 1994). However, a hydrolase isolated from *F. meningosepticum*, peptide-*N*-(*N*-acetyl- β -glucosaminyl)asparagine amidase (PNGase F), has activity on the pentasaccharide core region of asparagine-linked glycans, and is therefore referred to as a deglycosidase (Tarentino *et al.*, 1985).

IgG is a 150 kDa glycoprotein that is a key player in the human immune response. It is composed of two identical light chains and two identical heavy chains. The heavy chain comprises one variable domain (V_H) and three constant domains (C_H1–3), and the light chain consists of one variable domain (V_L) and one constant domain (C_L). The C_H1 and C_H2 domains are separated by a protease-sensitive hinge region. Papain, which generates the antigen-binding Fab fragments and the effector function-promoting Fc fragment, can cleave this region. The Fc region is the interaction site for a number of effector function-promoting molecules, including complement factor C1_q and Fc receptors on effector cells such as monocytes, macrophages, neutrophils and B cells (for a review see Burton, 1985). In the C_H2 domains, there is a conserved *N*-glycosylation site at Asn297. In human IgG, a complex biantennary oligosaccharide with a bisecting *N*-acetylglucosamine and a core fucose is attached at this site (Figure 1).

The functional relevance of this oligosaccharide has been implicated in several studies. For instance, murine IgG lacking the oligosaccharide does not activate complement, bind to Fc-receptors on macrophages, or induce antibody-dependent cellular cytotoxicity. Furthermore, such deglycosylated IgG impairs the elimination of antibody-antigen complexes from the circulation (Nose and Wigzell, 1983). It has also been shown that aberrant IgG glycosylation is associated with a number of autoimmune disorders, such as rheumatoid arthritis (RA) and Crohn's disease (Dube *et al.*, 1990). Agalactosyl IgG autoantibodies have also been demonstrated to be pathogenic in a murine model of collagen-induced arthritis (Rademacher *et al.*, 1994; for a review see Rahman and Isenberg, 1996).

Streptococcus pyogenes is a significant human pathogen that causes infections such as impetigo, scarlatina and pharyngitis, as well as severe invasive diseases such as necrotizing fasciitis and sepsis (Bisno and Stevens, 1996; Cunningham, 2000). Non-suppurative sequelae include glomerulonephritis and acute rheumatic fever with heart

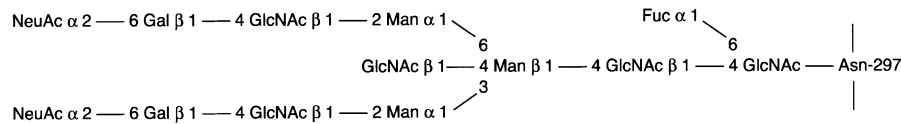


Fig. 1. Structure of the fully substituted complex biantennary type oligosaccharide attached to Asn297 of the IgG γ -chain. GlcNAc, *N*-acetylglucosamine; Fuc, fucose; Man, mannose; Gal, galactose; NeuAc, sialic acid.

complications. *Streptococcus pyogenes* expresses a number of proteins involved in virulence and manifestations of infection. One important protein is the secreted streptococcal cysteine proteinase, also known as the erythrogenic toxin B SpeB (Gerlach *et al.*, 1983). SpeB degrades several host proteins including vitronectin, fibronectin (Kapur *et al.*, 1993), fibrin and fibrinogen (Elliott, 1945; Matsuka *et al.*, 1999). In addition, SpeB releases pro-inflammatory kinins from kininogens (Herwald *et al.*, 1996). SpeB can also release biologically active fragments from the streptococcal surface, such as M protein, protein H and C5a peptidase (Elliott, 1945; Berge and Björck, 1995). The role of SpeB as an important virulence factor in *S.pyogenes* pathogenesis has been established using *in vivo* infection models (Lukomski *et al.*, 1997; Tsai *et al.*, 1998).

Other significant virulence factors of *S.pyogenes* are the cell wall-anchored M and M-like proteins. These are surface-exposed, dimeric, coiled-coil, α -helical structures associated with the binding of numerous plasma proteins, such as fibrinogen, fibronectin, plasminogen, immunoglobulins and human albumin (Whitnack and Beachey, 1982; Berge and Sjöbring, 1993; Cleary and Retnoningrum, 1994; Retnoningrum *et al.*, 1993; Frick *et al.*, 1995). In addition to expressing M1 protein, the AP1 strain of M1 serotype used in this study expresses the M-like protein H (Gomi *et al.*, 1990). This protein binds with high affinity to the Fc region of human IgG (Åkesson *et al.*, 1990) and has been shown to be important for survival in human blood (Kihlberg *et al.*, 1999).

In this report we describe a novel secreted endoglycosidase in *S.pyogenes*, EndoS, which specifically hydrolyzes the β -1,4-di-*N*-acetylchitobiose core of the asparagine-linked glycan of human IgG. Furthermore, we show that the secreted SpeB cleaves the hinge region of IgG in a papain-like manner and thus acts as an IgG protease. This synergistic action of EndoS and SpeB could influence the pathogenicity of *S.pyogenes* during acute infection as well as in sequelae such as acute post-streptococcal glomerulonephritis and rheumatic fever.

Results

Identification of a secreted protein in *S.pyogenes* with similarity to an endoglycosidase

When a group A streptococcal strain of M1 serotype, AP1, was grown in C-medium (CM) and the secreted proteins analyzed by SDS-PAGE, a prominent protein was observed in the culture supernatant (Figure 2, lane A). No band at a similar molecular weight was present in the EndoS-mutant strain MC14 (Figure 2, lane B; see below). This protein band was excised and sequenced by Edman degradation, revealing the amino acid sequence EEKTVQVQ. This sequence was used to search the

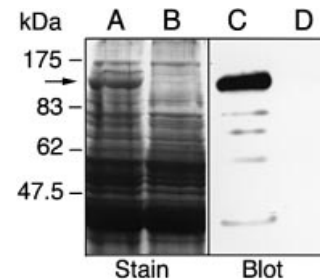


Fig. 2. Identification of a prominent, high molecular weight, secreted protein from *S.pyogenes* (indicated by an arrow). Proteins present in bacterial culture supernatants were separated by two identical 10% SDS-PAGE, one stained with Coomassie Blue (Stain), the other blotted to a membrane and detected with polyclonal rabbit anti-EndoS antiserum (Blot). Lanes A and C, AP1 (wild type); lanes B and D, MC14 (EndoS⁻).

