The streptococcal inhibitor of complement (SIC) protects Streptococcus pyogenes from bacteriocin-like inhibitory substance (BLIS) from Streptococcus salivarius

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Streptococcus pyogenes; streptococcal inhibitor of complement (SIC); Streptococcus salivarius; bacteriocin-like inhibitory substance (BLIS).

Abstract
Streptococcus salivarius inhibits the growth of Streptococcus pyogenes in vitro. Streptococcus pyogenes has various virulence factors, including the streptococcal inhibitor of complement (SIC). Although SIC inhibits the activity of the peptides LL-37 and NAP1, the relationship between SIC and the bacteriocin-like inhibitory substance (BLIS) has not been elucidated. Here, we evaluated whether S. salivarius BLIS affects S. pyogenes SIC. We created three Δsic mutant strains from three S. pyogenes strains and performed deferred antagonism assays. The test strains were BLIS-positive S. salivarius JCM5707 and BLIS-negative S. salivarius NCU12. Deferred antagonism assays with JCM5707 showed that the inhibitory zones in the three Δsic mutant strains were wider than those in the three wild-type strains. Streptococcus pyogenes was cultured in BLIS-containing broth and the change in SIC in the supernatant was assessed by two-dimensional gel electrophoresis (2-DE). The 2-DE analysis of S. pyogenes exoproteins with the JCM5707 supernatant showed reduced SIC compared with those without the JCM5707 supernatant. Changes in sic mRNA levels affected by S. salivarius BLIS were evaluated by a reverse transcriptase-PCR. The sic mRNA level was affected more by the BLIS-positive S. salivarius than by the BLIS-negative strain. Our result indicates that SIC plays a role in the inhibition of S. salivarius BLIS.

Introduction
The ability of normal bacterial flora of the upper airways to inhibit the growth of potential pathogens in vitro has been well described (Tano et al., 1999; Brook & Gober, 2000). Bacteriocins, proteinaceous antimicrobials, produced by bacteria, kill closely related bacteria, but do not kill the producer strain, which exhibits a degree of specific immunity to the bacteriocin (Jack et al., 1995). The term bacteriocin-like inhibitory substance (BLIS) is used to describe bacterial products that have inhibitory effects, similar to those of bacteriocins, before isolation and characterization of the active agent (Tagg & Bannister, 1979). BLIS may also be defined as an extracellularly released bacterial peptide or protein molecule, which is able to kill some closely related bacteria even at low concentrations by a mechanism against which the producer bacterium exhibits some specific immunity (Tagg, 1992). Most of this inhibitory activity has been attributed to α-haemolytic streptococci, and one streptococcus with a particularly high incidence of BLIS-producing strains is Streptococcus salivarius (Dempster & Tagg, 1982). This species is a numerically prominent colonist of human oral epithelial surfaces (Walls et al., 2003) and is one of the first organisms to have established itself in the mouths of neonates (Carlsson et al., 1970). Streptococcus salivarius is a commensal bacterium that colonizes in oral cavities and saliva and produces salivaricin A, salivaricin A2, salivaricin B or enocin, which have bactericidal effects (Sanders & Sanders, 1982; Upton et al., 2001; Hyink et al., 2007). Three salivaricin genes, salA, salA2 and sbo, encode salivaricin A, salivaricin A2 and salivaricin B, respectively (Upton et al., 2001; Hyink et al., 2007). The presence of...
S. salivarius strains producing salivaricin A has been shown to reduce the frequency of acquisition of Streptococcus pyogenes in the oral cavities of school children (Dierksen et al., 2000).

