New Gene Cluster for Lantibiotic Streptin Possibly Involved in Streptolysin S Formation

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Streptolysin S (SLS) is a serum-extractable and oxygen-stable hemolysin produced by Group A *Streptococcus*. A SLS-deficient mutant in which transposon Tn *916* was inserted in a locus distinct from the *sag* gene cluster [Nizet *et al.* (2000) *Infect. Immun.* 68, 4245–4254] was obtained by filter mating of the transposon-harbouring *Enterococcus faecalis* strain and *Streptococcus pyogenes* BL(T). This mutant, N22, had completely lost the hemolytic activity, in consequence of insertion of a single Tn *916* into a hitherto-unknown lantibiotic gene cluster composed of 10 open reading frames. The arrangement and sequence of this lantibiotic gene cluster were similar to those of nisin and subtilin, and so we designated this new lantibiotic as streptin. The bactericidal activity of streptin was abolished on treatment with trypsin or proteinase K. The different host range and nucleotide sequence clearly distinguished streptin from streptococcins. Streptin was not hemolytic and its bacteriocin activity was independent of carrier oligonucleotides effective for SLS. The fact that N22 also lost the anti-bacterial activity against indicator streptococci reveals that the factor(s) required for lantibiotic formation plays an important role in SLS formation as well.

Key words: hemolysin, lantibiotic, streptin, streptolysin S, transposon.

Two kinds of hemolysin are produced extracellularly by *Streptococcus pyogenes*: one is streptolysin S (SLS) and the other is streptolysin O (SLO). SLO is an oxygen-sensitive, thiol-activated, membrane-damaging protein, and its structural gene has been cloned and characterized (*1*, *2*). SLS is a serum-extractable, oxygen-stable, membrane damaging agent of a peptide nature, and is non-immunogenic (*3*). SLS is hemolytically active only when complexed with certain carrier substances, such as RNA core, serum components, nonionic detergents, or bisazobenzidine dyes (*3–5*). This exotoxin has a membrane damaging effect on not only erythrocytes, but also lymphocytes (*6*), neutrophils, platelets (*7*), tumor cells (*8*), and subcellular organelles (*9*, *10*).

Lai *et al.* (*11*) reported that active peptide of SLS consisted of 32 amino acid residues comprising tyrosine and phenylalanine, but was deficient in histidine, valine, leucine, cysteine, methionine, and arginine residues. According to Loridan and Alouf (*12*), carrier-free SLS is basic (pI 9.2) and the molecular weight of the denatured peptide is about 1,800. The amino acid sequence of SLS, however, is totally unknown, mainly because of the unavailability of homogeneously purified SLS, that is unstable and non-immunogenic. Even amino terminal determination by Edman degradation was unsuccessful for SLS (*13*).

Genetic analysis of SLS per se, and factors involved in its formation and secretion is essential. Because hemolytic streptococci are rather refractory to DNA transformation, transposon mutagenesis may be useful for the detection of genes encoding SLS or related factors. More recently, the *sag* gene cluster was found to be essential for SLS production, in Tn *916* mutagenesis experiments (*14*, and Karaya *et al.* unpublished data). Moreover, Nizet *et al.* (*15*) have reported that, upon transfer of the *sag* operon, β-hemolysis was elicited by *Lactococcus lactis* but not by *Escherichia coli*. This implies that some lactococcal factors, deficient in *E. coli*, are involved in expression of the β-hemolytic activity in the transformants. Although comparative analysis of nucleotide sequences indicates close relationships among *sag* gene product(s), SLS and bacteriocin, the structural gene for SLS apopeptide remains to be identified definitely.

During investigation on factors required for SLS formation, we found several genes besides *sag* in *S. pyogenes* strain Sa, mainly using Tn *916* mutagenesis. In this study, we characterize the gene cluster directing the production of a new bacteriocin, streptin. This lantibiotic belongs to Class AI and differs from Class AII members streptococcin A-FF22 (*16*) and streptococcin A-M49 (*17*). Streptin per se is devoid of hemolytic activity, and some factors required for the production of this lantibiotic may be involved in SLS formation.

