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Identification of β-haemolysin-encoding genes in *Streptococcus anginosus*

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SUMMARY

Streptococcus anginosus **is an emerging pathogen, but little is known about its virulence factors. To detect the genes responsible for β-haemolysis we performed genomic mutagenesis of the β-haemolytic *S. anginosus* type strain ATCC 12395 using the vector pGhost9:IS*S1*. Integration site analysis of 15 non-haemolytic mutants identified a gene cluster with high homology to the genes of the streptolysin S (SLS) encoding *sag* gene cluster of *S. pyogenes*. The gene cluster harbours 10 open reading frames displaying significant similarities to the *S. pyogenes* genes *sagA-sagI*, with the identities on protein level ranging from 38 to 87%. Complementation assays of *S. anginosus sagB* and *sagD* integration mutants with the respective genes confirmed their importance for β-haemolysin production and suggest the presence of post-translational modifications in *S. anginosus* SLS similar to SLS of *S. pyogenes*. Characterization of the *S. anginosus* haemolysin in comparison to the *S. pyogenes* SLS showed that the haemolysin is surface bound, but in contrast to *S. pyogenes* neither fetal calf serum nor RNA was able to stabilize the haemolysin of *S. anginosus* in culture supernatants. Inhibition of β-haemolysis by polyethylene glycol of different sizes was carried out, giving no evidence of a pore-forming haemolytic mechanism. Analysis of a whole genome shotgun sequence of *Streptococcus constellatus*, a closely related streptococcal species that belongs to the *S. anginosus* group, revealed a similar *sag* gene cluster. Employing a genomic mutagenesis strategy we were able to determine an SLS encoding gene cluster in *S. anginosus* and demonstrate its importance for β-haemolysin production in *S. anginosus.***

INTRODUCTION

Together with *Streptococcus constellatus* and *Streptococcus intermedius*, *Streptococcus anginosus* belongs to the *S. anginosus* group, which was formerly referred to as the *Streptococcus milleri* group. Streptococci of the *S. anginosus* group are commensals of mucosal membranes but can also be found as pathogens causing abscess formation and invasive infections (Whiley *et al.*, 1990, 1992). More recently, streptococci from the *S. anginosus* group have been reported to play a role as respiratory pathogens in cystic fibrosis patients (Parkins *et al.*, 2008). Invasive infections caused by the *S. anginosus* group appear to be under-recognized. In a large surveillance study from Canada the incidence rate of invasive infections with streptococcal species from the *S. anginosus* group was found to be 8.65/100,000, an incidence that was higher than the rates of invasive *Streptococcus pyogenes* or *Streptococcus agalactiae* infections (Laupland *et al.*, 2006). The failure to recognize the true pathogenic potential of *S. anginosus* strains may

be partly caused by their heterogeneous phenotype regarding Lancefield antigens and haemolysis (Facklam, 2002), which contributes to difficulties in correct species identification.

Very little is known about virulence factors in this group. The β-haemolysins represent classical bacterial virulence factors and a considerable proportion of strains in the *S. anginosus* group demonstrate a prominent β-haemolytic phenotype on blood agar plates. In a study conducted in southern India, where β-haemolytic group C and G streptococci have a high incidence rate, the species composition of this group was investigated (Reissmann *et al.*, 2010). β-haemolytic *S. anginosus* group strains accounted for 19% of the purulent infections in this group, which demonstrates their importance in human infections. In *S. intermedius* the β-haemolytic phenotype is caused by intermedilysin (Nagamune *et al.*, 1996), which belongs to the group of cholesterol-dependent cytolysins. However, a screen for the presence of intermedilysin genes in *S. anginosus* and *S. constellatus* was not able to identify intermedilysin homologues in these species (Nagamune *et al.*, 2000).

The prototypical β-haemolysin that can be found in many streptococcal species is streptolysin S (SLS), which was first discovered in *S. pyogenes* (Todd, 1938) and represents a well characterized virulence factor of *S. pyogenes*. SLS has not only haemolytic but also cytolytic functions (Bernheimer & Schwartz, 1964; Keiser *et al.*, 1964; Taketo & Taketo, 1966; Hryniewicz & Pryjma, 1977; Miyoshi-Akiyama *et al.*, 2005; Goldmann *et al.*, 2009). Contributions to virulence include soft-tissue damage (Datta *et al.*, 2005), an impact on host phagocytosis by destruction of neutrophils and macrophages (Miyoshi-Akiyama *et al.*, 2005; Goldmann *et al.*, 2009) and recruitment of the host protease calpain to the plasma membrane, enhancing the ability of *S. pyogenes* to cross the epithelial barriers (Sumitomo *et al.*, 2011). SLS is encoded in the *sag* gene cluster (Nizet *et al.*, 2000), which is present in many different streptococcal species (Fuller *et al.*, 2002; Humar *et al.*, 2002). Lately SLS-like gene clusters encoding for β-haemolysins have not only been found in streptococci (Fuller *et al.*, 2002; Humar *et al.*, 2002) but also in *Staphylococcus aureus*, *Clostridium botulinum* and *Listeria monocytogenes* (Cotter *et al.*, 2008; Lee *et al.*, 2008). In addition, homologues of the *sag* gene clusters producing thiazole/oxazole-modified microcins have been found in more distantly related phyla (Lee *et al.*, 2008; Haft *et al.*, 2010). A common feature of all these gene clusters is the production of cyclic peptides through the extensive post-translational modifications performed by enzymes encoded within the gene cluster (Molloy *et al.*, 2011).

To elucidate and characterize the genetic background and the biochemistry of the *S. anginosus* β-haemolysin we generated a mutant library of the haemolytic type-strain of *S. anginosus* (ATCC 12395) using the vector pGhost9:IS*S1*. Following a screen for non-haemolytic mutants we characterized the vector integration sites of 15 non-haemolytic mutants and detected a gene cluster showing considerable homologies to the *sag* gene cluster of *S. pyogenes*. Functional studies were carried out to characterize the properties of the *S. anginosus* SLS in comparison to the *S. pyogenes* β-haemolysin.

METHODS

Streptococcal strains and growth conditions

Streptococcal and *Escherichia coli* strains used in this study are listed in Table 1. *Escherichia coli* DH5 served as a host for recombinant pAT28 plasmids and *E. coli* EC101 as host for recombinant pGh9:IS*S1* plasmids. Streptococci were grown on tryptone soya agar plates with sheep blood (Oxoid, Basingstoke, UK) or in THY-broth [Todd–Hewitt broth (Oxoid) supplemented with 0.5% yeast extract (Difco, Sparks, MD)] at 37°C and 5% CO_2. Mutants with mobilized plasmids were grown at 30°C. Mutant strains harbouring chromosomally integrated pGh9:IS*S1* vectors were cultured in medium containing erythromycin (250 μg ml^{-1} for LB (lysogeny broth) medium and 1 μg ml^{-1} for THY-broth) at a temperature of 37°C. Mutant strains harbouring pAT28 plasmids in the cytoplasm were grown in medium containing spectinomycin (100 μg ml^{-1} for LB medium and 120 μg ml^{-1} for THY-broth).