Streptococcus pyogenes is a Gram-positive bacterium that infects the upper respiratory tract, including the tonsils and pharynx, and is responsible for postinfectious diseases, such as rheumatic fever and glomerulonephritis. In addition, S. pyogenes causes streptococcal toxic shock syndrome (Cunningham, 2000). The pathogenesis of S. pyogenes is unclear; however, many virulent proteins are considered to be causative factors. Among them, the streptococcal inhibitor of complement, the SIC protein, is unique (Akesson et al., 1996). The distribution of the sic gene is present in, but not limited to, the M1 serotype of S. pyogenes (Akesson et al., 1996). Although various roles of Sic have been reported, the precise functions of Sic have not been completely revealed. Originally, it was reported that Sic inactivated the complement system, which is an important host defence mechanism (Akesson et al., 1996). Another function of Sic is to interact with the host cell protein, serine protease (Hoe et al., 2002). Sic also inhibits the activity of the antibacterial peptides, LL-37 (Frick et al., 2003), NAP1 (Frick et al., 2003), β-defensin (Fernie-king et al., 2007) and CXC chemokine (Egesten et al., 2007).

Although Sic is associated with the inhibition of neutrophil-derived small peptides, there have been no reports on the relationship between Sic and bacteria-derived small peptides, especially BLIS. Streptococcus pyogenes colonizes within the throat before invading the host tissue. When S. salivarius subsequently colonizes in an S. pyogenes-invaded locus, further colonization of S. pyogenes might be inhibited by the S. salivarius-derived BLIS. However, the bacterial interface between Sic and S. salivarius-derived BLIS under throat-colonizing conditions is not clear. Here, we clarify the role of Sic against BLIS from S. salivarius.

Materials and methods

Bacterial strains and culture condition

M1 serotype S. pyogenes strains 1529, GT01 and MDMH used in this study were clinical isolates from hospital patients in Japan with invasive S. pyogenes infections. The S. salivarius JCM5707 strain was purchased (Riken Bio Resource Center, Tsukuba, Japan). The S. salivarius NCU12 was a clinical isolate from Nagoya City University Hospital. Streptococci were usually cultured in 20 mL of brain–heart infusion broth (Eiken Chemical Co., Tokyo, Japan) containing 0.3% yeast extract (Difco Laboratories, Detroit, MI) (BHI-YE) for 18 h at 37 °C without agitation. To assess the BLIS effect against Sic, S. salivarius was firstly cultured in 20 mL of BHI-YE for 18 h at 37 °C without agitation and subsequently an aliquot of the streptococcal culture was centrifuged at 8000 g for 20 min. A 4-mL aliquot of the supernatant was added to 16 mL of fresh BHI-YE. In this broth medium (BHI-YE with S. salivarius supernatant), S. pyogenes was cultured for 18 h at 37 °C without agitation and subsequently further proteome analysis was performed. Escherichia coli JM109 were maintained on Luria–Bertani (LB) agar (Difco Laboratories) or were grown in LB broth with aeration at 37 °C. The following antibiotic concentrations were used when appropriate: ampicillin (Sigma Chemical Co., St. Louis, MO), 100 mg L⁻¹ (E. coli); and spectinomycin (Sigma Chemical Co.), 100 mg L⁻¹ (E. coli) and 100 mg L⁻¹ (S. pyogenes).

Confirmation of sic sequence

The sequences of sic in S. pyogenes strains 1529, GT01 and MDMH were determined using an automated DNA sequencer, CEQ 2000XL (Beckman Coulter Inc., Fullerton, CA), according to the manufacturer’s instructions. The sic DNA was amplified with sic-n9 and sic-c3Eco oligonucleotides with pyrobest DNA polymerase (Takara Bio Inc., Otsu, Japan). Sequence reactions were carried out using sic-n3Bam and sic-c7Spe oligonucleotides. The alignment data of the sic genes sequenced were compared by the BLAST search with those already deposited in the DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/top-j.html). The sequences of the PCR primers used are shown in Table 1.