Streptococcal Genome Sequencing Project (SGSP) database (Ferretti *et al.*, 2001; Roe *et al.*, 2001) for similarities using the tBLASTn algorithm (Altschul *et al.*, 1990). A perfect match (8/8) was found in an open reading frame (ORF) of 995 amino acids (Figure 3). The putative protein encoded by this ORF has a predicted size of 108 kDa after removal of a potential signal peptide. This putative protein was then used to search the DDBJ/EMBL/GenBank databases using the BLASTp algorithm, which resulted in similarities with the endo- β -*N*-acetylglucosaminidase F₂ (EndoF₂) precursor from *F.meningosepticum* (Tarentino *et al.*, 1993). The similarity was confined to the first 444 amino acids of the query sequence, where amino acids 227–235 constitute a perfect chitinase family 18 active site, ending with a glutamic acid (Figure 3). The glutamic acid in the active site of chitinases is known to be essential for enzymatic activity (Watanabe *et al.*, 1993).

Based on this information, the protein was denoted EndoS, for endo- β -*N*-acetylglucosaminidase of streptococci, and the gene *ndoS*. The *ndoS* gene from strain AP1 was cloned and sequenced. It was 99% identical to *ndoS* in SF370, the strain of M1 serotype used by SGSP. The deduced amino acid sequence was also 99% identical to EndoS from SF370. These sequence data have been submitted to DDBJ/EMBL/GenBank under accession No. AF296340. This suggests that *S.pyogenes* bacteria are able to secrete a 108 kDa protein with similarities to endo- β -*N*-acetylglucosaminidases.

Generation of MC14, an isogenic mutant strain lacking EndoS expression

To investigate the properties of secreted EndoS further, an isogenic strain mutated in the *ndoS* gene of AP1 was constructed using an insertion–duplication strategy. An internal 534 bp fragment from the *ndoS* gene covering a part of the ORF ending upstream of the putative enzymatic

EndoS	1	MDKHLLVKRTLGCVCAATLMGAALATHHDSLNTVKA <u>EEKTVQVQ</u> KGLPSI	50
EndoF2	1	MKTANFSFALCLS	13
		KT . L	
EndoS	51	DSLHYLSENSKKEFKKEELSKAGQESQKVKEILAKAQQADKQAQELAKMKI	100
EndoF2	14	VVIMLFIKCTRSEQDLSVTKDAIAQ-----KSGVTVSAVNL	49
		. . . E . . K K	
EndoS	101	PEKIPMKPLHGPLYGGYFRTWHDKTSDPTEKDKVNSMGELPKEVDLAFIF	150
EndoF2	50	SNLIAYKNSDHQISAGYYRTWRDSATASG---NLPMSRWLPDSLDMVMVF	96
		I K . GY.RTW.D . . . SM LP .D. .F	
EndoS	151	HDWTKDYSLFWKELATKHVPKLNKQGTRVIRTIPWRFLAGGDNSGIAEDT	200
EndoF2	97	PDYTPPENAYWNTLKTNYVPYLHKRGTKVIITL-----GDLN---SAT	136
		D T .W L T VP L.K.GT.VI T. GD T	
		*	
EndoS	201	SKYPNTPEGNKALAKAIVDEYVYKYN LDGLDVDVE HDSIPKVDKKEDTAG	250
EndoF2	137	TTGGQDSIGYSSWAKGIYDKWVGEYN LDGIDIDIE --SSPSG-----AT	178
		. . G . AK I D V YN LDG.D.D.E S P A	
EndoS	251	VERSIQVFEEIGKLIQPKGVDKSRLFIMDSTYMADKNPLIERGAPYINLL	300
EndoF2	179	LTKFVAATKALSKYFGPK-SGTGKTFVYDTNQNPN--TNFFIQTAPRYNYV	225
	 K GPK . F. D. . . . AP N .	
EndoS	301	LVQVYGSQGEKGGWEPVSNRPEKTMEERWQGYSKYIRPEQYMIGFSFYEE	350
EndoF2	226	FLQAYG-----RSTTNLTTVSGLYAPYISMKQFLPGFSFYEE	262
		.Q YG R . . Y. YI Q. . GFSFYEE	
EndoS	351	NAQEGNLWYDINSRKDEDKANGINTDITGTRAERYARWQPKTGGVKGKGF	400
EndoF2	263	NGYPGNYWNDVRYQP-----NGTG-----RAYDYARWQPATG-KKGGVF	300
		N GN W D. . NG . RA YARWQP TG KGG.F	
EndoS	401	SYAIDRDGVAHQPKKYAKQKEFKDATDNIFHS-DYSVSKALKTVMLKDKS	449
EndoF2	301	SYAIERDAPLTS-----SNDNTLRAPNFRVTKDLIKIMN----	334
		SYAI.RD . . DN . . . V.K L .M	
EndoS	450	YDLIDEKDFDPKALREAVMAQVGTRKGDLERFNGTLRLDNPAIQSLEGLN	499
EndoF2	335	-----P	335
		P	

Fig. 3. EndoS is similar to EndoF₂. Sequence alignment of EndoS from *S.pyogenes* strain AP1 and EndoF₂ from *F.meningosepticum*. The N-terminal sequence of the protein band isolated from AP1 culture supernatant is underlined (EEKTVQVQ). The chitinase motif is indicated in bold (LDGLDVDVE) and the essential glutamic acid for activity is marked with an asterisk. The consensus sequence is shown under the alignment and similarities are indicated with dots.

motif was ligated into the streptococcal shuttle vector pFW13 (Podbielski *et al.*, 1996). This resulted in plasmid pMC11. pMC11 was electroporated into AP1 bacteria, and one mutant clone, denoted MC14, was chosen for further characterization (Figure 4A). PCR experiments on chromosomal DNA from AP1 and MC14 bacteria with vector- and *ndoS*-derived primers were used to verify the integration of pMC11 into the chromosome of MC14. This revealed that both a 558 and a 1010 bp product could be amplified using chromosomal DNA from MC14, but not from wild-type AP1 (Figure 4B), thus confirming disruption of the *ndoS* gene.