EXPERIMENTAL PROCEDURES

Strains, Media, and Growth Conditions—The bacterial strains, plasmid, and phages used in this study are listed in Table I. BL(T)^{N} is a norfloxacin resistant strain of BL(T), isolated by culturing in THY medium [3% Todd-Hewitt broth (Difco) supplemented with 1% yeast extract (Nihon Seiyaku)] containing norfloxacin (Kyorin Pharmaceutical,

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TABLE I. **Bacterial strains, plasmids, and phages used in this study.**

Bacterial strains, plasmids, phages	Relevant charasteristics	Source or reference
S. pyogenes BL(T)	SLS+, SLO−,	Taketo, Y.
S. pyogenes BL(T)^N	SLS+, SLO−, nor	This work
S. pyogenes N22	SLS−, SLO−, nor, tet [Tn *916*]	This work
E. faecalis CG110	tet [Tn 916] rif fus	Clewell, D.B.
E. coli XL1 Blue		Laboratory stock
E. coli LE392		Stratagene
Plasmid		
pUC19		Stratagene
Phage		
λEMBL4		Stratagene
N225L	18 kb insert containing Tn*916*-5′ end region of N22	This work
N2210	13 kb insert containing Tn*916*-3′ end region of N22	This work
N523	11 kb insert containing streptin gene cluster	This work
Indicator strains for bacteriocin activity		
Group A *Streptococcus* strain FF22 (Indicator 2)	M-type 52, T-pattern 3/13 streptococcin-producing strain	Tagg, J.R.
Group A *Streptococcus* strain W-1 (Indicator 8)	T-pattern 6	Tagg, J.R.
Group C *Streptococcus* strain T-148 (Indicator 9)		Tagg, J.R.

Tokyo). *E. faecalis* CG110 used for Tn *916* donor was generously provided by D.B. Clewell. The indicator strains used for checking the bacteriocin activity were generously provided by J.R. Tagg. λEMBL4 and pUC19 were purchased from Stratagene. *E. coli* XL1-Blue was used for subcloning and LE392 was the host strain for phage. Strains of *S. pyogenes* were grown aerobically without shaking in THY medium at 37°C. Cells of *E. coli* were grown in Luria broth (LB), supplemented with 100 μg/ml ampicillin (Wako) when required, at 37°C with shaking.

Filter Mating—Tn *916* donor (*E. faecalis* CG110) and recipient [*S. pyogenes* BL(T)^N] cells were collected by centrifugation and washed with THY medium. The donor and recipient cells (1–5 × 10^8 cfu of each strain) were mixed in the ratio of one to one and then collected on a membrane filter (0.22 μm: Sartorius). The other conditions for filter mating were essentially the same as those of Franke and Clewell (*18*). The transconjugants of *S. pyogenes* which did not show SLS activity were selected on THY agar plates containing 15 mg/ml norfloxacin, 10 mg/ml tetracycline, 0.4 mg/ml RNA core (Sigma), and 3% sheep erythrocytes. Single colony isolation of the selected SLS-deficient mutant was performed on a blood agar plate and its hemolytic activity was checked in liquid media.

Assaying of Hemolytic Activity—The hemolytic activity of extracellular, intracellular, and cell bound SLS was determined as described previously (*19–22*), except that sheep erythrocytes were used.

Genome Library Construction and Subcloning—Extraction and purification of *S. pyogenes* DNA were performed according to the methods of Nida and Cleary (*23*). A genomic DNA library was constructed using the λEMBL4 vector and genome DNA partially digested with *Sau*3AI (Roche Molecular Biochemicals) or completely digested with *Eco*RI (New England Biolabs). Gigapack II Gold Packaging Extract (Stratagene) was used to package the recombinant lambda phage. Extraction and purification of phage DNA, Southern blotting and plaque hybridization were performed according to standard molecular biological techniques (*24*). Restriction fragments of phage DNA were recovered from an agarose gel with JETSORB (Genomed) according to the manufacturer's instruction. DNA was inserted into the pUC19 vector treated with alkaline phosphatase (GibcoBRL). The ligation mixture was used to transform *E. coli* XL1-Blue.

DNA Sequencing—Purified plasmid DNA was prepared from overnight cultures of *E. coli* subclones with a JETquick Plasmid Miniprep Spin Kit (Genomed) or a Qiagen Plasmid Mini kit (Qiagen), using manufacturer's protocol. PCR was performed according to the protocol recommended by Toyobo for KOD or KOD Dash DNA polymerase, using primers designed by OLIGO (version 4.0) of programs from National Biosciences. The DNA sequences of recombinant plasmids and PCR products were determined with an Applied Biosystems 373A DNA Sequencer or ABI PRISM™ 310 Genetic Analyzer using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) or a DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech). DNA analysis and sequence alignment were performed using GENETYX-MAC of Software Development (version 10.1). Databank searches were carried out with FASTA (http://crick.genes.nig.ac.jp/homology/fasta-e.shtml) and SSEARCH (http://watson.genes.nig.ac.jp/homology/ssearch-e.shtml).