Creation of a sic-inactivated mutant

Escherichia coli JM109 was used to propagate plasmid constructions. Nonpolar inactivated mutants of the sic gene were constructed via double-crossover allelic replacement in S. pyogenes 1529, GT01 and MDMH chromosomes according to Lukomska et al. (2000) with some modifications. Briefly, to construct the plasmid for the sic-inactivated mutant, a sic DNA fragment was amplified using oligonucleotide primers sic-n10Sma, containing an SmaI restriction site, and sic-c7Spe, containing an SpeI restriction site (fragment 1). Another sic DNA fragment was amplified with

Table 1. Oligonucleotide primers used during the experiments

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>sic-n9</td>
<td>CGCCAGGTTTTCTATGGTTATG</td>
</tr>
<tr>
<td>sic-c3Eco</td>
<td>CGGATACTTCGTAAACGGCTGGTGTTCG</td>
</tr>
<tr>
<td>sic-n3Bam</td>
<td>GGGATCCGGTAAATGCTTATG</td>
</tr>
<tr>
<td>sic-c7Spe</td>
<td>GAGACTGATTAATGCTTATG</td>
</tr>
<tr>
<td>sic-n7Nhe</td>
<td>GGCAGTGCCGAAACTAAAGCCTACAGT</td>
</tr>
<tr>
<td>sic-n10Sma</td>
<td>TCCCCGGGCTAAATAAGGATGCGAT</td>
</tr>
<tr>
<td>sic-c5Spe</td>
<td>GGATGCTCGATGGTCCCTCC</td>
</tr>
<tr>
<td>mrg-n2</td>
<td>GGACTGATTTGCGGAATATGCTAAGGCTTTC</td>
</tr>
<tr>
<td>mrg-c2</td>
<td>TAAGCTCATCAAGGATGAC</td>
</tr>
</tbody>
</table>

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oligonucleotide primer sic-n7Nhe containing an Nhel restriction site and sic-cSBam containing a BamHI restriction site (fragment 2). The Smal- and Spel-digested fragment 1 was cloned into the Smal–SpeI site of the E. coli pFW12 plasmid (Podbielski et al., 1996). The resulting plasmid was then digested with Nhel and BamHI. Subsequently, the Nhel–BamHI-digested fragment 2 was inserted. Finally, this plasmid was digested with SspI and Smal, and an spc2 DNA fragment containing aad9 (a promoterless spectinomycin-resistant gene), which was obtained from an Smal-digested fragment of the E. coli plasmid pSL60-2 (Podbielski et al., 1996), was inserted. The constructed suicide vectors, sic-c::aad9/pFW12, were transformed into strains 1529, GT01 and MDMH as described elsewhere (Sawai et al., 2007). The sequences of the PCR primers used are shown in Table 1.

Deferred antagonism assay

A deferred antagonism assay method based on Tagg & Bannister (1979), with some modifications, was used to compare the relative susceptibilities of different bacterial strains with the BLIS activities produced in agar media. Briefly, the test S. salivarius strain was inoculated diametrically across the surface of a trypticase soy agar II with a 5% sheep blood agar plate (Nippon Becton Dickinson Co. Ltd, Tokyo, Japan) as a 5-mm-wide streak. After incubation, the visible growth of the test strain was removed using a cotton stick (Nihon Menbo, Tokyo, Japan), and the surface of the agar was sterilized by exposure to chloroform (Sigma Chemical Co.) vapours for 30 min. The plate was then exposed to air for 15 min before inoculation with 18-h BHI-YE cultures of the S. pyogenes indicator strains across the line of the original test strain growth. The plates were then incubated as before for 24 h and examined for zones of growth interference. Areas of inhibition of indicator strain growth were measured by the length of inhibition. All tests were performed in duplicate, and further examination was conducted if marked discrepancies were observed among the inhibition patterns obtained.