General DNA techniques

For DNA preparation and analysis, standard techniques were used. Polymerase chain reaction (PCR) was performed with *Taq* polymerase according to the manufacturer's protocol (Roche, Mannheim, Germany), with 30 cycles of amplification steps of

Table 1 Bacterial strains and plasmids

Strain or plasmid	Definition	Source
Strains		
Streptococci		
BSU 458	*S. anginosus* type strain ATCC 12395, Hly$^+$	ATCC
BSU 459	*S. constellatus* ATCC 27823	ATCC
BSU 877	*S. pyogenes* ATCC 12344, Hly$^+$	ATCC
BSU 649	BSU 458 derivative *sagB*::IS*S1*; Hly$^-$	This study
BSU 651	BSU 458 derivative *sagB*::IS*S1*; Hly$^-$	This study
BSU 653	BSU 458 derivative *sagB*::IS*S1*; Hly$^-$	This study
BSU 655	BSU 458 derivative *sagB*::IS*S1*; Hly$^-$	This study
BSU 657	BSU 458 derivative *sagB*::IS*S1*; Hly$^-$	This study
BSU 659	BSU 458 derivative *sagD*::IS*S1*; Hly$^-$	This study
BSU 661	BSU 458 derivative *sagD*::IS*S1*; Hly$^-$	This study
BSU 663	BSU 458 derivative *sagD*::IS*S1*; Hly$^-$	This study
BSU 665	BSU458 derivative *sagD*::IS*S1*; Hly$^-$	This study
BSU 592	BSU 458 derivative *sagD*::pGhost:IS*S1*; Hly$^-$	This study
BSU 603	BSU 458 derivative *sagH*::pGhost:IS*S1*; Hly$^-$	This study
BSU 607	BSU 458 derivative *sagD*::pGhost:IS*S1*; Hly$^-$	This study
BSU 608	BSU 458 derivative *sagD*::pGhost:IS*S1*; Hly$^-$	This study
BSU 609	BSU 458 derivative *sagD*::pGhost:IS*S1*; Hly$^-$	This study
BSU 612	BSU 458 derivative *sagH*::pGhost:IS*S1*; Hly$^-$	This study
Escherichia coli		
DH5α	*endA1 hsdR17 supE44* DlacU169(f80lacZDM15) *recA1 gyrA96 thi-1 relA1*	Boehringer
EC101	*E. coli* JM101 derivative with *repA* from pWV01 integrated into the chromosome	Law *et al.* (1995)
Plasmids		
pGhost9:IS*S1*	Eryr ori Ts	Maguin *et al.* (1996)
pAT28	Specr ori pUC ori pAmb1	Trieu-Cuot *et al.* (1990)
pAT28::sagB	pAT28 derivative carrying a 1556-base-pair *sagB* fragment from wild-type BSU458	This study
pAT28::sagD	pAT28 derivative carrying an 1622-base-pair *sagD* fragment from wild-type BSU458	This study
pBSU409	pAT28 derivative, carrying a promotorless *egfp* gene	Gleich-Theurer *et al.* (2009)
pBSU409::sagprom	pBSU409 derivative carrying a 441-base-pair *sag* promoter fragment from wild type *S. anginosus* ATCC 12395	This study
pBSU101	pAT28 derivative carrying *egfp* under the control of the *cfb* promoter	Aymanns *et al.* (2011)

1 min at 94°C, 1 min at 50–56°C, and 1–4 min at 72°C depending on primers (Table 2) and product size. If expected product sizes exceeded 4 kb and for inverse PCR the Expand Long Template PCR system (Roche) was used according to the manufacturer's protocol with 10 cycles of amplification steps of 10 s at 94°C, 15 s at 48–53°C and 4 min at 60°C, followed by 20 cycles of amplification steps of 15 s at 94°C, 15 s at 48–53°C and 4 min at 60°C. Inverse PCR was carried out to obtain additional sequence information of the *sag* gene cluster. Genomic DNA of wild-type *S. anginosus* was digested with diverse restriction enzymes. After ligation of genomic DNA, PCR was carried out with outward directed primers, located close to the restriction site in the region of the known sequence. Resulting PCR products were sequenced. Genomic streptococcal DNA was isolated as described elsewhere (Pospiech & Neumann, 1995). Plasmid DNA was isolated and purified using the Qiaprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Plasmids and PCR products were sequenced on an ABI 373 automated DNA sequencer using the ABI PRISM Dye terminator cycle sequencing kit (PE Applied Biosystems, Weiterstadt, Germany). Streptococcal strains were

