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Novel Twin Streptolysin S-Like Peptides Encoded in the *sag* Operon Homologue of Beta-Hemolytic *Streptococcus anginosus*

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Streptococcus anginosus **is a member of the anginosus group streptococci, which form part of the normal human oral flora. In contrast to the pyogenic group streptococci, our knowledge of the virulence factors of the anginosus group streptococci, including *S. anginosus*, is not sufficient to allow a clear understanding of the basis of their pathogenicity. Generally, hemolysins are thought to be important virulence factors in streptococcal infections. In the present study, a *sag* operon homologue was shown to be responsible for beta-hemolysis in *S. anginosus* strains by random gene knockout. Interestingly, contrary to pyogenic group streptococci, beta-hemolytic *S. anginosus* was shown to have two tandem *sagA* homologues, encoding streptolysin S (SLS)-like peptides, in the *sag* operon homologue. Gene deletion and complementation experiments revealed that both genes were functional, and these SLS-like peptides were essential for beta-hemolysis in beta-hemolytic *S. anginosus*. Furthermore, the amino acid sequence of these SLS-like peptides differed from that of the typical SLS of *S. pyogenes*, especially in their propeptide domain, and an amino acid residue indicated to be important for the cytolytic activity of SLS in *S. pyogenes* was deleted in both *S. anginosus* homologues. These data suggest that SLS-like peptides encoded by two *sagA* homologues in beta-hemolytic *S. anginosus* may be potential virulence factors with a different structure essential for hemolytic activity and/or the maturation process compared to the typical SLS present in pyogenic group streptococci.**

Streptococcus anginosus is a member of the anginosus group streptococci (AGS), which also include *Streptococcus intermedius*, *S. constellatus* subsp. *constellatus*, and *S. constellatus* subsp. *pharyngis* (1, 2). *S. anginosus* is an opportunistic pathogen and forms part of the normal flora in the human oral cavity, genitourinary tract, and gastrointestinal tract (3), and it is generally considered to have a relatively low pathogenic potential compared with other streptococci, in particular members of the pyogenic species group. However, *S. anginosus* is increasingly being recognized as being able to cause a wide range of purulent infections that commonly manifest as abscess formation, and its presence has also been detected in esophageal cancer (4–9). Despite the increased awareness of the clinical importance of *S. anginosus*, the molecular basis of pathogenicity of this species has not been determined. It has long been observed that some strains of *S. anginosus* give beta-hemolysis on blood agar, and it has been assumed that a beta-hemolytic reaction indicates production of a cytolytic factor(s) thought to be important in pathogenicity. To date, the only beta-hemolytic factor of AGS studied in detail is the toxin intermedilysin, a human-specific cholesterol (CHL)-dependent cytolysin (CDC) secreted from *S. intermedius* (10). Evidence to date indicates that the distribution of *cdc* gene homologues in AGS is limited to *S. intermedius* (11), and there are no reports describing other factors conferring beta-hemolytic capability on some strains of *S. anginosus*.

In the present study, identification of the factor for beta-hemolysis in strains of *S. anginosus* was conducted by a random gene knockout approach on the beta-hemolytic type strain of *S. anginosus*, NCTC10713. The gene(s) responsible for the production of the beta-hemolytic factor found in four nonhemolytic transformants shared high homology with the components of the *sag* operon, including *sagA*, present in pyogenic group streptococci, such as *S. pyogenes*, and encoding the cytolytic peptide streptolysin S (SLS). This is the first report on the distribution and characterization of the *sag* operon homologue in nonpyogenic, oral streptococci (i.e., *S. anginosus*). The specific gene structure of the *sag* operon homologue of *S. anginosus* (sag^{SA}) and the contribution of sag^{SA} to beta-hemolysis in beta-hemolytic *S. anginosus* are described in this article.

MATERIALS AND METHODS

Bacterial strains. The type strain, NCTC10713, was used for molecular analyses of the SLS homologue of *S. anginosus*, and a further 125 strains of *S. anginosus* from human clinical sources were tested for epidemiological purposes. Initial confirmation of these strains as belonging to *S. anginosus* was by the PCR-based method of Takao et al. (12). The type strain of *S. pyogenes* GTC262 and a streptolysin O gene (*slo*)-deleted mutant of *S. pyogenes* strain NIH35 were also used for the comparison of hemolytic characteristics of SLS and SLS homologues. The latter was a kind gift from T. Sumitomo (Osaka University Graduate School of Dentistry). All strains were cultured in brain heart infusion (BHI) broth (Becton, Dickinson and Company) or on BHI agar plates in 5% CO_2 (vol/vol) in air at 37°C. In order to observe beta-hemolysis, strains were spread onto or punctured

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into BHI agar plates containing 5% (vol/vol) human or horse blood and cultured as described above.

Transformation of *S. anginosus* by a competence-stimulating peptide. Transformation of *S. anginosus* NCTC10713^T was conducted using a competence-stimulating peptide (CSP) encoded by the *comC* gene of the *comCDE* system (13). An overnight culture of the target strain was incubated in BHI broth at 37°C in 5% (vol/vol) CO_2 for 3 h in the presence of 100 μg/ml each of CSP and the DNA fragment to be incorporated into the genomic DNA of the target strain. Further incubation was continued at room temperature for 2 h to help facilitate transformation, and the transformation mixture was plated onto BHI agar plates containing appropriate antibiotics for the selection of transformants.

Screening of beta-hemolysis factors by random gene knockout. In order to screen the factor(s) for beta-hemolysis of *S. anginosus* NCTC10713^T, random gene knockout was conducted using plasmid pGhost9:IS*S1* (14). The gene(s) encoding the beta-hemolytic factor was determined by plasmid rescue. Briefly, purified genomic DNA from candidate transformants which had lost their beta-hemolytic capability on BHI blood agar was digested with EcoRI and then self-ligated using DNA ligation Mighty mix (TaKaRa, Japan). Competent cells of *Escherichia coli* strain TG1 (14) were transformed with the self-ligated DNA according to a standard heating protocol and then plated onto LB agar containing 100 μg/ml erythromycin and incubated at 30°C. The genomic region disrupted by insertion with pGhost9:IS*S1* was amplified by PCR using primers pGh9 02 Eco and 5′ IS*S1*(rev) Eco listed in Table 1 and the purified self-ligated DNA as the template. Each amplicon was purified, and DNA sequence analysis was carried out by BEX Co. Ltd. (Tokyo, Japan) using an ABI Prism 3130xl genetic analyzer (Applied Biosystems) DNA sequencer.

Sequencing of the gene(s) encoding the beta-hemolytic factor(s) was carried out by primer walking based on the sequence information obtained by the method mentioned above. The primers used for primer walking are listed in Table 1 (primers 1 to 18). For the preparation of templates for sequencing, PCR was conducted using PrimeSTAR DNA polymerase (TaKaRa) and the genomic DNA prepared from *S. anginosus* NCTC10713^T as the template. The DNA sequencing of the amplicon was carried out as described above.

Phylogenetic analysis of beta-hemolytic factors. A phylogenetic tree of SagA homologues was constructed by Njplot (15) using microcin B17 as an out-group. The amino acid sequences (accession numbers in parentheses) of SagA and its homologues from the following organisms were used for this phylogenetic analysis: *Streptococcus pyogenes* MGAS5005 (CP000017), *Streptococcus dysgalactiae* subsp. *equisimilis* GGS_124 (AP010935), *Streptococcus equi* subsp. *equi* 4047 (NC_012471), *S. equi* subsp. *zooepidemicus* MGCS10565 (NC_011134), *S. equi* subsp. *zooepidemicus* H70 (NC_012470), *Streptococcus iniae* 9117 (AF465842), *Streptococcus pseudoporcinus* SPIN20026 (NZ_AENS01000034), *Streptococcus constellatus* subsp. *pharyngis* SK1060 (NZ_AFUP01000001), *Streptococcus anginosus* NCTC10713^T (SagA1 and SagA2; JN619420), *S. anginosus* R84/4972 (SagA1 and SagA2; JN619421), *Clostridium botulinum* Loch Maree (clostridiolysin S; CP000962), *C. botulinum* ATCC 3502 (AM412317), *C. botulinum* 657 (clostridiolysin S; NC_012658), *Listeria monocytogenes* F2365 (listeriolysin S; NC_002973), *Staphylococcus aureus* RF122 (staphylysin S; AJ938182) (16), and *Escherichia coli* MC4100 (microcin B17; M15469). Prior to Njplot analysis, the amino acid sequences described above were aligned by ClustalX (17).