Two-dimensional gel electrophoresis (2-DE) analysis

Exoproteins from the culture supernatants were prepared using Sawai’s method with some modification (Sawai et al., 2007). Whole streptococcal cell proteins were prepared as below. The 18-h streptococcal culture was centrifuged at 8000 g for 20 min, the pellet was dissolved in 3 mL of sample buffer, 7.8 M urea (Sigma Chemical Co.), 2 M thiourea (Sigma Chemical Co.) and 4% 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulphonic acid (CHAPS; Sigma Chemical Co.) and was sonicated thricely on ice for 30 s. This sample was then precipitated with trichloroacetic acid (final concentration, 10%) and washed with acetone. This procedure was performed again. All sample pellets were dissolved in 150 μL of rehydration buffer, 7.8 M urea, 4% CHAPS, 0.6% dithiothreitol (Sigma Chemical Co.) and 1% Bio-Lyte 3/10 buffer (Bio-Rad Laboratories Inc., Hercules, CA). Aliquots (120 μL) of the samples were loaded onto a 7-cm ReadyStrip IPG strip (pH 3–10; Bio-Rad Laboratories Inc.) for exoproteins or an 11-cm ReadyStrip IPG strip (pH 3–10; Bio-Rad Laboratories Inc.) for whole-cell proteins. The equilibrated strips were layered onto a 12% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) and electrophoretic separation was performed. Thereafter, the gels were stained with Coomassie brilliant blue. Amino acid sequences of protein spots were analysed by protein sequencer 49X-cLC ( Applied Biosystems Inc., Foster City, CA) as described previously (Nakamura et al., 2004). Protease inhibitor E-64 (Roche Diagnostics, Indianapolis, IN) was added to the BHI-YE broth during culture supernatant analysis as described by Aziz et al. (2004).

Assessment of mRNA levels by a semi-quantitative reverse-transcription (RT)-PCR

The deferred antagonism assay was modified to use a 10-h incubation period instead of the aforementioned 24 h. To confirm the influence of BLIS, a BLIS-positive strain S. salivarius JCM5707 was used as the test strain. The indicator strain S. pyogenes 1529 was swabbed from the adjunctive inhibitory lesion zone (BLIS-influenced site). As a control, a similar deferred antagonism assay was performed using the BLIS-negative S. salivarius strain NCU12. Upon assay completion, the collected bacteria were adjusted by addition of phosphate-buffered saline to 10⁶ CFU mL⁻¹ and total bacterial RNA was extracted using the Qiagen RNA extraction kit (Qiagen, Hilden, Germany). The RT-PCR procedure has been described previously (Tanaka et al., 2005). In brief, total RNA (200 ng) was reverse transcribed in the presence of sic-, mga- and gyrA-specific primers using the SuperScript first-strand synthesis system, according to the instructions of the manufacturer (Invitrogen Co.), in a 10 μL reaction volume. The primers used for amplification were sic-n6 and sic-c4 for sic, mga-n2 and mga-c2 for mga and gyrA-F and gyrA-R for gyrA. A total of 0.4 μL from the first 10-μL reverse transcriptase reaction was used for the next PCR (reaction volume, 10 μL). Amplification and detection of the specific products were performed with the following cycle profile: 23 thermal cycles of 30 s at 94°C, 30 s at 55°C for annealing and 30 s at 72°C for extension, with an additional extension of 300 s at 72°C. The amount of contaminating chromosomal DNA in each sample was determined in control reactions that did not contain reverse transcriptase. The intensities of the RT-PCR products in agarose gel were analysed using the public domain NIH IMAGE program (developed at the US National Institutes of Health and
available on the Internet at http://rsb.info.nih.gov/nih-image/) and transformed into a bar graph after analysis. The data are presented as relative transcription amounts normalized to the housekeeping gene gyrA.

Detection of salA, salA2 and sbo genes
Detection of salA, salA2 and sbo genes was performed by PCR and Southern blotting assays according to previously reported methods (Ross et al., 1993; Upton et al., 2001; Hyink et al., 2007). The salA probe was amplified using an S. pyogenes 1529 chromosome as a gene template. The streptococci chromosomes were extracted using Promega DNA extraction kits (Promega, Madison, WI) according to the manufacturer’s instructions.

Statistical analysis
The degrees of significance between means were determined by the paired t-test. A P-value of < 0.05 was considered significant. The compared experiments were repeated a minimum of five times to improve the precision of the resulting data.