Table 2 Primers used in this study

Name	Sequence 5′ to 3′	Annealing temperature in °C
sag.458.1 fw	TTTTTATGCCAGACAGGTTG	53
sag.458.1 rev	CCGCTGTACTAGATGAATCG	53
pre_sagA0_for	GCTTTGCACTATGTTACAATC	53
pre sagA for	ATGAAGCTGATTGAGGTGAA	53
sag A0 for	GTTCAACAACTGGATCCGTA	53
sag A for	GCTTCTCTGTAGCAGTAGGC	50
sagA inverse for	CGCCAATCAACTGTTTCCTC	50
sag A1 for	GCTTGTTGCTCAGCATCTCATGC	50
sag A2 for	GTCATCGTCAGTTCAAAGCG	50
sag A3 for	CTACCAGCATGCTTTGAAATGC	50
sag A4 for	GTTCGAATTGTCCAATTTAACG	50
sag A inverse rev	CCGCTGTACTAGATGAATCG	50
sagB_EcoR1_for	TAGAATTCTTTGGATGAAGCTAGGTTAG	56
sag B_Hind3_rev2	CGAAGCTTCAACAACCTGAAATAATT	56
sag D inverse for	GGGATTATGGAAGCTTCAGC	50
sag D inverse rev	GGATCAAGACCAGCTTGAAC	50
sag D rev	GTTCTGTGCTTTCGATATTGC	50
sag D1 rev	GACACTATTGCACATAGCTGC	50
sag D2	CGCCTAAGTACTTAATCAGC	50
sag D3 rev	GCTTGACTAACAGGCTGAGTAGC	50
sag D4 rev	ACTTCTGCTAATTTCAGAGCCG	50
sag D kompl rev 2	GCGCGCGGATCCGAATTGGTCTGCTATATCCT	56
sagE.458.1 fw	GCACTCAATCATCTTCTAATG	53
sagE.458.1 rev	CTTTCGATATTGCTTAAAGCC	53
sag E2 rev	CCATATAAAAGACCAGTCAG	50
sag F1 for	GCCAAGGTATTGAAATCATC	53
sag F2 for	TCCTTGCGTGTCTTACCTTT	53
sag F 3 for	CTTTCACTTAGAGAGGAACG	53
sag F1 rev	CCTAAGATTTCCACCAATGT	53
sag F2 rev	TCGTAGGGGCTCCAGACAC	53
sag F3 rev	CAAGCTTTCACGACAGCAAA	53
sag G1 for	GATTGAAGAAGGAAGCAAAG	53
sag F4 for	GATCAGCCAATAAAGGTGAT	53
sag H1 for	GAAGGAGCTGAAACAGATTAC	53
sag H2 for	GAATTTAGTTCTCTCAGACC	53
sag H3 for	CAACGACTTTACTCGTTAATC	53
sag H1 rev	CCCTCCAAATACAAGATAGA	53
sag H2 rev	GCATTGGCTTTAAACCATTC	53
sag H3 rev	GTTCCTAAGCCTAGATTAACG	53
sag I1 for	CTAGCAGTCAAGTGACAGAA	53
sag I2 for	CCTCTTGTTTAGTTTGCTCG	53
sag I3 for	CAGTAGCTGCTATACTGATTG	53
sag I1 rev	GCTGATGTTAACGACTTTCA	53
sag I2 rev	TAGCAGTTCTCCTCATCTATC	53
sag I3 rev	GATACTGGAAGCATATAAACAC	53
pAT28-2	CTCTTCGCTATTACGCCAGCT	53
pAT28-3	GTTGTGTGGAATTGTGAGCGG	53
pAT28-EGFP4	CCTTGAAGAAGATGGTGCGC	53
ISpGhost9P7	ATCTACTGAGATTAAGGTCTTAATGG	53
pGhost KS	CGAGGTCGACGGTATCG	53
sag prom for	GGGCCCGAATTCGGTTGGATTTGATAGTAATGTACG	53
sag prom rev	GGGCCCGGATCCGAAGAAAATTTTAACATAGTTTG	53

transformed according to the protocol of Ricci *et al.* (1994).

Construction of mutants and identification of chromosomal integration sites

For the construction of a genomic mutant library, the pGh9:IS*S1* (Maguin *et al.*, 1996) vector was integrated into the chromosome of the *S. anginosus* type strain ATCC 12395. This vector contains the insertion sequence IS*S1* together with the thermosensitive replicon pG1host, allowing the replication of plasmids in the streptococcal host at the permissive temperature of 30°C and chromosomal integration of the entire plasmid at temperatures $\geq$37°C. Construction of library was carried out as described previously (Spellerberg *et al.*, 1999).This library was screened for mutants showing a loss of the β-haemolytic phenotype on blood agar plates and 15 non-haemolytic mutants were selected for further investigation. Mobilization of the pGh9:IS*S1* vector from individual mutants leading to stable mutants harbouring a single copy of the IS*S1* insertion element at the original integration site were generated by induction of rolling circle replication at 30°C in the absence of antibiotic pressure. Chromosomal pGh9:IS*S1* integration sites were determined as described previously (Spellerberg *et al.*, 1999). Primers were designed on the basis of these sequences and inverse PCR was performed to obtain more sequence information on the surrounding genes.

Complementation of non-haemolytic mutants

To complement non-haemolytic mutants with an insertion of IS*S1* in *sagB* (*sagB*:IS*S1* mutant) or *sagD* (*sagD*:IS*S1* mutant) primers were designed to amplify *sagB* or *sagD* of *S. anginosus* and the resulting PCR products were cloned into pAT28. The vector pAT28:*sagB* was introduced in *sagB*:IS*S1* mutant and vector pAT28:*sagD* into the *sagD*:IS*S1* mutant by electroporation. As a negative control the vector pAT28:*sagB* was introduced into the *sagD*:IS*S1* mutant and the vector pAT28:*sagD* into the *sagB*:IS*S1* mutant. Resulting clones were selected on THY-agar plates supplemented with spectinomycin and checked for the presence of the recombinant pAT28 vector. Subsequently, the phenotype of complementation mutants was evaluated on blood agar plates.

Gene expression analysis of the sag gene locus

To investigate gene expression of the *sag* genes of *S. anginosus* the putative promoter region was cloned into a streptococcal enhanced green fluorescent protein (EGFP) plasmid. For this purpose the promoter region of the *sag* gene cluster was amplified with the primers sagprom forward and sagprom reverse. The resulting PCR products and the vector pBSU409 were digested with the enzymes *Bam*HI and *Eco*RI, ligated and transformed into DH5α cells. Correct construction of the plasmid was controlled through PCR with primers flanking the insertion site (pAT28-3 and pAT28-EGFP4) (Table 2) and sequencing of the PCR products. The recombinant plasmid harbouring the putative *sag* gene cluster promoter sequence upstream of the *egfp* gene was transformed into the wild-type *S. anginosus* type strain ATCC 12395. Spectinomycin-resistant clones were selected for further analysis. To investigate the expression or *S. anginosus* in different growth phases fluorescence-activated cell sorting analysis of *the S. anginosus* strain carrying the pBSU409::sagprom vector was carried out as described elsewhere (Aymanns *et al.*, 2011) at optical densities at 600 nm (OD_{600}) 0.2, 0.4, 0.6 and 0.8. Additionally, cultures for OD_{600} 0.6 were grown with 5% haemolysed human blood to investigate whether *S. anginosus* haemolysin is blood inducible. As a negative control the *S. anginosus* strain ATCC 12395 with the original vector was used. The *S. anginosus* ATCC 12395 strain with the vector containing the *cfb* (CAMP-factor gene) promoter of *S. agalactiae* was used as a positive control (Aymanns *et al.*, 2011).

Haemolytic assay

To determine the haemolytic activity, assays were carried out with *S. pyogenes* ATCC 12344 as a positive control. For the generation of haemolytic SLS extracts, the protocol of Loridan & Alouf (1986) was followed, with some modifications. Briefly, the strains were grown overnight in 10 ml THY-broth at 37°C and 5% CO_2. These cultures were centrifuged for 10 min at 4°C (2000 g) and 1 ml of the supernatant was collected and stored on ice. Pellets were then washed with 0.1 M potassium phosphate buffer and afterwards suspended in 6 ml induction buffer (IB, 100 mM KH_2PO_4, 2 mM $MgSO_4$, 30 mM maltose, pH

7.0) or 5 ml IB and 1 ml fetal calf serum (FCS) or 6 ml IB with 0.5 mg ml^{-1} RNA core (Sigma-Aldrich, St Louis, MO) and incubated at 37°C for 10 min. Cultures were centrifuged for 10 min at 4°C, 1 ml supernatant was collected and stored on ice and pellets were resuspended in 500 μl IB. Twofold dilution series of the pellets with and without FCS/RNA and the supernatants with and without FCS/RNA were prepared with phosphate-buffered saline (PBS) in 1.5-ml tubes. Pellets and supernatant with FCS of *S. pyogenes* were prediluted beforehand; then 500 μl PBS and 500 μl of a 0.5% sheep erythrocytes or human erythrocytes solution in PBS was added. The tubes were carefully mixed and incubated for 60 min at 37°C. After centrifugation the optical density of the supernatant was measured at 540 nm to determine haemolytic activity.