The mutation in MC14 did not affect the generation time, and the bacteria reached the same cell density as cultures of wild-type bacteria (data not shown). Secretion of EndoS could also be confirmed using a rabbit antiserum against full-length EndoS purified by preparative

SDS-PAGE. Secretion of EndoS was analyzed by western blotting using this EndoS-specific antiserum. Reactivity against a 108 kDa protein and some minor degradation products was apparent in the supernatant from AP1 bacteria (Figure 2, lane C). In contrast, no reactivity was detected in MC14 (Figure 2, lane D). This shows that the disruption of the *ndoS* gene completely abolished secretion of EndoS.

***Streptococcus pyogenes* has glycosidase activity on human IgG**

In other bacteria, such as *S.oralis*, it has been shown that endoglycosidases sequentially hydrolyze N-linked glycoproteins for nutritional purposes (Byers *et al.*, 1999). Therefore, we hypothesized that EndoS in *S.pyogenes* could act in a similar manner and release glycans. Wild-type and EndoS-mutant bacteria were inoculated into poor

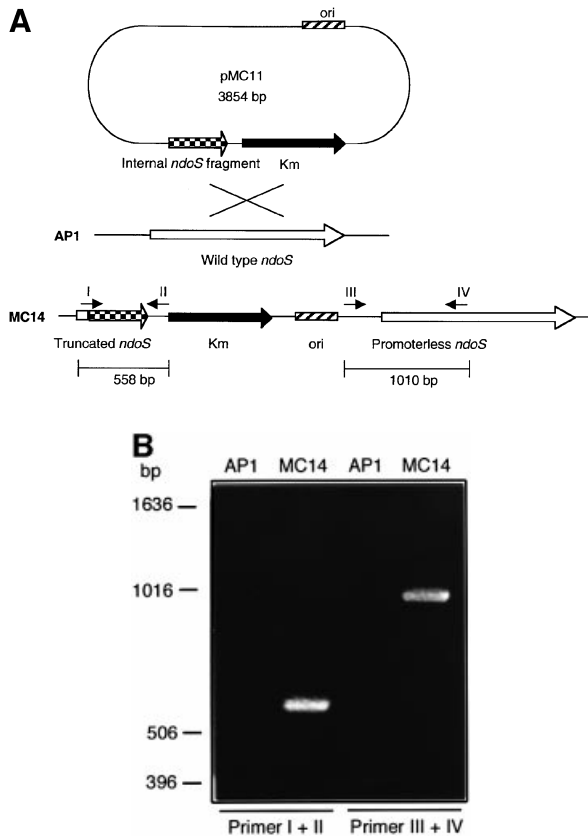


Fig. 4. (A) A schematic drawing of the strategy for mutagenesis of the *ndoS* gene in AP1 bacteria. An internal 534 bp fragment from the *ndoS* gene was cloned in the suicide plasmid pFW13, generating pMC11. This plasmid was electroporated into AP1 for homologous recombination. (B) PCR analysis of purified chromosomal DNA from AP1 and MC14. The vector- and *ndoS*-derived primers used are indicated with roman numerals I–IV in (A) and (B).

growth medium, diluted CM (12.5%), which did not promote bacterial growth unless 1% human plasma was added. Bacterial growth was monitored over time, and no significant difference in growth of the two strains was apparent (data not shown). This indicated that EndoS does not play a major role in nutrient acquisition, at least under these experimental conditions.

Since EndoS has similarities to endoglycosidases, we analyzed culture supernatants for degradation products of plasma proteins after bacterial growth. The plasma proteins in culture supernatants were separated by SDS-PAGE. This revealed some apparent differences in the pattern of plasma proteins in the sample from wild-type compared with EndoS-mutant bacteria. A distinct difference was observed in the culture grown in the absence of EndoS (MC14), where a band of ~31 kDa was detected (Figure 5, lane B). Interestingly, this 31 kDa band could not be detected in the presence of EndoS (AP1), where instead a distinct band of ~27 kDa was observed (Figure 5, lane A). The 31 kDa protein was isolated and subjected to N-terminal sequencing. The identified sequence, GPS-VFLFPP, was 100% identical to amino acids 236–244 in the hinge region of the IgG heavy chain (γ). Neither of these two bands was detected in the medium control

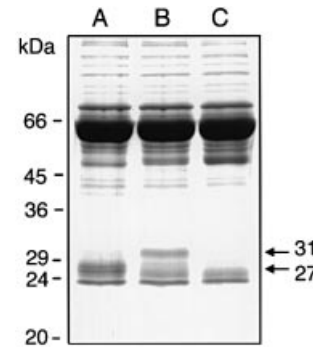


Fig. 5. EndoS has glycosidase activity on IgG in human plasma. *Streptococcus pyogenes* AP1 and MC14 were grown in diluted CM (12.5%) supplemented with 1% human plasma. After overnight growth, culture supernatants were analyzed by 10% SDS-PAGE. Lane A, proteins from growth with AP1 (wild type); lane B, proteins from growth with MC14 (EndoS⁻); lane C, medium alone. The 27 and the 31 kDa fragments of the γ -chains are indicated with arrows on the right.

(Figure 5, lane C). Interestingly, the cleavage site where papain and SpeB cleave human IgG differs in that papain cleaves at position 224 in human IgG₁ (Burton, 1985), while SpeB cleaves between Gly236 and Gly237.

The size difference between the 31 and the 27 kDa bands (Figure 5) most likely represents γ -chains with or without the complex biantennary oligosaccharide that results from inactivation of the *ndoS* gene in MC14. Plasma IgG contains a range of sugars in the Fc portion (Parekh *et al.*, 1985); the largest attached to the γ -chain at Asn297 is shown in Figure 1. This experiment suggested that the glycan moiety on human IgG present in the growth medium is hydrolyzed by EndoS. Furthermore, a proteolytic cleavage of the hinge region must have occurred in order to generate a Fc fragment. This suggested a protease present in the culture supernatant of both AP1- and MC14-cleaved IgG. A likely candidate for this activity was the streptococcal cysteine proteinase SpeB.