Results
Initially, the presence of the sic gene in three S. pyogenes strains was confirmed through sequence analysis. The results revealed that the SIC proteins in S. pyogenes 1529, GT01 and MDMH were Sic 1.90, Sic 1.291 and Sic 1.105, respectively. Although the SIC types in the three strains were different, the amino acid sequence changes observed in the three strains were subtle. The percentages of sequence identity in MDMH and GT01 compared with 1529 were 98.1% and 97.1%, respectively.

Next, we created independent isogenic Δsic mutant strains from the three S. pyogenes strains. Inactivation of the sic genes in these mutants was confirmed by 2-DE assays. Figure 1a and b show exoproteins of the supernatant in the 1529 wild type and Δsic mutants in BHI-YE only, respectively. SIC was analysed by 2-DE, followed by protein sequencing, to identify the spot of interest. In 2-DE gel, the circle shows the spot of the SIC protein. There is no protein spot in the circled area in (b) because SIC is absent. Molecular weights (M) are shown in kDa on the vertical axis, and approximate isoelectric point (pl) values are shown on the horizontal axis of each gel. Gels displayed were representative of at least three different experiments.

Fig. 1. Proteomic analysis of SIC in Streptococcus pyogenes 1529 wild-type and Δsic mutant supernatant. (a, b) The exoprotein presence of 1529 wild type and Δsic mutants in BHI-YE only, respectively. SIC was analysed by 2-DE, followed by protein sequencing, to identify the spot of interest. In 2-DE gel, the circle shows the spot of the SIC protein. There is no protein spot in the circled area in (b) because SIC is absent. Molecular weights (M) are shown in kDa on the vertical axis, and approximate isoelectric point (pl) values are shown on the horizontal axis of each gel. Gels displayed were representative of at least three different experiments.

Fig. 2. Deferred antagonism assays of (a) BLIS-positive and (b) BLIS-negative Streptococcus salivarius. (a) Inhibitory zones in a blood agar plate using BLIS-positive S. salivarius. The two arrows represent the inhibitory zone of Streptococcus pyogenes affected by S. salivarius BLIS.

Fig. 3. Comparison of Streptococcus salivarius inhibitory zones between Streptococcus pyogenes wild type and Δsic mutants. Solid and open columns show the inhibitory zone lengths for wild-type and Δsic mutant S. pyogenes, respectively. The experiments were performed in triplicate, each replicate repeated at least three times, and all with similar results. The results show means and SDs. *P < 0.05.
antagonist assays using NCU12 as the test strain showed no inhibitory zones among all S. pyogenes strains (data not shown).

Next, we assessed the effect of BLIS from JCM5707 against SIC by 2-DE. The 2-DE analysis of exoproteins from BHI-YE with the S. salivarius JCM5707 supernatant showed reduced SIC amounts compared with BHI-YE without the JCM5707 supernatant (Figs 4a and 1a). We also found similar amounts of SIC in exoproteins from BHI-YE with the S. salivarius NCU12 supernatant and from BHI-YE only (Figs 4b and 1a). Furthermore, we performed whole-cell proteome analysis of SIC-positive S. pyogenes in BHI-YE with S. salivarius JCM5707 to rule out the accumulation of SIC protein in the streptococcal cell. Because SIC is known to be a secreted protein (Akesson et al., 1996), we did not detect the accumulation of SIC in whole-cell analysis (Fig. 4c). Although we also performed these 2-DE analyses using a combination of E-64 and another protease inhibitor, phenyl methyl sulphonyl fluoride (PMSF), we did not find any differences in the 2-DE gel between E-64 only and an E-64–PMSF combination (data not shown).

We also evaluated the mRNA expression levels of SIC to determine whether JCM5707 BLIS affects the ability of SIC secretion in M1 serotype S. pyogenes strains. The mRNAs were extracted from S. pyogenes alongside those from BLIS-positive and BLIS-negative S. salivarius. The sic mRNA intensity level alongside the BLIS-positive S. salivarius mRNA was significantly higher than that alongside the BLIS-negative strain (P < 0.05; Fig. 5). We also assessed the expression level of mga that was associated with SIC. However, the level of mga mRNA alongside the BLIS-positive S. salivarius mRNA was slightly higher than that alongside the BLIS-negative one. However there were no significant differences compared with the level of SIC (data not shown).