To determine if haemolysis is regulated by cell density, the haemolytic assay was carried out with slight modifications. Overnight cultures of the wild-type *S. anginosus* were used to inoculate fresh THY-broth and the cultures were allowed to grow until they reached an OD_{600} of 0.2, 0.4, 0.6 and 0.8. Haemolysin assays were carried out as described above using pelleted bacteria.

Inhibition assay

Cultures of the wild-type *S. anginosus* and *S. pyogenes* strains were incubated overnight in 10 ml and 20 ml THY-broth at 37°C and 5% CO_2. The next day, cultures were pelleted and washed with PBS. Afterwards cells were resuspended in 500 μl PBS and were added to 500 μl polyethylene glycol (PEG) of different molecular mass (final concentration 10 mM) and 500 μl sheep erythrocytes (0.5%) in a total volume of 1.5 ml. Prediluted *S. pyogenes* cells were used as a positive control. After incubation for 1 h at 37°C the tubes were centrifuged and the supernatant was measured at an optical density of 540 nm to check for haemolytic activity.

Nucleotide sequence accession numbers

During the revision process a sequence of the *sag* gene cluster of *S. anginosus* strain ATCC12395 became available at DDBJ/EMBL/GenBank databases under accession no. JN619420.

RESULTS

Identification of the *S. anginosus sag* gene cluster

To identify the genes responsible for the haemolytic phenotype of *S. anginosus* we generated a mutant library of strain ATCC 12395 by chromosomal integration of the pGhost:IS*S1* vector, which is to our knowledge the first genetic mutant library of this streptococcal species. We screened 30 plates with approximately 500 mutants each and selected 15 non-haemolytic mutants for further analysis. Determination of the insertion sites of these non-haemolytic mutants revealed that 14 unique insertion sites were located within a gene cluster of about 9 kb with 10 open reading frames (ORFs) (Fig. 1). Two mutants, BSU608 and BSU609, harboured identical integration sites. The gene cluster shows a high resemblance to the *sag* gene cluster of *S. pyogenes*, with identities on protein level ranging from 38% (SagF) to 87% (SagA1) (Table 3). Although we were working on the assembly of the nucleotide sequence of the *sag* gene cluster of *S. anginosus* by genome walking, a whole genome shotgun sequence of the *S. anginosus SK52* strain became available online (Accession number NZ_AFIM01000032 and AICP01000041.1). Comparison of the nucleotide sequence of the *sag* gene cluster of *S. anginosus* ATCC 12395 with strain SK52 revealed a similar gene cluster in strain SK52. Whereas *sagB*, *sagC* and *sagG* had already been annotated in the shotgun sequence, ORFs for *sagD*, *sagE*, *sagF, sagH* and *sagI* are also present at the expected genetic loci. Interestingly both sequences, ATCC 12395 and strain SK52, harbour at the upstream region of the gene clusters two ORFs corresponding to the gene *sagA of S. pyogenes,* which were designated *sagA1* and *sagA2* (Fig. 1). The deduced amino acid sequences compared with other SLS-like structures are shown in Fig. 2.

To confirm that genes in the *sag* gene cluster of *S. anginosus* are essential for haemolytic activity and that the insertions of IS*S1* are the definite cause for loss of haemolysis in these mutants, we complemented the insertion mutants of *sagB* and *sagD* with the respective wild-type genes. Plasmids were created on the basis of pAT28, containing either the complete reading frame of *sagB* or *sagD* in trans. The vector pAT28::*sagB* was able to restore

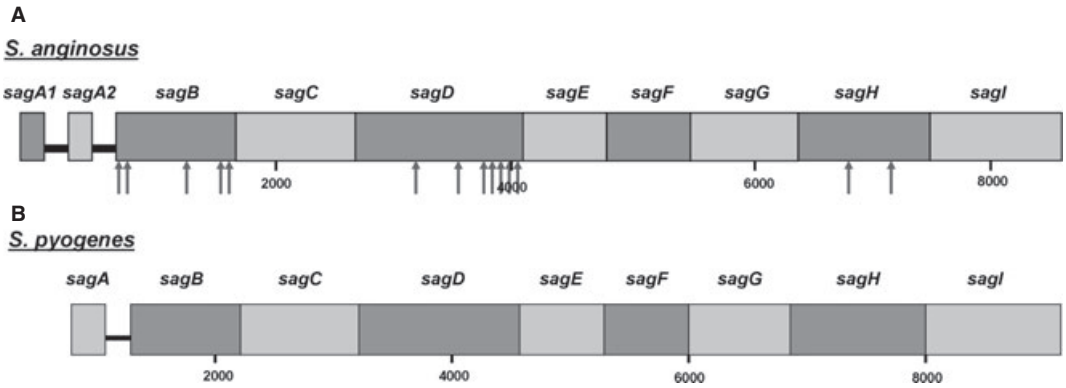

Figure 1 (A) Depicted is the *sag* gene cluster of *Streptococcus anginosus*, which consists of 10 open reading frames ORFs (*sagA1* to *sagI*). Approximate nucleotide positions are indicated. Grey arrows show pGhost9:IS*S1* integration sites. (B) Schematic representation of the *sag* gene cluster of *S. pyogenes*.

Table 3 Comparison of deduced amino acids from *sag* gene cluster of *Streptococcus anginosus* with sag proteins of *Streptococcus pyogenes*

Protein	Identity in %	Positives in %	Predicted function
SagA1	87	100	Haemolytic active protein
SagA2	83	92	Haemolytic active protein
SagB	64	80	Responsible for post-translational
SagC	69	85	modifications
SagD	81	89	
SagE	46	64	CAAX N-terminal protease family protein
SagF	38	61	Putative membrane protein
SagG	72	85	ABC-transporter
SagH	74	85	
SagI	69	84	

β-haemolysis in the *sagB*::IS*S1* mutant but failed to show any effect on haemolysis in the *sagD*::IS*S1* mutant. Conversely, vector pAT28::*sagD* restored haemolysis in the insertion mutant *sagD*::IS*S1* but not in *sagB*::IS*S1*. These results confirm the involvement of the *sag* gene cluster in the haemolytic activity of *S. anginosus* and underline the importance of the genes *sagB* and *sagD* (Fig. 3).