PCR amplification of the genes encoding beta-hemolytic factors. Each entire coding region for the beta-hemolytic factor was amplified by PCR with the primer sets *sagA1*-Fw and *sagA1*-Bw for *sagA1*, *sagA2*-Fw and *sagA2*-Bw for *sagA2*, *sagA1*-Fw and *sagA2*-Bw for the region containing *sagA1* and *sagA2*, and *sagA1*-Fw and *sagB*-A2-Bw for the region from *sagA1* to *sagB* and with the template genomic DNA prepared by the method described previously (18). The reaction mixture (total volume, 10 μl), containing 0.25 U of *GoTaq* DNA polymerase (Promega), 0.2 mM deoxynucleoside triphosphates (dNTPs) (Promega), 1.5 mM $MgCl_2$ (Promega), 0.25 μM primers, and genomic DNA, was pretreated for 2 min at 94°C. Subsequently, the mixture was amplified (30 cycles) using the following parameters: 98°C for 30 s, 55°C for 30 s, and 72°C for 10 s, before a final heating step at 72°C for 5 min. The amplification of target genes was confirmed by agarose gel electrophoresis using TBE buffer system (89 mM Tris, 89 mM borate, 2 mM EDTA [pH 8.0]).

Measurement of cytolytic activity for culture supernatant. The culture supernatants of tested strains were prepared by centrifugation (9,000 × *g*, 5 min) of mid- to late-logarithmic-phase cultures in BHI (optical density at 660 nm [OD_{660}] of 1.0) incubated at 37°C in 5% (vol/vol) CO_2. Human erythrocytes washed with phosphate-buffered saline (PBS) were incubated with serially diluted culture supernatant with PBS at 37°C for 1 h and then centrifuged (750 × *g*, 5 min). Subsequently, the precipitated intact erythrocytes were washed twice with PBS (750 × *g*, 5 min) and resuspended in sterilized deionized water. The OD_{540} of each suspension was measured in order to calculate the hemolytic activity according to the method previously reported (10). To assay susceptibility to inhibitors, prior to the interaction with erythrocytes the culture supernatant was preincubated for 5 min at room temperature with (i) lecithin at a final concentration of 0.02% (wt/vol), (ii) cholesterol at a final concentration of 1 μM, or (iii) both.

Construction of deletion mutants for the genes encoding the beta-hemolytic factor. The deletion mutants for *sagA1* and/or *sagA2* were constructed by double-crossing-over homologous recombination with an erythromycin resistance marker for selection. The fragments for transformation were generated by PCR amplification using PrimeSTAR DNA polymerase (TaKaRa). A PCR primer set with individual restriction sites at each 5′ end was used in amplification of the erythromycin resistance gene cassette (*erm*) in order to secure effective ligation and the desired direction (Table 1).

In order to construct several mutants used in this study, a strain with *erm* and without *sagA1* and *sagA2* (template mutant) was first constructed as follows. Each component of the insert fragment was amplified by PCR using the following primer sets: (i) up-*sagA2*-fus-Fw and *sagC*-inv-Bw for the amplification of the region from *sagB* to *sagC*, (ii) up-*sagA2*-Fw-(BamHI) and up-*sagB*-fus-Bw for the promoter region of *sagA2*, (iii) up-operon-Fw1 and up-operon-Bw(PstI) for the upstream region of *sagA1*, and (iv) *erm*-Fw(BamHI) and *erm*-Bw(PstI) for the erythromycin-resistant gene cassette. Amplicons were purified using a QIAEX II gel extraction kit (Qiagen). The purified fragments of *sagB* to *sagC* and the *sagA2* promoter region were used as a template for fusion PCR carried out using *Ex Taq* DNA polymerase (TaKaRa) with the following reaction conditions: denaturation for 2 min at 94°C, followed by 30 cycles of 30 s at 98°C, 30 s at 55°C, and 10 s at 72°C before a final heating at 72°C for 5 min. The fusion PCR product, the fragment upstream of *sagA1*, and the *erm* fragment, were digested by restriction enzymes (PstI for upstream of *sagA1*, BamHI for the fusion PCR product, and both PstI and BamHI for *erm*), purified, and then ligated using DNA ligation Mighty mix (TaKaRa). The ligated mixture was used as the template in nested PCR with primer set up-operon-Fw2 and *sagB*-inv-Bw (Table 1), and the purified DNA fragment was used for transformation by CSP as described above. The clone with the desired mutation in the genomic DNA was screened for growth in the presence of 1 μg/ml erythromycin and the formation of a zone of beta-hemolysis on blood agar. The deletion of the target gene was confirmed by DNA sequencing of the amplified fragment of the target region with PrimeSTAR DNA polymerase (TaKaRa). The genomic DNA of the constructed template mutant was used as the template for PCR amplification of the *erm* gene fragment to generate the *sagA1* deletion mutant, the *sagA2* deletion mutant, and the control mutant with the *erm* gene upstream of *sagA1*.

Next, in order to prepare the remaining fragment for construction of these mutants, PCR amplification was conducted using the genomic DNA of *S. anginosus* NCTC10713^T as a template using primers up-*sagA2*-Fw-(BamHI) and *sagC*-inv-Bw for construction of the *sagA1* deletion mutant, up-operon-Fw(SalI), *sagA1*-Bw, up-*sagB*-Fw, and *sagC*-inv-Bw for construction of the *sagA2* deletion mutant, and up-operon-Fw(SalI) and