Finally, we attempted to elucidate, through PCR and Southern blot analysis, whether the BLIS from JCM5707 was the same as salivaricin A, salivaricin A2 or salivaricin B. However, neither PCR nor Southern blot showed the
presence of sal A, sal A2 or sbo genes in the S. salivarius JCM5707 strain (data not shown).

Discussion

This is the first report on SIC secreted from three M1 serotypes of S. pyogenes inhibiting BLIS derived from S. salivarius. The inhibitory zone differences observed between each of the wild-type M1 serotypes and each of the isogenic sic mutant strains support our conclusion of SIC inhibition of BLIS.

We could not find SIC protein in the supernatant SIC-positive S. pyogenes treated with the culture supernatants of BLIS-positive S. salivarius. This result indicated that S. salivarius BLIS affects S. pyogenes and results in the reduction of SIC production. We evaluated SIC by 2-DE because we could not identify SIC by SDS-PAGE only, nor did we have adequate anti-SIC antibodies. However, we could identify the protein SIC clearly using 2-DE.

The supernatant of S. pyogenes contains an abundance of cysteine protease SpeB, which degrades SIC (Aziz et al., 2004). In this study, we added a specific cysteine protease inhibitor, E-64, to broth cultures to inhibit the SpeB proteolytic activity. We thus concluded that the reduction of SIC spot intensity is not the result of the effect of SpeB serine protease. A possible reason for the reduction is that the SIC protein bound with BLIS, and that this complex was then degraded by a streptococcal protease other than SpeB. Although 2-DE gel showed a decrease in SIC in the supernatant with BLIS, SIC production might increase in response to S. salivarius BLIS. This observation is supported by the increase observed in sic mRNA expression in S. pyogenes in a deferred antagonism assay with BLIS-positive S. salivarius compared with the expression using a BLIS-negative strain. Our results have revealed that antibacterial peptides derived from not only humans but also bacteria are inhibited by SIC.

We attempted to create a mutant complemented with a sic gene to eliminate the possibility that the inhibitory zone increases in Δsic mutant strains were caused by polar effects or secondary mutations. However, we were not successful. Because, in our experiment, we could not create the plasmid harbouring the intact sic gene for complement study, this result indicated that the expression of the sic gene might cause toxicity for E. coli. Instead of the complement study, we created three sic-inactivated strains independently using a nonpolar effect method (Lukomski et al., 2000); however, the phenotypes of these strains, determined by a deferred antagonism assay, were similar.

In this study, we did not confirm the presence of salivaricin A, salivaricin A2 or salivaricin B in BLIS-positive S. salivarius JCM5707 by PCR or Southern blot analysis. The BLIS proteins secreted by streptococci are generally relatively small compounds that can diffuse through agar and produce inhibition well away from the location of the organism that secreted them (Tagg, 1992). This kind of inhibition, at a distance from the original organism, makes other causes of inhibition less likely and suggests that the activity is due to BLIS (Dempster & Tagg, 1982). The deferred antagonism test used in this study has been used for over 25 years to test for BLIS production by streptococci, and is unique in that the bacteria being tested are never in direct contact with each other. This excludes substances that are integral components of the cell wall of the producer strain as causes of inhibition. As the deferred antagonism assay with JCM5707 as a test was positive, we speculate that the inhibitory substance secreted from JCM5707 was a BLIS other than salivaricin A, salivaricin A2 or salivaricin B. The supernatant from S. salivarius JCM5707, filtered through a 0.45-μm pore filter, did not produce a reduction of SIC protein in 2-DE analysis (data not shown). Thus, this novel BLIS may be easily adsorbed on membrane filters. Based on these findings, further investigation, including purification, is required.

In summary, SIC from M1 serotypes of S. pyogenes plays a role in inhibition of S. salivarius BLIS. Further analysis of the relationship between SIC and S. salivarius BLIS may promote the development of new anti-S. pyogenes therapies.

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References


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