SLS gene expression analysis

To obtain gene expression data on the *sag* gene cluster in *S. anginosus* we cloned the putative *sag* promoter in an EGFP reporter plasmid and transformed it in the wild-type *S. anginosus* strain ATCC 12395. Fluorescence-activated cell sorting analysis was carried out at different growth phases (Fig. 4). High fluorescence could be observed as early as OD 0.2 and remained on that level for all ODs. Addition of haemolysed human blood during mid-logarithmic growth phase (OD 0.6) did not show any significant effect.

To correlate the data obtained through the EGFP-*sag* gene reporter construct with β-haemolytic activity of *S. anginosus*, haemolysin assays were carried out at different growth phases (Fig. 5). A considerable amount of haemolytic activity was already observed at an OD of 0.2 and persisted until OD 0.8. No significant differences could be observed for these ODs.

Streptolysin S of *S. anginosus* is associated with the bacterial surface

Streptolysin S of *S. pyogenes* is located at the bacterial surface but can be released into the supernatant by the addition of FCS or RNA core (ribonuclease-resistant fraction of yeast RNA) to haemolytic extracts (Alouf, 1980). To investigate if the haemolysin of *S. anginosus* shows similar characteristics, we carried out haemolysin assays with the wild-type *S. anginosus* strain, using *S. pyogenes* and a non-haemolytic mutant of the *S. anginosus* type strain with integration in *sagB* as controls. The *S. anginosus* haemolysin was able to lyse sheep erythrocytes and human erythrocytes when associated with the bacterial surface. (Fig. 6). The culture supernatant did not show any haemolytic activity. As expected the *S. anginosus sagB* mutant showed no haemolytic activity in either whole bacteria cells or the culture supernatant. In line with previous publications (Weld,

S. aureus RF 122	MMKINNHTINGYSDINSSEAMQYAA**G**	CCSCSCSCSCSCSCSCTSASTAEQ
L. monocytogenes F2365	MNIKSQSSNGYSNNAVGSEAMNYAA**G**	CCSCSCSTCTCTCTCASSAATKM
S. pyogenes MGAS8232	MLKFTSNILATSVAETTQVAP**GG**	CCCCCTTCCFSIATGSGNSQGGSGSYTPGK
C. botulinum ATCC 3502	MLKFNEHVLTTTNNSNNKVTVAP**G**	SCCCCSCCCCVSVSGGGSASTGGGAAAGQGGN
S. dysgalactiae subsp. equisimilis GGS_124	MLQFTSNILATSVAETTQVAP**GG**	CCCCCTTCCFSINVGGGSAQGGSGSYTPGK
Streptococcus equi subsp. zooepidemicus MGCS10565	MLQFTSNILATSVAETTQVAP**GG**	CCCCCSCCCCVSASWGNTTINNNYAEQPKA
S. iniae (Fuller *et al.*, 2002)	MLQFTSNILATSVAETTQVAP**GG**	CCCCCCTCCVAVNVGSGSAQGGSGTPAPAPK
S. constellatus	MLKFSSNVLATSVADTTQVAP**GG**	CCCCSCTCCFSISTGGNSTGGSTTLPSPGK
S. anginosus SagA1	MLKFSSNVLATSVADTTQVAP**GG**	CCCCCCTCCFSVAVGGNATGGSTTGSVAPTK
SagA2	MLKLDSHIMATSVAETTQVAP**GG**	CCCCCCTCCFSVAVGGNATGGSTNIKP

Figure 2 Deduced amino acid sequences of *sagA* of different bacterial species. On the left side the N-terminal leader region is shown, to the right side the C-terminal core region is displayed, which later becomes the haemolytic active unit. The glycine residues (G) at the possible cleavage sites are depicted in bold letters and are underlined. The FXXXB motif is shaded in grey. Sites for post-translational modifications are illustrated in bold grey letters.

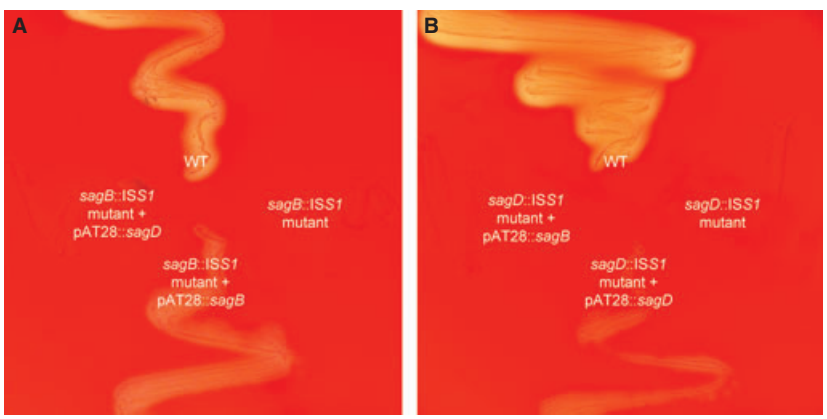

Figure 3 Complementation of a *sagB*::IS*S1* mutant (A) and a *sagD*::IS*S1* mutant (B). To check for haemolysis the strains were streaked on a blood agar plate. (A) Wild-type *Streptococcus anginosus* (WT), the *sagB*::IS*S1* mutant and its complementation with the vectors pAT28::*sagB* and pAT28::*sagD.* (B) Wild-type *S. anginosus* (WT), the *sagD*::IS*S1* mutant and its complementation with the vectors pAT28::*sagD* and pAT28::*sagB* are depicted.

1934; Bernheimer & Rodbart, 1948; Duncan & Mason, 1976; Loridan & Alouf, 1986) FCS or RNA core addition to the supernatant stabilized the haemolytic activity in the supernatant of *S. pyogenes*. However, in the case of *S. anginosus* the culture supernatant remained non-haemolytic upon the addition of FCS or RNA core.