TABLE 1 Primers used in this study

No.	Name	Sequence (5′→3′)[a]	Target	Use
1	pGh9 02 Eco	GGTATACTACTGACAGCTTCC	pGhost9 IS*S1*	Sequencing
2	5′ IS*S1*(rev) Eco	CGTAGATAATAACCAACAGCG	pGhost9 IS*S1*	Sequencing
3	up-*sagA2*-inv-Bw	TTGAGTTGTTTCTGCCACGC	*sagA2*	Sequencing
4	*sagB*-inv-Bw	AGTCCTTCAACCTGTCTGGC	*sagB*	Sequencing
5	*sagC*-Fw1	ATGAAATATCAATTAAATAG	*sagC*	Sequencing
6	*sagC*-inv-Bw	ATTGTTCGCCCAGCCTCTGC	*sagC*	Sequencing
7	*sagC*-Fw2	CACAAGGTGCGCTGGCAAAA	*sagC*	Sequencing
8	*sagC*-Fw3	CCCGTGAAATGGTAAAAGAC	*sagC*	Sequencing
9	*sagD*-inv-Bw	AAAATCCCTGTCCGATTGCC	*sagD*	Sequencing
10	*sagD*-Fw	CCAATTGATATGACCATCGG	*sagD*	Sequencing
11	*sagE*-Fw	TATTGATGTTGTGCTGACGC	*sagE*	Sequencing
12	*sagF*-Fw	TTTGGTGTATGGTAGCCACC	*sagF*	Sequencing
13	*sagH*-Bw	GCTCCTTCAAGCTGATAGCG	*sagH*	Sequencing
14	*sagH*-Bw2	CCTAAGCCTAGATTAACGAG	*sagH*	Sequencing
15	*sagI*-down-Bw1	CAGCTGGCTGCTTTGTTGGC	*sagI*	Sequencing
16	*sagI*-inv-Fw	AAGGGAGTTTATATGCTTCC	*sagI*-down	Sequencing
17	*sagI*-down-Bw2	GGCACTATTAAAAATAGCAT	*sagI*-down	Sequencing
18	*sagI*-down-Bw3	GCTACAGACTGTTGACGAAC	*sagI*-down	Sequencing
19	*sagA1*-Fw	ATGTTAAAATTTTCTTCAAACG	*sagA1*	Detection
20	*sagA1*-Bw	TTATTTTGTAGGTGCTACGG	*sagA1*	Detection
21	*sagA2*-Fw	ATGCTTAAATTAGATTCACATATTATGG	*sagA2*	Detection
22	*sagA2*-Bw	TTAAGGTTTAATGTTTGTTGAACC	*sagA2*	Detection
23	up-operon-Fw1	GACGAGCAGATGTAGATGCC	up-operon	Mutant construction
24	up-*sagA2*-fus-Fw	ATGAAAAATTATGAGAATGC	up-*sagA2*	Mutant construction
25	*erm*-Fw(BamHI)	AATGGATCCCCCGATAGCTTCCGCTATTG	*erm*	Mutant construction
26	*erm*-Bw(PstI)	GTACTGCAGCTAATAATTTATCTACATTCC	*erm*	Mutant construction
27	*erm*-Fw(SalI)	CGCGTCGACCCCGATAGCTTCCGCTATTG	*erm*	Mutant construction
28	up-operon-Fw2	TGTCGCACGGAACCATTCGC	up-operon	Mutant construction
29	up-operon-Bw(PstI)	GCGCTGCAGGCGCGTTCTTATAGCATTTG	up-operon	Mutant construction
30	up-operon-Fw(SalI)	CGCGTCGACGATGGTTATATTTGTGAAATAGG	up-operon	Mutant construction
31	up-operon-Bw	GCGTAAACATTTTCAAACTAC	up-operon	Mutant construction
32	up-*sagA2*-Fw(BamHI)	CAGGATCCTCATGGATTGCTAGC	up-*sagA2*	Mutant construction
33	up-*sagB*-Fw	AATTTTGTTTATAGGAATTAG	up-*sagB*	Mutant construction
34	up-*sagB*-fus-Bw	GCATTCTCATAATTTTTCATC	up-*sagB*	Mutant construction
35	*sagA1*-comp-Fw1(EcoRI)	GGGAATTCGGATTTGATAGTAATGTACG	up-*sagA1*	Complementation
36	*sagA1*-comp-Fw2(EcoRI)	GGGAATTCAGTGTAAAGATTAGCTAGCC	up-*sagA1*	Complementation
37	*sagA1*-comp-Bw(PstI)	GCGCTGCAGGCTTCATCCAAAGAATCGTC	*sagA1*-down	Complementation
38	*sagA2*-comp-Fw(EcoRI)	GGGAATTCGTTCATGATGAGTTAAAAAC	up-*sagA2*	Complementation
39	*sagA2*-comp-Bw(PstI)	GCGCTGCAGAGATGAATCGATGCTTTACC	*sagA2*-down	Complementation
40	*sagB*-S1-Fw	AGCTTGTTGCTCAGCATCTCATGCC	*sagB*	5′-RACE
41	*sagB*-A1-Bw	GATGATACTCTGAGAAAGAGAAGTGGTTGG	*sagB*	5′-RACE
42	*sagB*-S2-Fw	TTCTGAATTTTTCACAGATTCAGCTGTAGC	*sagB*	5′-RACE
43	*sagB*-A2-Bw	GCTTCGTCTCTTGTCAGGTGAGAGG	*sagB*	5′-RACE
44	*sagB*-RT-Bw(phospho)	Phospho-AGACAGAGAAGCTGC	*sagB*	5′-RACE
45	M13 Forward primer	GTAAAACGACGGCCAGT	pUC18	Sequencing
46	M13 Reverse primer	CAGGAAACAGCTATGAC	pUC18	Sequencing

[a] Restriction sites are underlined.

sagC-inv-Bw for construction of the control mutant. The prepared components were digested with BamHI (for the *sagA1* deletion mutant) or SalI (for the *sagA2* deletion mutant and control strain) and then ligated, and the mixture was used as the PCR template with the primer set up-operon-Fw2 and *sagB*-inv-Bw (Table 1). The purified amplicon was used for transformation.

In order to use a host strain for complementation experiments of *sagA1* and *sagA2* described below, another double-deletion mutant for *sagA1* and *sagA2* was constructed as follows. Each fragment required was amplified by PCR using the following primer sets and templates: primer set up-operon-Fw1 and up-operon-Bw and the genomic DNA prepared from the control mutant for amplification of the region containing the *erm* gene and primer set up-*sagB*-Fw and *sagC*-inv-Bw and the genomic DNA prepared from *S. anginosus* NCTC10713^T for the amplification of the region containing *sagB* to *sagC*. The purified fragments were phosphorylated and ligated using DNA ligation Mighty mix (TaKaRa), and nested PCR was conducted using primer set up-operon-Fw2 and *sagB*-inv-Bw to prepare the fragment for transformation.

The purified DNA fragments prepared by nested PCR were used for transformation with CSP. The clone with the desired mutation in the genomic

DNA was also screened by the method described above. The construction of the mutants was also confirmed by DNA sequencing of the amplified fragment of the target region using PrimeSTAR DNA polymerase (TaKaRa).

Complementation experiment of beta-hemolysis in the *sagA1* and *sagA2* double-deletion mutant. The plasmids for complementation experiments were constructed as follows. DNA fragments for insertion were amplified by PCR with primer sets (i) *sagA1*-comp-Fw1(EcoRI) and *sagA2*-comp-Bw(PstI) for amplification of the region of *sagA1* plus *sagA2* with three potential prokaryotic promoters for *sagA1*, (ii) *sagA1*-comp-Fw1(EcoRI) and *sagA1*-comp-Bw(PstI) for amplification of the region of *sagA1* with three potential prokaryotic promoters for *sagA1*, and (iii) *sagA2*-comp-Fw(EcoRI) and *sagA2*-comp-Bw(PstI) for amplification of the region of *sagA2* with their own promoter, using genomic DNA from *S. anginosus* NCTC10713^T as the template. Other DNA fragments were also amplified with primer sets (iv) *sagA1*-comp-Fw2(EcoRI) and *sagA2*-comp-Bw(PstI) for amplification of the region of *sagA1* plus *sagA2* with the latter two of the three potential prokaryotic promoter sequences for *sagA1* and with (v) *sagA1*-comp-Fw2(EcoRI) and *sagA1*-comp-Bw(PstI) for amplification of the region of *sagA1* with the latter two of the three potential prokaryotic promoter sequences for *sagA1*. These amplicons were digested with both EcoRI and PstI, ligated into pSETN1 (19) digested with the same restriction enzymes. Competent cells of *Escherichia coli* DH5α Z1 (20) were transformed with these plasmids according to the standard heating method and then plated on LB agar containing 40 μg/ml chloramphenicol and incubated at 37°C. After sequence confirmation, the constructed plasmids were used for complementation experiments. Each plasmid for complementation was introduced into the *sagA1* and *sagA2* double-deletion mutant by the CSP described above and plated onto BHI agar containing 3 μg/ml chloramphenicol and 1 μg/ml erythromycin. The complementation of target genes was evaluated by the formation of beta-hemolysis on blood agar.

Investigation of transcription of the *sag* operon homologue of *S. anginosus*. The investigation of transcription for *sag*^{SA} was conducted by 5′ rapid amplification of cDNA ends (5′-RACE). Briefly, the total RNA was prepared from the early-log-phase (8 h) culture of *S. anginosus* NCTC10713^T by NucleoSpin RNA II (Macherey-Nagel) according to the manual appended to the kit with a modification for the cell lysis condition using lysozyme and mutanolysin with an RNase inhibitor. 5′-RACE was conducted with the 5′-Full RACE Core set (TaKaRa) according to the manual appended to the kit using primer *sagB*-RT-Bw(phospho) for reverse transcription. The PCR enzyme used was *Ex Taq* DNA polymerase (TaKaRa), the primer set for the first PCR was *sagB*-S1-Fw and *sagB*-A1-Bw, and that for the second PCR was *sagB*-S2-Fw and *sagB*-A2-Bw (Table 1).