SpeB cleaves IgG in the hinge region and EndoS hydrolyzes the glycan on IgG

Our finding that *S.pyogenes* bacteria can both hydrolyze the glycan moiety and proteolytically cleave IgG in the γ -chain is novel. *Streptococcus pyogenes* secretes a cysteine proteinase, SpeB. Recently, the three-dimensional structure of SpeB was solved, and this revealed that it is related in three-dimensional structure to papain (Kagawa *et al.*, 2000). As it is also well established that papain cleaves human IgG into two identical Fab (antigen binding) fragments and an Fc fragment (Porter, 1973), we investigated the ability of SpeB to cleave the hinge region of IgG. Thus, an isogenic mutant strain, AL1, devoid of SpeB expression was constructed.

Purified human IgG was incubated with culture supernatant from wild-type (AP1), EndoS-mutant (MC14) and SpeB-mutant bacteria (AL1), in the absence or presence of the reducing agent dithiothreitol (DTT), and analyzed by SDS-PAGE. The rationale for using a reducing agent is that the activity of SpeB depends on reducing conditions (Liu *et al.*, 1963). Analysis of IgG incubated in the presence of SpeB but in the absence of EndoS under non-reducing conditions (–DTT) revealed a 50 kDa band consistent with fully glycosylated γ -chains and a 25 kDa

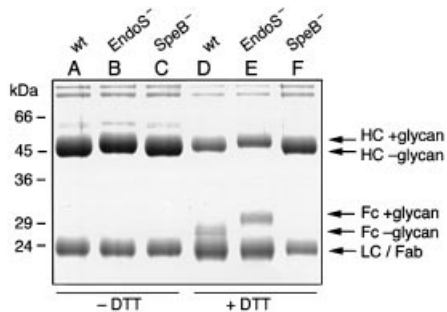


Fig. 6. Removal of Fc glycans by EndoS and cleavage of the hinge region by SpeB. SDS-PAGE analysis of purified human IgG incubated with supernatants from API (wt), MC14 (EndoS⁻) and AL1 (SpeB⁻). Samples were incubated under non-reducing (-DTT) or reducing (+DTT) conditions, as indicated. Lanes A and D, wild type (wt); lanes B and E, EndoS⁻; lanes C and F, SpeB⁻. Arrows on the right indicate the position of heavy chains with glycan (HC +glycan) or without glycan (HC -glycan), Fc fragment with glycan (Fc +glycan) or without glycan (Fc -glycan), light chains (LC) and Fab fragment of heavy chains (Fab).

band representing the light chains (Figure 6, lane B). Incubation with EndoS, both in the presence and absence of SpeB, led to a shift of ~4 kDa in the size of the γ -chains (Figure 6, lanes A and C). This suggested that the glycan moiety on Asn297 was removed.

However, incubation of purified human IgG with culture supernatant containing both EndoS and SpeB under reducing conditions (+DTT) resulted in partial processing of the γ -chains into a band at 27 kDa (Figure 6, lane D). This corresponds to proteolytically processed γ -chains without the glycan. Furthermore, incubation without EndoS in the presence of SpeB results in a partial processing of the heavy chains into a 31 kDa band (Figure 6, lane E). This most likely represents the Fc fragment of the γ -chains with the glycan still attached. It should also be noted that when incubating in the presence of SpeB under reducing conditions, the generated γ -chain Fab fragment migrated at approximately the same molecular weight as the light chains (Figure 6, lanes D and E). However, in the absence of SpeB (Figure 6, lane F), no proteolytic cleavage of the γ -chains could be detected even under reducing conditions. Together, these data demonstrate that SpeB cleaves IgG in the hinge region and that EndoS hydrolyzes the γ -chains of human IgG.

EndoS cleaves the chitobiose core of the glycan on human IgG

To confirm that the size alteration of IgG was caused by EndoS activity and resulted from removal of the glycan moiety on γ -chains rather than proteolytic degradation, three experiments were set up. In a first approach, a lectin blot analysis was performed. Culture supernatants from wild-type API and EndoS-mutant MC14 bacteria were incubated with purified IgG and separated by SDS-PAGE. Proteins were then analyzed by staining or using a lectin from *Galanthus nivalis* (GNL) that preferentially recognizes α -1,3 mannose residues found in the biantennary glycan on γ -chains (Figure 1). In the stained gel, a clear difference in the size of the γ -chains was observed in the sample treated with EndoS compared with non-treated IgG (Figure 7A, lanes A and B). Lectin blot analysis of the

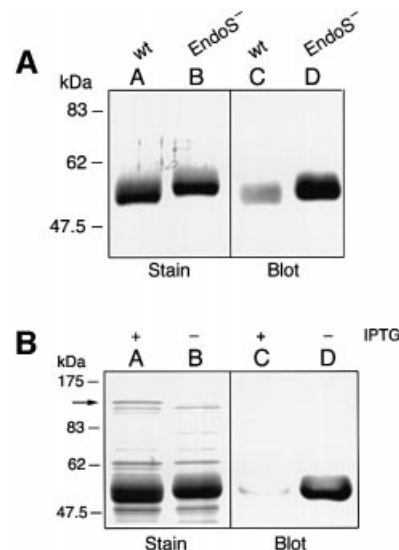


Fig. 7. SDS-PAGE and lectin blot analysis of IgG incubated with and without streptococcal or recombinant EndoS and separated by 10% SDS-PAGE. Gels were analyzed by Coomassie Blue staining (Stain) or by blotting to a membrane that was probed with GNL lectin (Blot). (A) Lanes A and C, IgG incubated with streptococcal EndoS; lanes B and D, IgG incubated without EndoS. (B) IgG incubated with extracts from *E.coli* expressing EndoS. Lanes A and C, induced cells (+IPTG); lanes B and D, non-induced cells (-IPTG). The arrow on the left indicates the position of recombinant EndoS.

same samples with the GNL lectin revealed a significantly reduced signal when incubated with EndoS (Figure 7A, lane C). In contrast, the γ -chains were still glycosylated when incubated in the absence of EndoS (Figure 7A, lane D). These data support the proposal that EndoS has the ability to remove structures containing α -1,3 mannose from the γ -chains of human IgG.