Inhibition assay

To further characterize the membrane damage induced by *S. anginosus* SLS, inhibition experiments using PEG were carried out with *S. pyogenes* SLS as a control. This test can be used to detect membrane pores of a defined size (Lang & Palmer, 2003). Carr

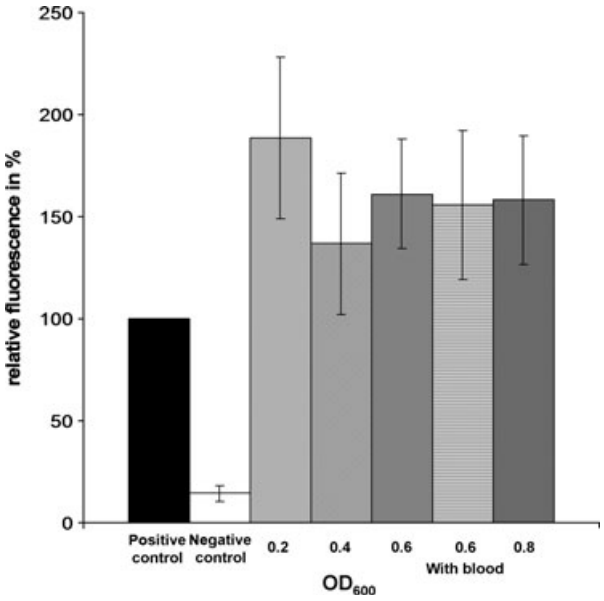

Figure 4 Fluorescence-activated cell sorting analysis of strain ATCC12395 carrying plasmid pBSU409::*sagprom*. Relative fluorescence is shown as a percentage of the positive control strain (ATCC12395 carrying plasmid pBSU101). Measurements were obtained for bacteria grown to an optical density at 600 nm (OD_{600}) of 0.2, 0.4, 0.6, 0.8. The values represent mean values and standard deviations of six independent experiments.

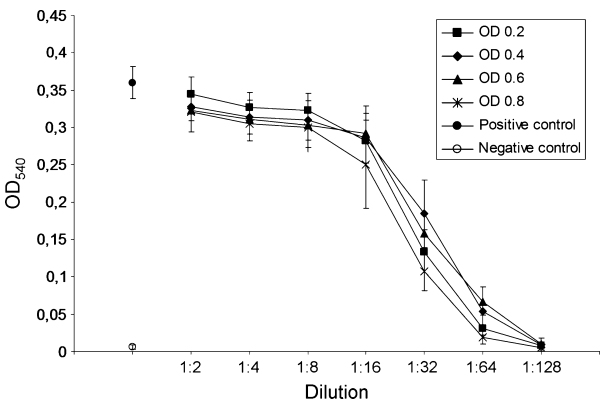

Figure 5 Haemolytic assay of wild-type *Streptococcus anginosus* strain ATCC 12395 at an optical density at 600 nm (OD_{600}) of 0.2, 0.4, 0.6 and 0.8. Assays were carried out with human blood as detailed in the Methods. Haemolysis was measured by quantifying haemoglobin release at 540 nm. Data represent mean values and standard deviations of five independent experiments.

et al. (2001) studied the mechanism of haemolysis exerted by SLS of *S. pyogenes* and suggested that the haemolysin integrates into the erythrocyte membrane where it forms lytic transmembrane pores. Therefore we should see an inhibiting effect with growing molecular mass of PEG. We observed an inhibiting effect of PEG as haemolytic activity of *S. anginosus* SLS decreased with increasing PEG size (Fig. 7). The decrease was not sharp but was a steady one, leaving us unable to determine a definite pore size for the *S. anginosus*-induced membrane damage. For *S. pyogenes* a more abrupt decrease between PEG 4000 and PEG 6000 could be observed.

The *sag* gene cluster of *S. constellatus*

Primers based on the *sag* gene cluster of *S. anginosus* were used to look for corresponding genes in *S. constellatus*. We were able to amplify parts of the region between *sagG* and s*agI*, which presumably encode an ABC type transporter. In the meantime a whole genome shotgun sequence of *S. constellatus* subsp. *pharyngis* SK1060 strain (Accession Number NZ_AFUP01000001) became available in the NCBI database. An ORF analysis of the corresponding region revealed ORFs of all nine *sag* genes (*sagA* to *sagI*). When compared with each other, the *sag* gene clusters of *S. anginosus* and *S. constellatus* had an overall identity of 85% in the nucleotide sequence, whereas at the protein level the identities ranged from 73 to 93%. In contrast to *S. anginosus* the *sag* gene cluster of *S. constellatus* harboured only a single copy of the *sagA* gene. Interestingly the deduced amino acid sequence of SagA of *S. constellatus* is very similar to the two copies of the *S. anginosus* SagA, with the N-terminal leader region being identical in SagA of *S. constellatus* and in SagA1 of *S. anginosus*. The C-terminal part of the protein demonstrates higher sequence heterogeneity, but it contains numerous possible sites for post-translational modifications of the SagA peptide (Fig. 2). We cannot provide a mutational analysis of the *sag* genes in *S. constellatus*, but the high similarities of the *sag* genes and the conserved structure of the gene cluster strongly suggest that the β-haemolytic activity of *S. constellatus* is also encoded in a *sag* gene cluster with considerable homologies to *S. anginosus.*

DISCUSSION

By integration of the vector pGhost:IS*S1* we were able to establish a mutant library of the *S. anginosus* type strain ATCC 12395. To our knowledge a