Nucleotide Sequence Accession Number—The nucleotide sequence of the deduced streptin gene cluster and its flanking region has been deposited in DDBJ under accession number AB030831.

Assaying of Bacteriocin Activity—Bacteriocin activity was detected as the inhibitory effect of a culture supernatant on the growth of an indicator bacteria (*25, 26*). The conditions and procedure were as for streptococcin titration (*25, 27*). Pyogenic streptococci were incubated at 32°C for 24 h, without shaking, in TSYG medium [Tryptic Soy Broth (Difco) supplemented with 2% glucose and 1% yeast extract (Nihon Seiyaku), pH 6.7]. A supernatant was obtained by centrifugation, heated at 80°C for 30 min, and then concentrated with a centrifugal concentrator (Taitec) to 1.2 ml (7.5-fold concentration). Overnight cultures of indicator strains in THY medium were streaked, 1 cm wide, across the surface of Tryptic Soy Broth agar plates, using sterilized cotton swabs. After 30 min, the concentrated culture supernatant was repeatedly spotted onto the indicator streak lines, and the plates were incubated at 37°C for 24 h.

RESULTS

Intracellular, Cell-Bound, and Extracellular SLS Activities of Wild Type and Mutant Streptococci—*S. pyogenes*

BL(T) is a strain producing SLS but not SLO. BL(T)^N, a norfloxacin-resistant derivative, was obtained by serial subculture of BL(T) in the presence of norfloxacin. Tn *916* was introduced by filter mating of BL(T)^N and *E. faecalis* CG110. Transconjugants without hemolytic activity were selected on THY plates containing norfloxacin, tetracycline, and sheep erythrocytes. Non-hemolytic colonies appeared in the ratio of one to 3,000 transconjugants. N22, one of the non-hemolytic transconjugants, was compared on intracellular, extracellular, and cell-bound SLS activities with the wild type (Table II). BL(T)^N exhibited high specific activity of hemolysis, whereas N22 exhibited no detectable hemolytic function in the intracellular, cell-bound, and extracellular fractions. Southern blotting definitely indicated that SLS activity was abolished by a single Tn *916* insertion into the N22 genome (data not shown).

Sequence Analysis around the Tn 916 Insertion Point in the BL(T) Genome—An about 12 kb region around the Tn *916* insertion site of the BL(T) genome was sequenced to determine the gene responsible for the inactivation of hemolysis. Thus, N22 genomic DNA partially digested with *Sau*3AI was cloned into λEMBL4. The recombinant phage clone, N225L, containing the Tn *916* fragment was selected using a Tn *916*–specific probe and the sequence of the region adjacent to Tn *916* was determined. Clone N523 was selected from the BL(T) genome–*Eco*RI library in λEMBL4, using a probe designed from the Tn *916* flanking region of N225L, and the *Eco*RI fragment cloned in N523 was sequenced. The N225L sequence adjacent to N523 was confirmed by direct sequencing of the PCR product amplified from BL(T) genome DNA.

Based on the 12,040 bp sequence around the Tn *916* insertion point in the BL(T) genome, 10 putative open reading frames (ORFs) were determined. These ORFs formed a cluster, and Tn *916* was located in the seventh ORF in the N22 strain (Fig. 1). ORF-5 and ORF-8 contained ATP binding motif in common with ABC transporter protein. In con-

TABLE II. **SLS activity in culture supernatants of streptococci and mutants.**

Strain	Fraction	SLS activity (HU/OD_{660})
S. pyogenes BL(T)	Extracellular	1,200
	Cell bound	550
	Intracellular	7.4
S. pyogenes BL(T)^N	Extracellular	950
	Cell bound	1,100
	Intracellular	9.0
S. pyogenes N22	Extracellular	<0.1
	Cell bound	<0.1
	Intracellular	<0.1