In a second approach, cloning and expressing the gene encoding EndoS in *Escherichia coli* using pET-30a Xa/LIC expression system provided additional evidence that EndoS has glycosidase activity. Partially purified extracts from isopropyl- β -D-thiogalactopyranoside (IPTG)-induced or non-induced cells were incubated with purified IgG, and analyzed by SDS-PAGE and lectin blotting. This revealed that only the extract prepared from induced cells had the ability to alter the size of the γ -chains (Figure 7B, lanes A and B). Furthermore, analysis using the GNL lectin revealed that only the induced cell preparation had glycosidase activity on IgG (Figure 7B, lanes C and D).

In a third approach, we confirmed that EndoS is an endoglycosidase that cleaves the chitobiose core of human IgG, using a lectin from *Ulex europaeus* I (UEA-I). UEA-I recognizes α -linked fucose-containing oligosaccharides. Thus, we cleaved human IgG with PNGase F, a highly specific deglycosidase that cleaves the bond between asparagine and the innermost *N*-acetylglucosamine of the glycan (Tarentino *et al.*, 1985). Purified human IgG was treated with either EndoS or PNGase F, followed by separation by SDS-PAGE, or blotting and probing with the UEA-I lectin in blot analysis. In the stained gel, a clear difference in the size of the γ -chains was observed in the sample treated with EndoS compared with non-treated IgG (Figure 8, lanes A and B). Furthermore, a decrease in size was also apparent after PNGase F treatment (Figure 8, lane C). The lectin blot revealed a signal of equal intensity

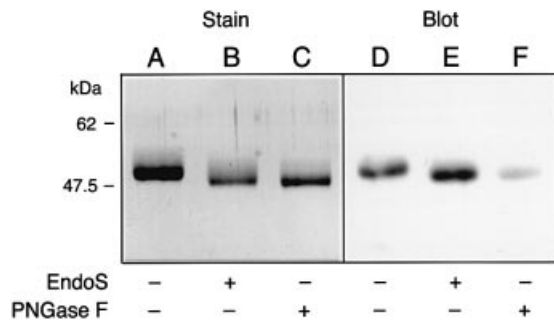


Fig. 8. EndoS is an endoglycosidase. Purified human IgG was incubated with either EndoS or PNGase F (PNGase F), separated by 10% SDS-PAGE. Gels were analyzed by Coomassie Blue staining (Stain) or by blotting to a membrane that was probed with UEA-I lectin (Blot). Lanes A and D, untreated IgG; lanes B and E, EndoS-treated IgG; lanes C and F, PNGase F-treated IgG.

when the UEA-I lectin bound to untreated or EndoS-treated IgG, suggesting that the fucose group was still attached to the latter (Figure 8, lanes D and E). However, when IgG was incubated with PNGase F, which removes the entire glycan moiety, the UEA-I lectin gave a significantly lower signal (Figure 8, lane F). This suggests that EndoS cleaves the chitobiose core of human IgG, leaving an *N*-acetylglucosamine with an α -linked fucose. Finally, the α -linked residue's presence on the glycan moiety did not influence EndoS activity, as analyzed by α -fucosidase treatment prior to incubation with EndoS (data not shown).

Thus, partially purified recombinant EndoS has the capacity to hydrolyze human IgG to the same extent as streptococcal culture supernatants containing EndoS. Taken together, these data clearly show that *S.pyogenes* is able to secrete an enzyme, EndoS, with endoglycosidase activity on the γ -chains of human IgG.

EndoS has endoglycosidase activity on IgG when bound to the bacterial surface

Wild-type AP1 bacteria express the cell wall-anchored protein H, which binds human IgG with high affinity in the interface between C_H2 and C_H3 (Åkesson *et al.*, 1990; Frick *et al.*, 1992). We thus addressed whether EndoS affected protein H binding to IgG. This was analyzed by incubating purified IgG in the presence or absence of EndoS, and analyzing the ability of protein H to bind IgG in a ligand blot experiment. Treated IgG was applied to a membrane in serial dilutions and probed with radiolabeled, purified protein H. This revealed that protein H bound IgG to the same extent irrespective of EndoS treatment and indicated that the Fc-binding capacity of protein H is not influenced by EndoS (Figure 8A). This was also the case for staphylococcal protein A, which bound to both intact and EndoS-treated IgG (data not shown), as previously described for aglycosyl mouse IgG (Leatherbarrow and Dwek, 1983; Nose and Wigzell, 1983).

AP1 bacteria have the capacity to absorb IgG on their surface via protein H. We thus asked whether IgG bound to the surface of AP1 could be hydrolyzed by EndoS. Purified IgG was absorbed to the surface of washed AP1 bacteria grown in THY. The rationale for using THY is to suppress SpeB activity (Elliott, 1945), which could otherwise

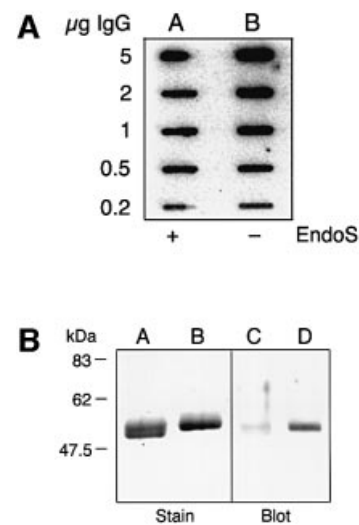


Fig. 9. (A) Ligand blot analysis of protein H binding to glycosylated or EndoS-treated IgG. Purified IgG was incubated with culture supernatant with or without EndoS, applied to a membrane in serial dilutions, and probed with ¹²⁵I-labeled protein H. Lane A, IgG incubated with EndoS; lane B, IgG incubated without EndoS. (B) EndoS has endoglycosidase activity on IgG when bound to the bacterial surface. SDS-PAGE and GNL lectin blot analysis of IgG, bound to the bacterial surface via protein H, treated with or without EndoS prior to elution at low pH. Lanes A and C, surface-bound IgG incubated in the presence of EndoS; lanes B and D, surface-bound IgG incubated without EndoS.

remove protein H from the cell wall (Berge and Björck, 1995). Unabsorbed IgG was washed away, followed by incubation with or without EndoS. After incubation, the bacteria were carefully washed and bound IgG was eluted at low pH. The IgG was then analyzed by 10% SDS-PAGE and by lectin blot analysis. As observed previously with soluble IgG (Figures 5 and 6), the γ -chains eluted from bacteria incubated in the presence of EndoS migrated as a protein band ~4 kDa smaller than the γ -chains eluted from bacteria incubated in the absence of EndoS (Figure 9B, lanes A and B). Furthermore, lectin blot analysis of the same IgG samples revealed that γ -chains incubated in the presence of EndoS were almost completely hydrolyzed. In contrast, the lectin reacted strongly with the γ -chains incubated in the absence of EndoS (Figure 9B, lanes C and D). Thus, EndoS has endoglycosidase activity on the γ -chains of IgG when bound to the bacterial surface as well as in solution.