In order to determine the start point of each *sag*^{SA} transcript, sequencing of the products obtained in 5′-RACE analysis was carried out as follows. The 5′-RACE product fractions were purified from the agarose fragments (two fractions: upper region from around 1,000 bp to 800 bp and the lower region from around 500 bp to 200 bp) after agarose gel electrophoresis and phosphorylated by T4 polynucleotide kinase (Roche) at 37°C for 1 h. Plasmid pUC18 was cut by SmaI, and both resultant blunt ends were dephosphorylated using shrimp alkaline phosphatase (Roche). Subsequently, the purified dephosphorylated pUC18 and each phosphorylated 5′-RACE product fraction were ligated using DNA ligation Mighty mix (TaKaRa). The competent cells of *E. coli* JM109 were transformed by each recombinant plasmid and screened on an LB plate containing 50 to 100 μg/ml of ampicillin, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 0.004% (wt/vol) 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). After an overnight incubation at 37°C, the plasmids were purified from white colonies, and the size of each cloned fragment was confirmed by double digestion with EcoRI and SalI. Finally, DNA sequence analysis of each insert was carried out using sequence primers, M13 Forward primer and M13 Reverse primer (Table 1).

Nucleotide sequence accession numbers. Nucleotide sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession no. JN619420 for the *sag* operon homologue of *S. anginosus* NCTC10713^T and JN619421 and JN619422 for the regions around *sagA1* and *sagA2* of *S. anginosus* R84/4972 and R87/1657, respectively.

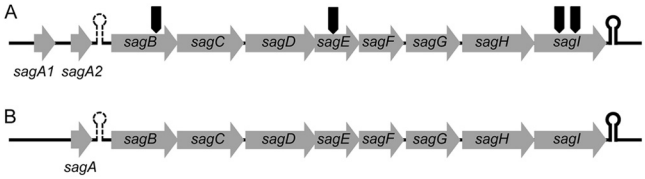

FIG 1 Genetic structure of *sag*^{SA} (A) and the "typical" *sag* operon from other pyogenic group streptococci (B). *sag*^{SA} is characterized by the presence of twin *sagA* homologues (*sagA1* and *sagA2*). The arrows in panel A indicate the insertion sites of pGhost9:IS*S1* in four nonhemolytic transformants obtained in random gene knockout experiments.

RESULTS

Screening and identification of the gene encoding the beta-hemolysis factor of *S. anginosus*. Screening of the gene encoding the beta-hemolytic factor was carried out by random gene knockout with pGhost9:IS*S1* in *S. anginosus* strain NCTC10713^T. Previously, successful random gene knockout with this vector system was reported in *Streptococcus agalactiae* for screening the genetic locus essential for the production of hemolysin (21). Four nonhemolytic clones were detected and revealed that the genes with insertion of pGhost9:IS*S1* were homologues of *sagB*, *sagE*, and *sagI* (2 clones), corresponding to genes coding for components of the *sag* operon of pyogenic group streptococci (Fig. 1).

The entire DNA sequence of the *sag*^{SA} was determined by primer walking using the primers listed in Table 1 and based on the partial DNA sequence information of the *sagE* and *sagI* genes. In comparison to the nucleotide sequencing of the shotgun sequence of *S. anginosus* SK52 (NCTC10713^T) in the NCBI database (BioProject PRJNA64677), our sequence data in the present study differed by 2 nucleotides in the 9210 base sequence of the *sag*^{SA}: one located in the *sagB* homologue and the other located in the *sagI* homologue. The nucleotide difference in *sagI* leads to a silent mutation. However, the nucleotide difference in the ORF of *sagB* is important because 1 base deletion is found in the BioProject database, and this nucleotide deletion results in a frameshift mutation. If the BioProject data are correct, this mutation results in a truncation of the *sagB* homologue to make a short product of 16 amino acid residues, whereas based on our sequencing results, SagB is predicted to code for 316 amino acid residues (i.e., the same number as SagB of *S. pyogenes*). This suggests that the data registered in the BioProject database for the *sag* operon homologue from *S. anginosus* SK52 should be corrected.

It was determined that *sag*^{SA} consisted of functional genes homologous to the components of the *sag* operon of *S. pyogenes* by gene analysis using GENETYX software. The arrangement of the genes from *sagB* to *sagI* of *sag*^{SA} was very similar to that of *S. pyogenes* (Fig. 1). However, a significant difference was observed around the *sagA* gene. Interestingly, two *sagA* homologues existed in tandem at the upstream region of the *sagB* gene in *sag*^{SA}. No such tandem structure was found in the *sag* operons of pyogenic group streptococci with a single *sagA* gene (Fig. 1). As shown in Fig. 1A, the two *sagA* homologues in *S. anginosus* are designated *sagA1* and *sagA2*, respectively.

A phylogenetic analysis for the transcriptional products of

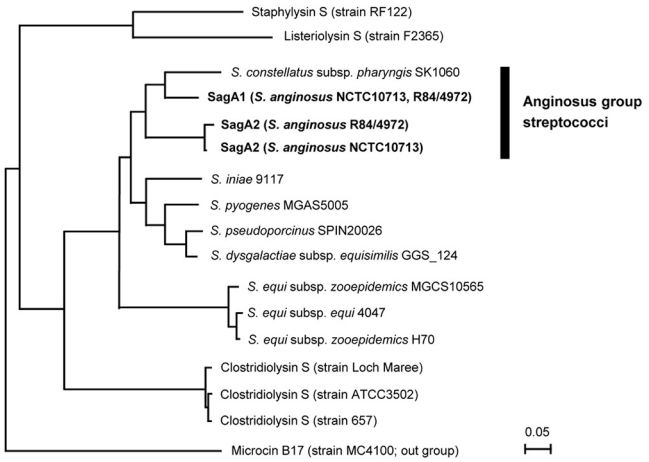

FIG 2 Phylogenetic tree of SagA of streptococci and their homologues in other bacterial genera. The amino acid sequences were aligned by ClustalX, and the phylogenetic tree was constructed by Njplot using microcin B17 as an outgroup. SagA1 and SagA2 from *S. anginosus* strains are indicated in boldface.

sagA1 and *sagA2* was carried out (Fig. 2). The phylogenetic tree shows that SagA homologues of *S. anginosus* (SagA1 and SagA2) were clustered separately from a pyogenic streptococcal SagA (Fig. 2). Recently, whole-genome shotgun sequencing of *S. constellatus* subsp. *pharyngis* strain SK1060 (BioProject PRJNA67177) was reported, and it was revealed that this subspecies also has a homologue of the *sag* operon: SagA deduced from the *sagA* gene in this homologue was more similar to SagA1 than was SagA2.

The DNA sequence of the genes making up sag^{SA} was compared with their homologues in the *sag* operons found in other pyogenic group streptococci, including *S. pyogenes* MGAS5005 (GenBank accession no. CP000017), *S. dysgalactiae* subsp. *equisimilis* GGS_124 (GenBank accession no. AP010935), *S. equi* subsp. *zooepidemicus* MGCS10565 (GenBank accession no. CP001129), and *S. iniae* strain 9117 (GenBank accession no. AF465842). The sequence identity of each gene is listed in Table 2. The components of sag^{SA}, *sagA* encoding the cytolytic peptide protoxin (22), *sagB*, *sagC*, and *sagD* contributing to heterocycle formation of protoxin (23), and *sagG*, *sagH*, and *sagI* contributing to extracellular transport of the mature toxin (22) shared higher DNA sequence identity (>66%) with those of the pyogenic group species *sag* operon, although the sequence homologies of *sagE* and *sagF* were found to be relatively low (Table 2). These DNA homology results indicate that the product of each component of sag^{SA} may have a similar function to the corresponding product derived from *sag* operons in pyogenic group species (*sagBCD* to heterocycle formation and *sagGHI* to extracellular transport) and that *sagA1* and *sagA2* will be processed and exported to the extracellular milieu to function in the same way as SLS.