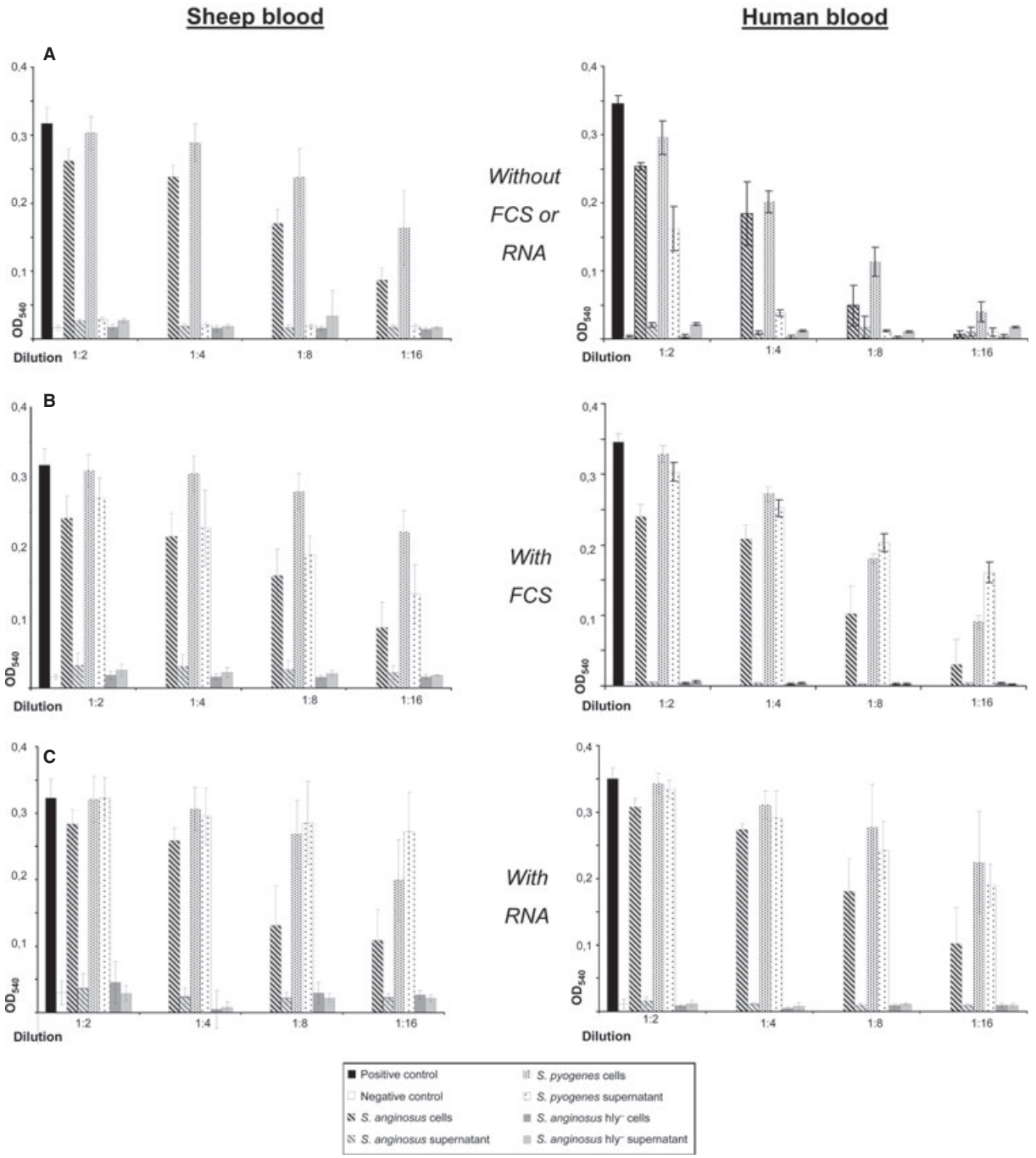

Figure 6 Haemolytic assay of wild-type *Streptococcus anginosus*, *S. pyogenes* and an *S. anginosus sagB* mutant strain. Sheep erythrocytes and human erythrocytes were used at a final concentration of 0.17%. Cells and supernatant were prepared as detailed in the Methods. Haemolysis was measured by quantifying haemoglobin release at 540 nm. (A) Haemolytic activity of bacterial cells and the supernatant of the wild-type *S. anginosus*, *S. pyogenes* and the *S. anginosus sagB* mutant is shown. (B) Haemolytic activity of bacterial cells and supernatant in the presence of FCS. (C) Haemolytic activity of bacterial cells and supernatant following the addition of RNA. Data represent mean values and standard deviations of five independent experiments.

chromosome-wide genetic mutation of *S. anginosus* has not previously been reported. Analysis of the integration sites of non-haemolytic mutants revealed the presence of 14 unique integration sites in a *sag* gene cluster of about 9 kb. These data indicate that pGhost:IS*S1* represents an efficient mutagenesis tool

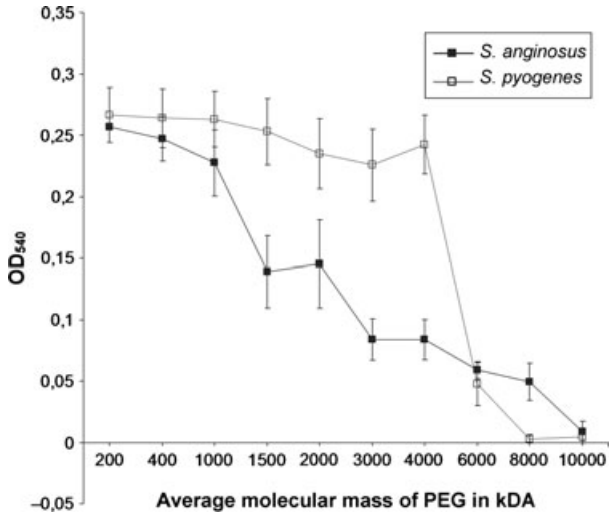

Figure 7 Inhibition assay of wild-type *Streptococcus anginosus* and *Streptococcus pyogenes*. Haemolytic activity of *S. anginosus* and *S. pyogenes* was measured upon addition of polyethylene glycol of different molecular mass as indicated. Streptolysin S (SLS) preparations were generated from 10 ml of overnight culture. Data represent mean values and standard deviations of five independent experiments.

for *S. anginosus*, as has been reported for other streptococcal species (Maguin *et al.*, 1996; Spellerberg *et al.*, 1999; Biswas & Scott, 2003; Smith *et al.*, 2003; Fontaine *et al.*, 2004; Biswas & Biswas, 2011). However, a more detailed analysis of the integration sites within the *sag* gene cluster revealed that the observed mutations did not occur randomly in all genes present within the 9 kb cluster. Mutations preferentially occurred in the genes *sagB*, *sagD* and *sagH* (Fig. 1). A possible explanation may be that insertions in other genes of the *sag* gene cluster do not result in a non-haemolytic phenotype and were therefore not selected in our screen. It may also provide an explanation for the fact that we did not find mutants with integrations in one of the *sagA* genes. It is possible that inactivation of only one of the two potential haemolysin genes may not eliminate haemolysis completely. However, in this genetic cluster three genes for an ABC transporter, which most probably is responsible for the export of the β-haemolysin, are present (*sagG*, *sagH*, *sagI*). Insertion of pGhost9:IS*S1* into the *sagH* gene of the putative transporter resulted in a non-haemolytic phenotype. It appears unlikely that insertions in other parts of the transporter would not affect haemolysis. Moreover research in *S. pyogenes* showed that allelic exchange mutants for each single *sag* gene resulted in a non-haemolytic phenotype (Datta *et al.*, 2005). A more likely explanation for the observed mutation sites may be that the genes *sagB*, *sagD* and *sagH* represent 'hot spots' for the insertion of the pGhost9:IS*S1* vector into the *S. anginosus* genome.