Discussion

In this report we show that the human pathogen *S.pyogenes* secretes a novel enzyme, EndoS, which removes the glycan moiety from the γ -chains of human IgG. EndoS has similarities to endo- β -*N*-acetylglucosaminidases that cleave the β 1-4 linkage between the two *N*-acetylglucosamines found in the core of the *N*-linked glycan of IgG. Furthermore, we show that the streptococcal cysteine proteinase SpeB specifically cleaves in the hinge region of IgG. By utilizing dilute growth medium with small amounts of plasma and an isogenic EndoS-mutant strain, endoglycosidase activities on IgG by EndoS as well as the papain-like cleavage of IgG by SpeB were detected. This probably could not have been achieved by analyzing purified native or recombinantly expressed EndoS.

Thus, these two enzymes act synergistically as an IgG endoglycosidase and IgG protease. This is the first described mechanism where *S.pyogenes* can both cleave and alter the glycan on one of the most significant molecules in the human immune system, IgG.

IgG in human serum appears in different glycoforms. This means that the glycan moiety terminates with different carbohydrate structures, and these various forms are important in the development of disease. For example, in the so-called agalactosyl IgG glycoforms, the glycan moiety terminates with two *N*-acetylglucosamine residues, instead of the fully substituted oligosaccharide with two galactose residues preceding two terminating sialic acids (Figure 1). This agalactosyl form is often found in patients with rheumatoid arthritis (RA) (Parekh *et al.*, 1985), Crohn's disease (Dube *et al.*, 1990), mycobacterial infections and systemic lupus erythematosus (SLE) with Sjögren's syndrome (Parekh *et al.*, 1989). The production of an agalactosyl form of IgG in RA patients, for example, is a result of impaired glycosyltransferase activity in their B cells (Axford *et al.*, 1987). Taken together, numerous observations have shown that the so-called agalactosyl form of IgG plays a role in various human diseases, of which several involve autoimmunity. Aseptic sequelae of *S.pyogenes* infections are also associated with autoimmunity where the glycosylation state of IgG may be of importance.

Hayano and Tanaka (1967) were the first to describe a neuraminidase that altered immunoglobulins in patients suffering from glomerulonephritis caused by this bacterium. In another report, McIntosh *et al.* (1972) suggested that a streptococcal neuraminidase activity produced by these strains removed the sialic acid from IgG. This, in turn, led to autoantigenicity and thereby gave rise to nephritogenicity. Thus, the neuraminidase activity augmented the pathological effect seen among patients with glomerulonephritis. Acute post-streptococcal glomerulonephritis is known to be a sequelae to infections by *S.pyogenes* of several M serotypes, including M1 (Bisno, 1991). This may be a result of delayed clearance of immune complexes formed by deglycosylated IgG from the circulation (Nose and Wigzell, 1983). Thus, EndoS endoglycosidase activity on IgG molecules could possibly enhance the formation of immune complexes that subsequently deposit in the kidney. EndoS can therefore be a factor that contributes to the onset of glomerulonephritis caused by these bacteria.

The API strain used in this study is unique because it expresses protein H, an effective binder of human IgG. API also secretes the cysteine proteinase SpeB, which degrades matrix and plasma proteins (for a review see Musser, 1997). It also releases biologically active fragments of proteins from the bacterial surface, such as protein H (Berge and Björck, 1995). The released protein H can form soluble complexes with IgG that activate complement distant from the bacterium (Berge *et al.*, 1997).

In addition, here we report yet another factor that contributes to the complex pathogenicity of this microorganism, namely the expression of EndoS, an enzyme capable of cleaving the glycan moiety of human IgG. EndoS may function to fine-tune the number of 'functional' IgG molecules, both in circulation and when bound

in an immune way via the Fab fragment at the bacterial surface. Thus, secreted EndoS may hydrolyze and neutralize IgG molecules that are bound to the bacteria, and impair Fc-mediated effector functions of IgG, such as complement binding and activation or opsonization.

The ability of the streptococcal cysteine proteinase SpeB to cleave IgG γ -chains in the hinge region in a papain-like manner adds a new activity for this enzyme, and to the extensive list of its biological functions. IgG-degrading activity by bacterial enzymes has previously been described for the human pathogen *Pseudomonas aeruginosa*, where an elastase cleaves both human and mouse IgG (Holder and Wheeler, 1984). In addition, the causative agents of chronic adult periodontitis, *Prevotella intermedia* and *Prevotella nigrescens*, have a cysteine proteinase that non-specifically degrades IgG (Jansen *et al.*, 1995). In contrast, specific IgA protease activity is a well established feature of *S.pneumoniae* and *Haemophilus influenzae* (Mulks *et al.*, 1980) as well as oral streptococci (Kilian and Holmgren, 1981). However, the specific IgG protease activity of SpeB that mimics papain cleavage, where the γ -chain is cleaved in the hinge region into two distinct fragments, presents an additional example of how a bacterial pathogen has evolved a mechanism to interfere with immune response molecules such as IgG.

The activity of EndoS is specific for IgG in that neither human IgA nor IgM was affected by EndoS (our unpublished observations). In human serum IgM, there are five N-linked glycosylation sites on the μ -chains, but the precise details of the oligosaccharide structure have only been solved for parts of the IgM molecule (Wormald *et al.*, 1991). Human serum IgA₁ has two N-linked glycosylation sites, on the α -chain of which the attached oligosaccharides are highly sialylated and galactosylated (Baenziger and Kornfeld, 1974).