Structural comparison of *sagA* and the translation product in *S. anginosus* and *S. pyogenes*. DNA sequence analysis of the region around *sagA1* and *sagA2* in *S. anginosus* revealed that *sagA1* and *sagA2* had three prokaryotic promoter sequences and one potential prokaryotic promoter sequence, respectively, with an additional promoter located between *sagA2* and *sagB* for the *sagB* gene and other downstream genes in the operon (Fig. 3). The *sag* operon of *S. pyogenes* also had the potential prokaryotic promoter sequences upstream of *sagA*, although the positions of these promoters differed from those of the promoters for sag^{SA} (Fig. 3). Interestingly, a terminator-like structure was found upstream of *sagB* in both *S. anginosus* and *S. pyogenes*. However, in *S. anginosus* the terminator-like structure was located between the *sagB* promoter and its Shine-Dalgarno (SD) sequence, in contrast to *S. pyogenes*, where it partially overlapped the potential promoter sequence of *sagB* (Fig. 3). Alignment of the regions upstream of *sagB* for *S. anginosus* and *S. pyogenes* showed that the position of *sagA* in the latter species corresponded to the position of *sagA1* in *S. anginosus* and not to that of *sagA2* (Fig. 3). Overall, the region within the operons from *sagB* to *sagI* exhibited a much higher degree of conservation between *S. anginosus* and pyogenic group streptococci than was observed in the region around *sagA*. Consequently, the alignment of the deduced amino acid sequences of *sagA1* and *sagA2* products, SagA1 and SagA2 of *S. anginosus* $NCTC10713^T$ (Fig. 4A), shows that the primary structures of SagA1 and SagA2 are highly conserved, although several amino acid deletions and substitutions were observed, especially in the C-terminal region of SagA2. Moreover, the alignment of SagA1, SagA2, and SLS/SagA of *S. pyogenes* revealed sequence conservation between the peptides overall (Fig. 4B), but with considerable diversity in the C-terminal region, including a notable deletion of a single amino acid (corresponding to S39 in SLS/SagA of *S. pyogenes*) observed in both SagA1 and SagA2 of *S. anginosus* (Fig. 4B). The amino acids potentially contributing to heterocycle formation are also shown in Fig. 4C, and the number and location of candidate amino acids concerned with oxazoline/thiazoline formation were observed to vary between SagA1 and SagA2 of *S. anginosus* and SagA of *S. pyogenes*.

Transcription of the *sag* operon of *S. anginosus*. The transcription of sag^{SA} was confirmed with particular regard to the function of the promoter(s) in the operon. Since sag^{SA} had several predicted promoters (shown in Fig. 3), 5′-RACE analysis was carried out. Partial cDNAs covering from the 5′ end to midway

TABLE 2 Sequence homology of the genes composing sag^{SA} of strain $NCTC10713^T$ against the corresponding genes of other streptococcal *sag* operons

Species[a]	% identity[b]									
	sagA1[c]	*sagA2*[c]	*sagB*	*sagC*	*sagD*	*sagE*	*sagF*	*sagG*	*sagH*	*sagI*
S. pyogenes	69	70	69	69	74	56	53	71	70	67
S. dysgalactiae subsp. *equisimilis*	71	68	71	69	75	57	57	72	71	68
S. equi subsp. *zooepidemicus*	73	67	71	68	74	60	57	69	73	68
S. iniae	70	68	71	70	75	62	55	70	72	66

[a] Shown are results from *S. pyogenes* MGAS5005, *S. dysgalactiae* subsp. *equisimilis* GGS_124, *S. equi* subsp. *zooepidemicus* MGCS10565, and *S. iniae* 9117.
[b] The results represent the percentage of identity of each gene in the sag^{SA} of strain $NCTC10713^T$ versus the corresponding gene in other streptococcal *sag* operons.
[c] Both *sagA1* and *sagA2* genes were compared to the *sagA* genes of other streptococci.

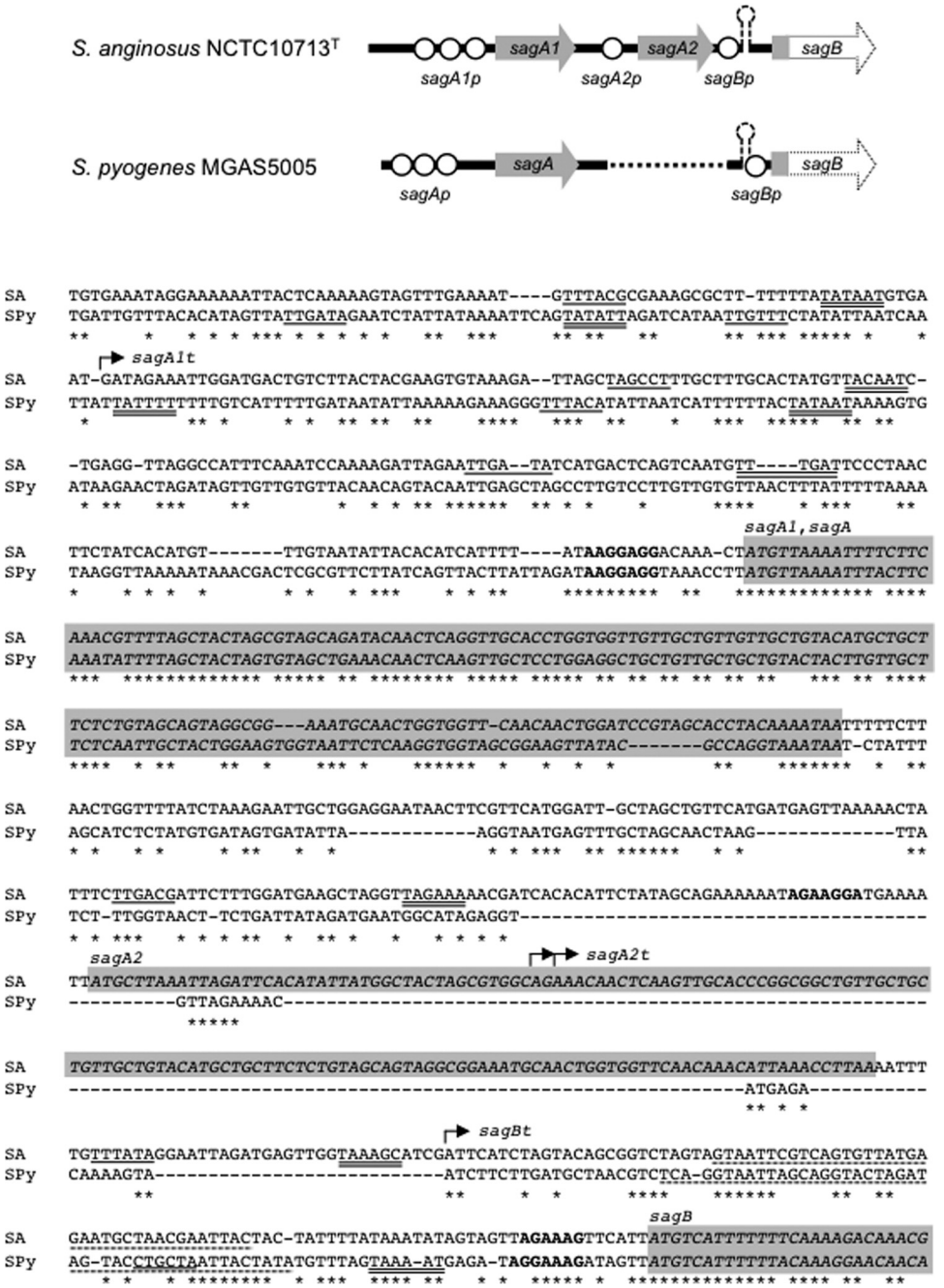

FIG 3 Genetic structure of the promoter regions of *sagA1* to *sagB* in *S. anginosus* and *sagA* to *sagB* in *S. pyogenes*. The typical prokaryotic promoters predicted from their DNA sequences are shown with suffix "p" in the scheme at the top. The nucleotide sequence alignments of the region from the promoter of *sagA1* to the head part of *sagB* ORF of *S. anginosus* NCTC10713^T (SA) and from the promoter of *sagA* to the head part of *sagB* of *S. pyogenes* MGAS5005 (SPy) are also shown. The deduced −35 and −10 regions are single and double underlined, respectively. The potential promoters with a score value above 57.00 predicted in the analyses using the program "Search for Promoter Sequence" within GENETYX software are shown. Moreover, a deduced promoter for *sagB* of *S. anginosus* is also shown, although the score value in this case was 53.25. Rho-independent terminators are indicated by a broken line, and SD sequences shown in bold. The ORF of each gene is shown italicized with a shaded background. The start point of each transcript of *sag*^{SA} revealed by 5′-RACE analysis (*sagA1t*, *sagA2t*, and *sagBt*) is indicated by an arrow.