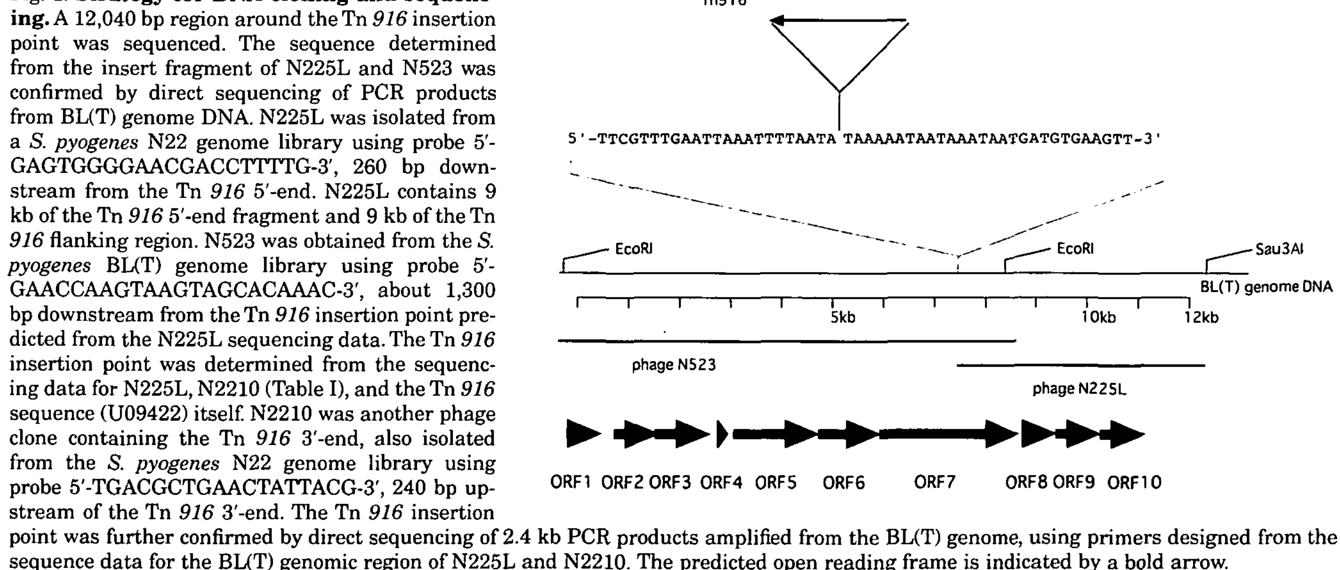

Fig. 1. **Strategy for DNA cloning and sequencing.** A 12,040 bp region around the Tn *916* insertion point was sequenced. The sequence determined from the insert fragment of N225L and N523 was confirmed by direct sequencing of PCR products from BL(T) genome DNA. N225L was isolated from a *S. pyogenes* N22 genome library using probe 5'-GAGTGGGGAACGACCTTTTG-3', 260 bp downstream from the Tn *916* 5'-end. N225L contains 9 kb of the Tn *916* 5'-end fragment and 9 kb of the Tn *916* flanking region. N523 was obtained from the *S. pyogenes* BL(T) genome library using probe 5'-GAACCAAGTAAGTAGCACAAAC-3', about 1,300 bp downstream from the Tn *916* insertion point predicted from the N225L sequencing data. The Tn *916* insertion point was determined from the sequencing data for N225L, N2210 (Table I), and the Tn *916* sequence (U09422) itself. N2210 was another phage clone containing the Tn *916* 3'-end, also isolated from the *S. pyogenes* N22 genome library using probe 5'-TGACGCTGAACTATTACG-3', 240 bp upstream of the Tn *916* 3'-end. The Tn *916* insertion point was further confirmed by direct sequencing of 2.4 kb PCR products amplified from the BL(T) genome, using primers designed from the sequence data for the BL(T) genomic region of N225L and N2210. The predicted open reading frame is indicated by a bold arrow.

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                           leader peptide                    propeptide

subtilin              MSKFDDFDLDVVKVSKQDSKITPQ    WKSESLCTPG-CVTGALQTCFLQTLTCNCKISK
                       :::::  :  :  :    ::      :  ::::: :     :  ::  :
streptin             MNNTIKDFDLDL-KTNKKDT-ATPY    VGSRYLCTPGSCWK--L-VCFTTTVK
                       : ::: :::      ::: : :      :  ::::: :     :  :   :
nisin                 MST-KDFNLDLVSVSKKDSGASPR    ITSISLCTPG-CKTGALMGCNMKTATCHCSIHVSK

consensus peptides          FNLDV   S  DS   PR
                            D       T  KT   Q
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Fig. 2. **Alignment of putative streptin leader- and prepro-peptides in comparison with those of Class AI lantibiotics.** The amino acid sequence of nisin A of *L. lactis* is cited from Kuipers *et al.* (*28*), and that of subtilin spaS of *B. subtilis* from Chung *et al.* (*29*); Klein *et al.* (*30*). Identical amino acid residues among streptin, nisin, and subtilin were indicated by colons. Consensus peptides indicate consensus sequences among Class AI lantibiotics leader peptides containing an FNLDV box and several other residues (*31*).