The ability of EndoS to act on other plasma or matrix proteins has not yet been fully investigated. However, EndoS has no activity on the highly N-linked plasma glycoprotein α_1 -acid glycoprotein (data not shown). This is in contrast to the activity described for endoglycosidases of *S.oralis* (Byers *et al.*, 1999). Furthermore, a cluster-dispersing endoglycosidase from *S.aureus* affects the growth morphology of the bacteria. However, an electron microscope examination of wild-type and EndoS-mutant bacteria did not reveal any alteration of shape or dispersal of the classical chain-forming growth morphology of the bacteria (data not shown).

In summary, our data reveal the first example of a human pathogen that secretes an enzyme, EndoS, that specifically acts as an endoglycosidase of human IgG both at the bacterial surface and in solution. This, together with the finding that SpeB has IgG-protease activity, adds important new aspects to the molecular pathogenesis of *S.pyogenes* that could be relevant both during acute infection and in aseptic sequelae.

Materials and methods

Growth conditions

Bacterial strains and plasmids used in this study are described in Table I. For maximal EndoS expression, CM was used. CM consists of 0.5% (w/v) Proteose Peptone No. 2 (Difco, Detroit, MI) and 1.5% (w/v) yeast extract

Table I. Bacteria and plasmids used in this study

Strain or plasmid	Relevant genotype	Characteristics	References
Bacteria			
<i>Streptococcus pyogenes</i>			
API	wild type	M1 serotype, IgG-binding via protein H	WHO Prague collection ^a
MC14	<i>ndoS</i> ::Km	<i>ndoS</i> inactivated	this study
AL1	<i>speB</i> ::Km	<i>speB</i> inactivated	this study
<i>Escherichia coli</i>			
DH5 α	<i>recA1 endA1 hsdR17</i>	cloning strain	BRL ^b
BI21(DE3)pLysS	F ⁻ <i>ompT hsdS_B</i>	cloning strain	Novagen ^c
Plasmids			
pFW13	<i>kan</i> ^R	streptococcal shuttle vector	Podbielski <i>et al.</i> (1996)
pMC11	<i>kan</i> ^R	plasmid construct to inactivate <i>ndoS</i>	this study
pJRS233	<i>erm</i> ^R	temperature-sensitive shuttle vector	Perez-Casal <i>et al.</i> (1993)
pJRS233-kana- <i>speB</i>	<i>kan</i> ^R , <i>erm</i> ^R , <i>speB</i> ::Km	plasmid construct to inactivate <i>speB</i>	Svensson <i>et al.</i> (2000)
pET-30a Xa/LIC	<i>kan</i> ^R	ligation-independent expression vector	Novagen ^c
pET Δ EndoS	<i>kan</i> ^R	recombinant EndoS clone	this study

^aWHO Collaborating Center for Reference and Research on Streptococci, Institute of Hygiene and Epidemiology, Prague, Czech Republic.

^bGibco-Bethesda Research Laboratories Inc., Gaithersburg, MD.

^cNovagen, Madison, WI.

(Oxoid, Basingstoke, UK) dissolved in CM buffer (10 mM K₂PO₄, 0.4 mM MgSO₄, 17 mM NaCl pH 7.5) (Gerlach *et al.*, 1983). To make a poor CM, it was diluted with CM buffer to 12.5% of the original concentration. All culture supernatants were centrifuged at 3800 g for 10 min and sterile-filtered through a 0.22 μ m membrane (Millipore, Bedford, MA) prior to use. For absorption experiments, streptococci were cultured in Todd-Hewitt broth (Difco) supplemented with 0.2% yeast extract (THY) at 37°C in a 5% CO₂ atmosphere. *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth at 37°C. When appropriate, 50, 150 or 200 μ g/ml kanamycin was added to cultures of *E. coli* carrying pFW13 and *S. pyogenes* mutant strains MC14 or AL1, respectively. Preparation of plasmid DNA from *E. coli* strains was carried out using a standard rapid alkaline extraction method (Birnbiom and Doly, 1979). Chromosomal DNA from *S. pyogenes* was prepared according to Pitcher *et al.* (1989) with some minor modifications, including the addition of 1000 U/ml mutanolysin (Sigma, St Louis, MO) in the lysis buffer.

Generation of an EndoS-specific antiserum

A culture supernatant from API grown in CM was precipitated with 5% trichloroacetic acid and separated by 10% preparative SDS-PAGE. The 108 kDa EndoS band (Figure 1) was excised from the gel and used for immunization of rabbits according to standard protocols (Harlow and Lane, 1988).

SDS-PAGE and N-terminal sequencing

To identify secreted proteins, bacterial culture supernatants were precipitated with a final concentration of 5% trichloroacetic acid, separated by 10% SDS-PAGE and stained with Coomassie Blue. Protein samples for N-terminal sequencing were separated by 10% SDS-PAGE and electroblotted to Immobilon-PTM (Millipore) as described (Matsudaira, 1987). Bands of interest were excised and subjected to Edman degradation (Edman and Begg, 1967).

For growth-promotion experiments and the identification of plasma protein substrates for EndoS, API and MC14 bacteria were cultured for 24 h at 37°C in 12.5% CM containing 1% heparinized human plasma. After growth, bacteria were pelleted by centrifugation at 15 000 g for 5 min and 1 μ l of each supernatant was separated by 10% SDS-PAGE. For analysis of EndoS and SpeB activity on purified IgG, culture supernatants from API, MC14 and AL1 bacteria were incubated for 2 h at 37°C with an equal amount of purified human IgG [2 mg/ml in phosphate-buffered saline (PBS)] in the absence or presence of a final concentration of 10 mM DTT. Two microliters of the samples were separated by 10% SDS-PAGE, followed by staining or blotting to membranes and analysis with a lectin as described below.