through the *sagB* ORF of each transcript were amplified by PCR. The result shows that two major bands were amplified: an upper band of approximately 1,000 bp and a lower band of below 500 bp (Fig. 5A). The length of both amplicons (shown in Fig. 5A) corresponded well with the sizes predicted from the sequence information of *sag*^{SA}, i.e., with larger and smaller amplicons transcribed from the *sagA1* promoter(s) and from the *sagA2* promoter, respectively. A small band of approximately 250 bp in size, predicted as the transcript from the *sagB* promoter, was detected as a faint band in this analysis.

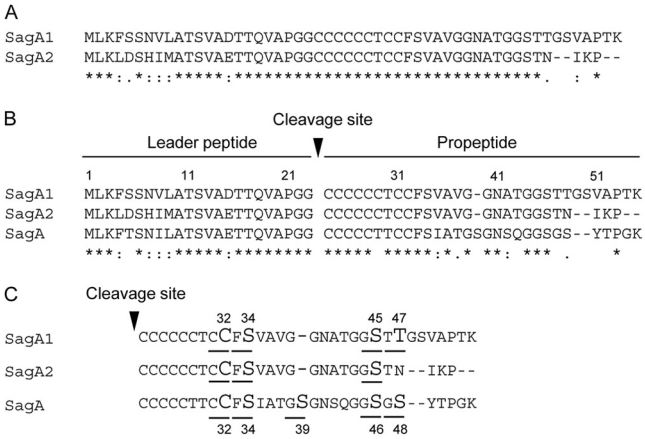

FIG 4 Alignments of amino acid sequences of SagA and homologues. (A) Identity of SagA1 with SagA2 of *S. anginosus* NCTC10713^T. (B) Alignment of the amino acid sequences of SagA homologues: SagA1 and SagA2 of *S. anginosus* NCTC10713^T and typical SagA of *S. pyogenes* MGAS5005. The alignment analyses were conducted using ClustalX. The symbols indicating identity/homology of primary sequence are as follows: *, identical amino acid, ":," amino acid highly conserved; "." amino acid somewhat similar. The amino acids deduced to contribute to heterocycle formation in SagA1, SagA2, and SagA are underlined, and the amino acids predicted to be indispensable residues for heterocycle formation according to the analysis in SagA are shown in large font (C).

Subsequently, the start points of the *sag*^{SA} transcripts were also investigated after cloning of the 5′-RACE products. Although the transcript induced by the *sagB* promoter was relatively faint in Fig. 5A, we succeeded in cloning the cDNAs with the 5′-end sequence information of the transcripts from three gene promoters (Fig. 5B). Sequencing of the cDNA clones revealed that the promoters for *sagA1*, *sagA2*, and *sagB* were all functional *in vivo*, and the start points of *sag*^{SA} transcription are shown in Fig. 3. Among the three potential prokaryotic promoters for *sagA1*, it was revealed that the first promoter (−35 sequence, TTTACG; −10 sequence, TATAAT; score, 67.46) was preferentially used for the transcription from *sagA1* onward. The promoter for *sagA2* (−35 sequence, TTGACG; −10 sequence, TAGAAA; score, 60.36) was also functional; however, the cloned transcript cDNAs were partially deleted in their 5′-terminal region, including midway through the *sagA2* gene with their SD sequence. This fact suggests the transcript induced by this promoter may be unstable, and the intracellular level of intact transcript may be low. Interestingly, the predicted promoter for *sagB* (−35 sequence, TTTATA; −10 sequence, TAAAGC; score, 53.25) also functioned *in vivo*. Nevertheless the latter was difficult to detect, as shown in Fig. 5A, perhaps because of low transcription frequency.

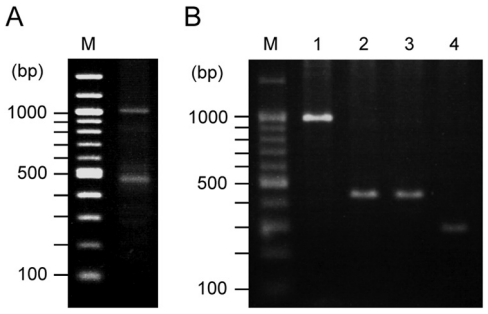

FIG 5 (A) 5′-RACE analysis of *sag*^{SA}. Total RNA from early-logarithmic-phase cells was prepared and used for 5′-RACE analysis. (A) The resulting DNA fragments containing the 5′ terminus of cDNA from mRNA sequence(s) of *sag*^{SA} were amplified by nested PCR and analyzed by agarose-gel electrophoresis. M, hundred-base-pair ladder marker. (B) Cloned 5′-RACE products for sequencing. The 5′-RACE product cloned in the SmaI site of pUC18 was obtained by double digestion with EcoRI and SalI and separated by agarose gel electrophoresis. M, hundred-base-pair ladder size marker. Lanes 1 to 4 show the 5′-end part of cDNA deduced to be the transcripts from *sagA1p* (lane 1), *sagA2p* (lanes 2 and 3), and *sagBp* (lane 4).

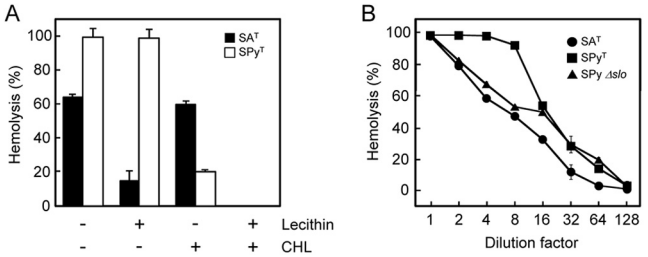

FIG 6 (A) Effect of lecithin and cholesterol on hemolysis induced by the hemolysin secreted by *S. anginosus* and *S. pyogenes* type strains. Culture supernatants were preincubated with 0.02% (wt/vol) lecithin or 1 μM cholesterol (CHL) and then incubated with human erythrocytes. Control reaction mixtures contained no lecithin or cholesterol. All results are shown as mean values ($n = 3$) with standard deviations (SD). (B) Dose dependency of hemolysis by culture supernatant of the *S. anginosus* type strain, *S. pyogenes* type strain, and the *S. pyogenes slo*-deleted mutant (Δ*slo*). The supernatants from mid- to late-logarithmic-phase culture (OD_{660} of 1.0) of the *S. anginosus* type strain (circles), *S. pyogenes* type strain (squares), and the *S. pyogenes* Δ*slo* mutant (triangle) were 2-fold serially diluted and then incubated with human erythrocytes. All results are shown as mean values ($n = 2$) with differences.