By using the pGhost9:IS*S1* vector system for the screening of haemolysin genes in *S. anginosus* we identified a homologue of the classical SLS-encoding gene cluster with 10 ORFs (Fig. 1). Gene clusters for *sag* have been identified as genetic background for β-haemolysins in many streptococci (Nizet *et al.*, 2000; Fuller *et al.*, 2002; Humar *et al.*, 2002;) and other bacteria (Cotter *et al.*, 2008; Lee *et al.*, 2008). Significant homologies of the *S. anginosus* gene cluster could be observed with SLS genes of *S. pyogenes*, *Streptococcus iniae* and *Streptococcus dysgalactiae* subsp. *equismilis*. These results were somewhat surprising as another member of the *S. anginosus* group, *S. intermedius*, harbours a β-haemolysin called intermedilysin, which belongs to the group of thiol-activated cytolysins that also includes streptolysin O (Nagamune *et al.*, 1996). Intermedilysin was the first β-haemolysin described in the *S. anginosus* group and no homologues were found in *S. anginosus* and *S. constellatus* (Nagamune *et al.*, 2000). However, the SLS haemolysins for which we identified corresponding genes in *S. anginosus* and *S. constellatus* belong to a different group of β-haemolysins, the SLS-like peptides. These molecules require extensive post-translational modifications to act as virulence factors and they are part of the thiazole/oxazole-modified microcin molecule group (Lee *et al.*, 2008; Molloy *et al.*, 2011). Due to its wide distribution among different bacteria and even Archaea (Lee *et al.*, 2008; Haft *et al.*, 2010) this group has recently been discussed as promising targets for drug and vaccine developments (Molloy *et al.*, 2011).

One remarkable difference between the *sag* cluster of *S. anginosus* and the SLS genes of other streptococcal species is the presence of two ORFs encoding SagA in *S. anginosus*, which may be a result of gene duplication. As the two copies are not completely identical, we compared them to the haemolytic peptides of other bacteria with SLS-like gene clusters (Fig. 2). Both copies of the *S. anginosus* SagA have a double glycine, which presumably represents the cleavage site for a protease, as has been shown in other bacteria (McAuliffe *et al.*, 2001). In *S. pyogenes*

SagE is suggested to be responsible for the cleavage of the SagA protoxin (Molloy *et al.*, 2011). Numerous sites for post-translational modifications are located in the C-terminal part of the peptides, which is thought to represent the haemolytically active unit. In SagA1 we found the FXXXB motif, where the SagBCD complex is supposed to bind, with X being any amino acid and B being a branched-chain amino acid (Mitchell *et al.*, 2009). This motif cannot be found in SagA2, and is also absent in SagA homologues of *Staphylococcus aureus* and *L. monocytogenes*. Except for the amino acids forming the FXXXB motif, the N-terminal leader regions of SagA1 and SagA2 are identical and very similar to the one of *S. pyogenes*. In the C-terminal core regions identity can be observed for the first 23 amino acids, which includes the vast majority of the possible sites for post-translational modifications (Fig. 2). The amino acid data suggest that both copies could be active as haemolytic SagA peptides, but as we were not able to isolate pGhost9:IS*S1* mutants of these genes, we do not know if both copies are required for haemolysis or if a single SagA peptide is sufficient. Interestingly the nucleotide data from *S. constellatus* show only a single copy of SagA harbouring an FXXXB motif (Fig. 2), suggesting that this closely related species produces a classical SLS.

Complementation of mutants with integration in *sagB* or *sagD* with the corresponding wild-type genes restored the haemolytic phenotype (Fig. 3). Previous studies in *S. pyogenes* (Lee *et al.*, 2008; Mitchell *et al.*, 2009) have shown that SagC, a cyclodehydratase, generates heterocycles within the SagA protoxin, and SagB, acting as dehydrogenase, removes two electrons to afford aromatic cycles. The incorporation of these aromatic heterocycles restricts backbone flexibility, which provides the mature SLS with a more rigid tertiary structure (Haft *et al.*, 2010). SagD seems to act as a docking scaffold protein and may be needed for assembly of the complex and regulation of activity (Haft *et al.*, 2010). As integration of IS*S1* in *sagB* or *sagD* leads to a loss of haemolysis and complementation with the wild-type genes to a recovery of haemolysis, these two genes appear to be crucial for putative post-translational modification steps in the *S. anginosus* SLS.

To characterize the membrane damage induced by *S. anginosus* SLS, we carried out inhibition assays with PEGs of different size hoping to predict the size of membrane damage induced by *S. anginosus* SLS. But as there was no sharp decrease in haemolytic activity at a specific molecular mass of PEG, it was not possible to determine a definite pore size (Fig. 7). The data are compatible with the hypothesis that the *S. anginosus* SLS may not induce classical haemolytic pores of a well-defined size but induces β-haemolysis via a different, perhaps detergent-based, mechanism. The difference in the results for *S. anginosus* and *S. pyogenes* may be because an active haemolysin cannot be released from the *S. anginosus* surface (Fig. 5) and therefore would require a mode of action different from *S. pyogenes*. To our knowledge the mechanism within which *S. pyogenes* SLS acts is unknown, whereas for streptolysin O (the cholesterol-dependent haemolysin of *S. pyogenes*) electron microscographs have been published showing defined membrane pores (Sekiya *et al.*, 1996).

Contrary to intermedilysin of *S. intermedius*, which acts exclusively on human erythrocytes (Nagamune *et al.*, 1996), we found *S. anginosus* SLS to lyse sheep erythrocytes as well as human erythrocytes (Fig. 6). It is well known that SLS of *S. pyogenes* is functional only when bound to bacterial cells, unless it is stabilized by a carrier molecule such as FCS or RNA core (Alouf, 1980). We were able to reproduce this effect in our haemolysin assay, as the culture supernatant of *S. pyogenes* did not show any haemolytic activity on sheep blood. Upon addition of FCS or RNA core to stabilize SLS, strong haemolysis could be detected. For other SLS-like peptides, such as listeriolysin S from *L. monocytogenes*, the same effect could be observed (Cotter *et al.*, 2008). However, for *S. anginosus* SLS the result was different. Neither FCS nor RNA core was able to stabilize the haemolysin. The culture supernatant remained non-haemolytic. Comparing this result with the classical features of *S. pyogenes* SLS and other SLS-like structures such as listeriolysin S, *S. anginosus* SLS appears to be unique in this regard. It is difficult to speculate about the reason for this difference but it has to be noted that there is also a distinct variation in the setup of the gene clusters as *S. anginosus* harbours two copies of *sagA*.

Haemolytic activity of *S. anginosus* does not seem to be regulated by cell density (Figs 4 and 5). Haemolysis could already be observed at OD_{600} 0.2, showing a very early onset in the expression of haemolysin genes that did not change up to OD_{600}

0.8 (Fig. 5). Addition of haemolysed human blood did not lead to an increase in fluorescence, which suggests that the *sag* gene locus is not blood-inducible.

The members of the *S. anginosus* group are emerging pathogens with a high pathogenic potential in infection. Not much is known about their virulence factors. As β-haemolysins are classical virulence factors in many bacteria (Nagamune *et al.*, 2000; Fuller *et al.*, 2002; Humar *et al.*, 2002; Datta *et al.*, 2005; Cotter *et al.*, 2008), the discovery of the genetic background for *S. anginosus* and *S. constellatus* β-haemolysins may be a first step to improve our understanding of the pathogenicity of these bacteria.

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