Western- and lectin-blotting experiments

For western blotting experiments, proteins were blotted to an Immobilon-PTM PVDF membrane (Millipore) according to Towbin *et al.* (1979). After blotting, membranes were blocked in PBS containing 0.1% Tween-20 and 5% skim milk (PBSTs) for 20 min at room temperature. After addition of the primary EndoS antibodies, diluted 1:1000 in PBSTs,

membranes were incubated for an additional 30 min at 37°C. Membranes were washed three times for 5 min each in PBST, incubated for 30 min at 37°C with peroxidase-labeled protein A (Sigma), diluted 1:5000 in PBSTs, and washed as above. After incubation, immunoreactive proteins were detected by the Immunoprint method (Nesbitt and Horton, 1992) and exposed on Cronex X-ray film (Sterling Diagnostic Imaging, Newark, DE).

For lectin analysis, treated or untreated IgG preparations were separated by 10% SDS-PAGE and electroblotted to PVDF membranes as above. Membranes were blocked with Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST), and incubated with 0.5 μ g/ml biotinylated GNL or UEA-I lectin (Vector Laboratories, Burlingame, CA). After washing in TBST, the membranes were incubated with 1 μ g/ml peroxidase-labeled streptavidin (Vector Laboratories). Membranes were washed in TBST and developed with the Immunoprint method as described above.

Mutagenesis of *ndoS* and *speB* in API

For mutagenesis of the *ndoS* gene in API, plasmid pFW13, which does not replicate in streptococci, was used for genomic integration of a truncated *ndoS* gene. A PCR product that covers bp 305–3232 of the *ndoS* gene was generated from API chromosomal DNA using oligonucleotide primers 5'-CTGTACATATGGAGGAGAAGACTG-3' and 5'-TTAATCTCGAGGTTGCTATCTAAG-3'. This product was cloned using the pGEMTM-T TA cloning vector system (Promega, Madison, WI) according to the manufacturer's instructions. One TA plasmid was digested with *SpeI*, which cleaves in the pGEM-T vector, and *HindIII*, which cleaves in the *ndoS* gene, to generate a fragment representing bp 305–839 of the *ndoS* gene (DDBJ/EMBL/GenBank accession No. AF296340). This fragment was ligated into *XbaI*- and *HindIII*-digested pFW13. *Escherichia coli* strain DH5 α was transformed using standard protocols (Sambrook *et al.*, 1989) and transformants were selected for kanamycin resistance. One clone, pMC11/DH5 α , was isolated for further analysis. Purified plasmid pMC11 was electroporated into competent API as described (Schalén *et al.*, 1995). Recombinants were selected on THY plates containing 150 μ g/ml kanamycin. Mutants were analyzed using PCR with vector- and *ndoS*-specific oligonucleotide primers, and one mutant, designated MC14, was chosen for further analysis.

An isogenic *speB* mutant was generated using the temperature-sensitive shuttle vector pJRS233 (Perez-Casal *et al.*, 1993) containing a cloned *speB* fragment interrupted by a kanamycin resistance gene, generating the plasmid pJRS233-kana-*speB* (Svensson *et al.*, 2000). This plasmid was electroporated into API strain according to Hanski *et al.* (1995), generating a mutant strain designated AL1. Lack of cysteine proteinase activity was confirmed using an assay with azocasein as the substrate, as previously described (Collin and Olsén, 2000).

α -fucosidase and PNGase F treatment of human IgG

Human IgG (10 μ g) was incubated either with 0.01 U of α -fucosidase from bovine kidney (Calbiochem, Darmstadt, Germany) in 50 mM sodium phosphate buffer pH 5.0 for 18 h, or with 1 U of PNGase F from *Chryseobacterium meningosepticum* (Calbiochem) in 50 mM sodium

phosphate buffer pH 7.5 for 18 h. After incubation, the hydrolyzed IgG was separated, blotted and probed with UEA-I lectin as described above.

Ligand blot experiments

Purified human IgG (Sigma) was incubated for 2 h with culture supernatants from API and MC14 bacteria grown in CM. After incubation, 0.2–5 µg of the treated IgG were applied to a PVDF membrane using a MilliBlot™ apparatus (Millipore). The membrane was blocked in PBST with 0.25% bovine serum albumin, washed with PBST and probed with 4×10^5 c.p.m./ml recombinant protein H labeled with ¹²⁵I according to Bolton and Hunter (1973). After incubation, the membranes were washed extensively with PBST and scanned using a Bio-Imaging Analyzer BAS-2000 (Fuji Photo Films Co. Ltd, Japan).

Bacterial absorption experiments

API bacteria were grown in THY for 16 h and harvested by centrifugation. Bacteria were washed three times with PBST containing 0.02% azide (PBSAT). Approximately 2×10^8 bacteria in 100 ml of PBSAT were incubated with 100 ml of purified human IgG (2 mg/ml in PBS) for 30 min at room temperature. Bacteria were washed as above, resuspended in 200 ml of PBSAT and incubated for 2 h at 37°C with 100 ml of culture supernatants from API (EndoS⁺) or MC14 (EndoS⁻) bacteria. After washing in PBSAT, bound IgG was eluted with 50 ml of 0.2 M glycine pH 2.0 and neutralized with Tris base prior to analysis of 10 ml by 10% SDS–PAGE.

Recombinant expression of EndoS in E.coli

The *ndoS* gene was cloned in *E.coli* using the pET-30a Xa/LIC ligation-independent cloning vector system according to the manufacturer's instructions (Novagen, Madison, WI). A PCR product covering bp 316–3195 of the intact *ndoS* gene was generated using oligonucleotide primers 5'-GGTATTGAGGGTCGCGAGGAGAAGACTGTT-3' and 5'-AGAGGAGAGTTAGAGCCTTATTTTTTATAGCAG-3' (vector compatible overhangs are underlined). This fragment was annealed to digested pET-30a vector generating pET*ndoS*. *Escherichia coli* strain BL21(DE3)pLysS was transformed with pET*ndoS* using standard protocols (Sambrook *et al.*, 1989). Lysates from cells induced with 1 mM IPTG (Sigma) and non-induced cells were prepared by freezing of the bacterial pellets at –70°C, followed by resuspension in PBS. Lysates were cleared of cell debris by centrifugation at 15 000 g for 10 min. Supernatants were incubated with purified IgG and separated by 10% SDS–PAGE for analysis of the activity of recombinantly expressed EndoS.

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