Characterization of beta-hemolysis induced by *S. anginosus*. As a result of the random gene knockout experiment indicating that *sag*^{SA} is responsible for beta-hemolysis in *S. anginosus*, the hemolytic activity was characterized. It was reported that the hemolytic activity of SLS secreted by *S. pyogenes*, which is a typical mature hemolysin product of the *sag* operon, is inhibited by the membrane lipid component lecithin (24). Therefore, the hemolytic activity shown by the culture supernatant of *S. anginosus* NCTC10713^T was investigated in the absence or presence of lecithin. It was observed that the hemolysis induced by *S. anginosus* NCTC10713^T culture supernatant decreased markedly in the presence of lecithin (Fig. 6A), suggesting that the SLS-like peptide secreted from *S. anginosus* NCTC10713^T has similar biochemical properties and biological function to the SLSs of pyogenic group streptococci, such as *S. pyogenes*. On the other hand, obvious inhibition of the hemolytic activity in the presence of lecithin was not shown for culture supernatant of *S. pyogenes* GTC262^T (Fig. 6A). However, the hemolytic activity of culture supernatant of *S. pyogenes* GTC262^T was significantly inhibited in the presence of CHL and completely inhibited by the combination of CHL and lecithin. These results suggest that a large part of the hemolysis by *S. pyogenes* GTC262^T depends on the hemolysin with susceptibility to CHL (i.e., SLO).

The dose dependency of hemolytic activity shown by culture supernatants of *S. anginosus* NCTC10713^T and *S. pyogenes* GTC262^T was also investigated. Similarly to the result shown in Fig. 6A, the hemolytic activity of *S. pyogenes* GTC262^T was higher than that of *S. anginosus* NCTC10713^T (Fig. 6B). In order to exclude the involvement of SLO, a Δ*slo* mutant of *S. pyogenes* was

TABLE 3 Detection of the *sagA1* and *sagA2* genes by PCR amplification in *S. anginosus* clinical isolates

Strain type	Positivity of PCR amplification (no. of positive strains/total)		
	Only *sagA1*	Only *sagA2*	*sagA1* and *sagA2*
Beta-hemolytic	0/24	0/24	24/24
Nonhemolytic	0/102	0/102	0/102

also investigated. It was revealed that the dose dependency of hemolytic activity of *S. anginosus* NCTC10713^T was nearly the same as that of the Δ*slo* mutant of *S. pyogenes* secreting only SLS. From the data shown in Fig. 6A and B, we conclude that hemolysis by beta-hemolytic *S. anginosus* NCTC10713^T is induced by a similar hemolysin to the SLS of *S. pyogenes*.

Contribution of the *sagA1* and *sagA2* genes to beta-hemolysis of *S. anginosus*. The correlation between the presence of the *sag*^{SA} operon and beta-hemolysis was also investigated genotypically. One hundred twenty-six strains of *S. anginosus* were assayed for possession of the *sagA1* and *sagA2* genes by PCR amplification, and it was revealed that, without exception, all beta-hemolytic *S. anginosus* strains possessed both *sagA1* and *sagA2* genes, and all nonhemolytic strains had neither *sagA1* nor *sagA2* genes (Table 3). The results of testing of 3 representative strains of beta-hemolytic *S. anginosus* (including NCTC10713^T) and 4 nonhemolytic *S. anginosus* strains are shown in Fig. 7. All beta-hemolytic *S. anginosus* strains had the characteristic tandem organization of *sagA1* and *sagA2* upstream of *sagB* seen with NCTC10713^T (Fig. 1 and 3).

In order to investigate the contribution of both *sagA1* and *sagA2* expression to beta-hemolysis, targeted gene deletion was conducted in *S. anginosus* NCTC10713^T. Figure 8 shows a schematic representation of the genetic structures of the deletion mutants constructed. The mutated strains were a *sagA1 sagA2* double-deletion mutant (Fig. 8B), a *sagA2* single-deletion mutant (Fig. 8C), and a *sagA1* single-deletion mutant (Fig. 8D). Moreover, a mutant with only an erythromycin-resistant gene cassette inserted just upstream of the region of *sag*^{SA} was constructed as the control strain for these deletion mutants (Fig. 8E). The hemolytic phenotype of each mutant was investigated on human blood agar plates by stab inoculation (Fig. 8, right-hand side). The double-deletion mutant showed a complete loss of beta-hemolytic activity, whereas both *sagA2* and *sagA1* single-deletion mutants still

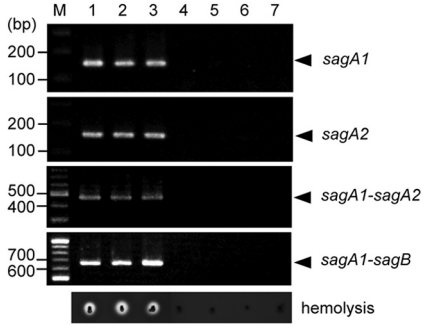

FIG 7 Relationship between the presence of the *sagA1* and *sagA2* genes and beta-hemolysis. Typical results of PCR amplification obtained in three beta-hemolytic strains and four nonhemolytic strains of *S. anginosus* are shown. Beta-hemolysis on blood agar plates for these strains is also indicated.

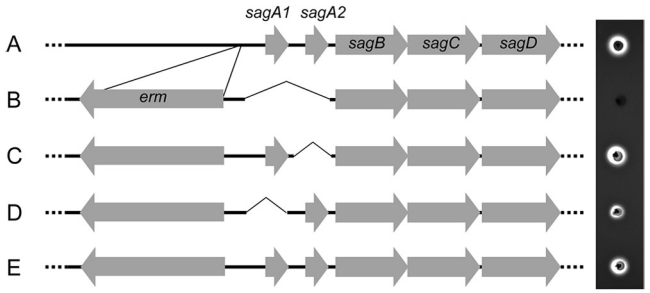

FIG 8 Relationship between the presence of *sagA* homologues and beta-hemolysis from gene deletion experiments. Schemes of the genetic structure of parent strain *S. anginosus* NCTC10713^T (A), the *sagA1 sagA2* double-deletion mutant (B), the *sagA2* single-deletion mutant (C), the *sagA1* single-deletion mutant (D), and a control mutant containing an erythromycin resistance gene cassette in the reverse direction just upstream of the intact *sag*^{SA} (E) are shown together with their beta-hemolytic activity on human blood agar plates.

gave beta-hemolysis, demonstrating that *sagA1* and *sagA2* each contributed to the beta-hemolytic phenotype. It was noted that the beta-hemolytic zone caused by the *sagA1* single-deletion mutant was smaller than that caused by the parent strain (Fig. 8).

Subsequently, complementation experiments of the *sagA1* and *sagA2* genes using their own promoters were also carried out, as shown in Fig. 9. The hemolytic activity of the complemented strain with both *sagA1* and *sagA2* genes was successfully restored to virtually the original levels of the control and parent strains (Fig. 9B). Moreover, complementation with either the *sagA1* or *sagA2* gene also gave beta-hemolytic activity, indicating that both *sagA1* and *sagA2* genes can function individually to confer beta-hemolytic activity on *S. anginosus* (Fig. 9D and F). Similarly to the *sagA1* single-deletion mutant (Fig. 8), the beta-hemolysis observed in the *sagA2*-complemented strain was, again, weaker than that of the control strain (Fig. 9F). Interestingly, the promoter region for the *sagA1* gene lacking the first promoter preferentially used *in vivo* also induced transcription of both *sagA1* and *sagA2* or *sagA1* (Fig. 9C and E).

DISCUSSION

Beta-hemolysis on blood agar plates is of significance to the medical microbiologist, being taken as an indication of the production of cytolytic factor(s) such as cytolysins. A significant proportion of clinical isolates of *S. anginosus*, including the type strain, NCTC10713, produce beta-hemolysis on blood-agar plates. However, the factor causing beta-hemolysis in this species has not previously been identified and characterized in detail.

In this study, we investigated beta-hemolysis in *S. anginosus* NCTC10713^T and demonstrated that the translational products of the *sagA* genes in *sag*^{SA} are responsible for this phenotype. The *sag* operon is a gene cluster composed of 9 genes and is present in the pyogenic group streptococci (22). In *S. pyogenes*, the effector molecule, streptolysin S (SLS), functions as the beta-hemolysin and is the product of the *sagA* gene within the *sag* operon. The other genes in the operon contribute to the maturation (*sagB*, *sagC*, and *sagD*) and secretion (*sagG*, *sagH*, and *sagI*) of SLS (22, 23). The genetic structure of the *sag* operon is highly conserved in the pyogenic group species (25, 26). Recently, the presence of *sag* operon homologues has been reported in other Gram-positive human pathogens: for example, *Clostridium* spp. (27), *Listeria monocytogenes* (28), and *Staphylococcus aureus* (29). The products of these

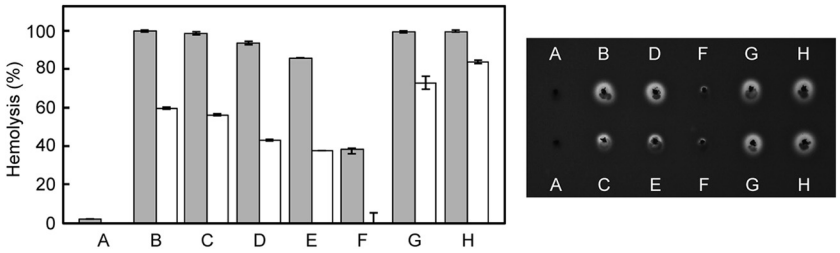

FIG 9 Complementation of *sagA* homologues in the *sagA1 sagA2* double-deletion mutant. (Right) Hemolysis on human blood agar after 1 day of cultivation. (A) The *sagA1 sagA2* double-deletion mutant. (B to F) Complementation of strains with both *sagA1* and *sagA2* transcribed by the intact promoter of *sagA1* with three potential promoters (Fig. 3) (B), by the truncated promoter of *sagA1* with the second and the last potential promoters (see Fig. 3) (C), with only *sagA1* transcribed by the intact promoter of *sagA1* (D) or by the truncated promoter of *sagA1* (E), or with only *sagA2* transcribed by its own promoter (F). Hemolytic patterns of a control mutant with an erythromycin-resistance gene cassette just upstream of intact sag^{SA} (G) and the parent strain (H) are also shown. (Left) Graph showing the hemolytic activity of the culture supernatant for each strain. Gray bars indicate the hemolytic activity of the original culture supernatant, and white bars indicate the hemolytic activity of culture supernatant diluted 3-fold with PBS. Results are shown as mean values ($n = 2$) with the differences between duplicates indicated.

operons, SLS-like peptides, are now categorized in a family named the thiazole/oxazole-modified microcins (TOMMs) (16). The typical operon structure encoding the TOMMs in *Streptococcus* spp. has been shown to be composed of 9 genes (Fig. 1B). However, it was demonstrated that the sag^{SA} operon in *S. anginosus* was composed of 10 genes, including two *sagA* homologues (designated *sagA1* and *sagA2*) and conserved *sagB* to *sagI* genes (Fig. 1A). This operon structure is specific for *S. anginosus*, and it has not been observed in other bacterial *sag* operons. Interestingly, our investigation clearly shows that only beta-hemolytic strains of *S. anginosus* possess sag^{SA} and that the presence of sag^{SA} in *S. anginosus* is not universal, as is the case in *S. pyogenes* and other pyogenic group streptococci, which all possess the *sag* operon. The DNA sequence of *sagA* in pyogenic group streptococci showed higher homology with *sagA1* than with *sagA2* of *S. anginosus* (Fig. 3). The reason why *S. anginosus* has two *sagA* homologues is unclear; however, the tandem arrangement of *sagA1* and *sagA2* in sag^{SA} may be due to gene duplication of *sagA* in an "*S. pyogenes*-type" *sag* operon with a single *sagA* mutation and subsequent mutations in *sagA2*. Alternatively the previous loss of *sagA2* from an original sag^{SA} operon might have resulted in the operon arrangement observed in *S. pyogenes*.

In the gene deletion experiments, the *sagA1* single-deletion mutant showed weaker beta-hemolysis than its parent strain and the *sagA2* deletion strain (Fig. 8D). A similar tendency was observed from the complementation experiments: the *sagA2*-complemented strain also showed weaker restored beta-hemolysis than that of the *sagA1*-complementated strain (Fig. 9F). These results suggested that the contribution to beta-hemolysis by *S. anginosus* of SagA1 was stronger than that of SagA2. We hypothesize that the weaker beta-hemolysis observed in the *sagA1* single-deletion strain than was observed in the *sagA2* single-deletion strain is due to transcript stability, as indicated by the results of the 5′-RACE analysis (Fig. 3 and 5); the amount of intact/functional mRNA for SagA2 would be lower than that for SagA1, resulting in less hemolytic activity by the former. Because it is well known that the immunogenicity of SLS is quite low and the antibody usable for the detection of SLS is difficult to raise (30), we have not yet succeeded in quantifying SagA1 and SagA2 individually in order to obtain a definite conclusion to this question.

In the complementation experiments, the beta-hemolytic activity of the *sagA2*-complemented strain was reduced and was not restored to the level of the *sagA1* single-deletion mutant (Fig. 9F). DNA sequence analysis of sag^{SA} demonstrated not only a deduced promoter sequence upstream of the *sagB* ORF but also the presence of a terminator-like structure between the promoter and SD sequence (Fig. 3). This terminator-like structure did not stop the transcription induced by the promoters of the *sagA1* and *sagA2* genes, as shown in Fig. 5A. Moreover, the transcription induced by the *sagB* promoter occurred as shown in Fig. 3 and 5B, and resulted in the maturation and transport of SagA homologues complemented in *trans*, as shown in Fig. 9, although the transcript induced by the *sagB* promoter was faintly detectable in 5′-RACE analysis, as shown in Fig. 5A. The terminator-like structure could not prevent mRNA elongation from *sagA1* and *sagA2*; however, this may cause reduced function of the *sagB* promoter at the initial step in transcription, and the components for maturation and transport of SagA1 and SagA2 (i.e., SagB to SagI) may not be produced efficiently in the *sagA1* and *sagA2* double-deletion mutant. Therefore, the low level of SagB to SagI and the low stability of transcriptional product from the *sagA2* promoter mentioned above, working together, would induce a reduced beta-hemolysis phenotype in the *sagA2*-complemented strain.

Interestingly, the data also suggested that a certain essential difference(s) might exist between the molecular characters of SagA1 and SagA2 of *S. anginosus* and the typical SagA of pyogenic group streptococci. This derives from the fact that a critical/important amino acid (S39) for the heterocycle formation and the cytolytic activity of *S. pyogenes* SagA is deleted in both *S. anginosus* SagA1 and SagA2 (Fig. 4C). Moreover, the numbers of heterocycles deduced in SagA1 and SagA2 may differ from that of SagA of *S. pyogenes*. These differences in amino acid sequence and predictable heterocycle formation are more significant than the intraoperon difference in SagA homologues of *S. anginosus* NCTC10713^T (i.e., between SagA1 and SagA2) and suggest that mature SagA homologues of *S. anginosus* NCTC10713^T may differ in the structure essential for cytotoxicity and/or the maturation detail, compared to SagA in *S. pyogenes*. Furthermore, the structural differences between SagA1 and SagA2 suggest potential differences in their interaction with other molecules (e.g., enzymes) responsible for their maturation and/or with target molecules, such as membrane phospholipids affecting maturation efficiency and specific activity. Therefore, the structure-activity relationship of each SagA homologue is of great interest.

In the present study, the novel factors responsible for the beta-hemolysis induced by *S. anginosus* have been revealed and their properties investigated. In contrast to a typical *sag* operon of pyogenic group streptococci, the presence of twin *sagA* genes (*sagA1* and *sagA2*) is a significant characteristic of beta-hemolytic *S. anginosus*. The data obtained here demonstrate the twin *sagA* homologues to be fundamental units for beta-hemolysis within *S. anginosus* and suggest that SagA1 and SagA2 in *S. anginosus* might have distinctive characteristics compared to SagA in pyogenic group streptococci. Thus, the elucidation of the mechanisms of transcription, expression, and maturation of SagA1 and SagA2 may be important for understanding the pathogenicity of beta-hemolytic *S. anginosus*. Further investigation is currently proceeding focused on comparing the properties of SagA1 and SagA2 of beta-hemolytic *S. anginosus* and pyogenic group streptococcal SagA (SLS).

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