sequence of the insertion of Tn *916*, ORF-7, -8, -9, and ORF-10 were probably not expressed in N22. The insertional inactivation of hemolysis indicates that at least one of these ORFs has an essential role in the synthesis, modification, regulation or secretion of SLS.

Detailed Analysis of the ORFs' Sequence—A DDBJ database search using programs FASTA and SSEARCH revealed that ORF4 exhibits significant homology with *nisA*, the precursor of nisin (*28*), and *spaS*, precursor of subtilin (*29*, *30*), as shown in Fig. 2. Alignment data on leader peptides strongly suggest that the putative ORF-4 peptide belongs to Class AI of group A lantibiotics, as shown by its high homology with nisin and the subtilin leader peptide, and the presence of an FNLDV box (*31*) characteristic of Class AI lantibiotics. Both nisin and subtilin are well known lantibiotics, *i.e.* antibiotics containing lanthionine. Besides the ORFs for precursors, several genes forming a cluster are required for the production of a mature peptide (Fig. 3). The putative amino acid sequences of ORF-1 to -10 deduced from the nucleotide sequences show significant homology with those of the nisin and subtilin gene clusters (Fig. 3 and Table III). These data indicated that the gene cluster around the Tn *916* insertion site in N22 specifies a lantibiotic in *S. pyogenes* BL(T). The occurrence of another lantibiotic, streptococcin, a Class AII lantibiotic, is known in several strains of *S. pyogenes* (*16*, *17*, *32*). Homology was, however, not detected between the ORFs in Table III and the streptococcin gene cluster (data not shown). This thus indicates that the lantibiotic of BL(T) is a hitherto unknown bacteriocin of *S. pyogenes*, and thus we designated this lantibiotic as "streptin."

The streptin gene cluster is composed of 9 open reading frames, encoding candidate proteins for precursor, modification, regulation, and transport, as deduced from the homology data with nisin and subtilin gene clusters (Table III). As far as homology is concerned, this cluster is apparently lacking in a locus (*srtI*) necessary for the production of an immunity protein against streptin itself. Actually, there is a single ORF (ORF-1 in Fig. 1) upstream of *srt*R in the streptin gene cluster, and the hydrophobicity profile of the putative amino-acid sequence of this ORF is quite similar to those of NisI and SpaI. The two immunity factors (NisI and SpaI) exhibit no homology, and it is probable that *srtI* encodes the immunity protein of streptin. Regulation factor (*srtR*, *srtK*) and transport factor (*srtT*, *srtF*) exhibited higher homology, whereas modification factor (*srtB*, *srtC*) and transport/immunity factor (*srtE*, *srtG*) showed a relatively limited homology with those of nisin and subtilin. *srtE* encodes a predominantly hydrophobic protein, which is similar to the C-terminal part of SpaF, and SrtF resembles the N-terminal part of SpaF. The sequences of NisF-NisE and SrtF-SrtE joined together resemble single SpaF protein probably evolved by fusion. There is a serine protease-coding gene, *nis*P, in the nisin gene cluster, but a corresponding gene is absent in the streptin and subtilin clusters. NisP is a subtilisin-like protease and *B. subtilis* is known to secrete many kinds of protease. An enzyme encoded by a gene other than the subtilin cluster may function as the protease factor and subtilisin is the most probable candidate. The gene encoding the protease factor is not located in a region flanking the streptin gene cluster (data not shown). Like *B. subtilis*, *S. pyogenes* also produces proteases, one of which might substitute for the NisP-equivalent protease factor.

Anti-Bacterial Activity of the Wild Type and Non-Hemolytic Mutant—The Tn *916* insertion in the streptin gene cluster is probably responsible for inactivating SLS activity. Is this gene cluster actually expressed and does it contribute to the anti-bacterial activity of lantibiotics? In order to answer these questions, anti-bacterial activity was com-

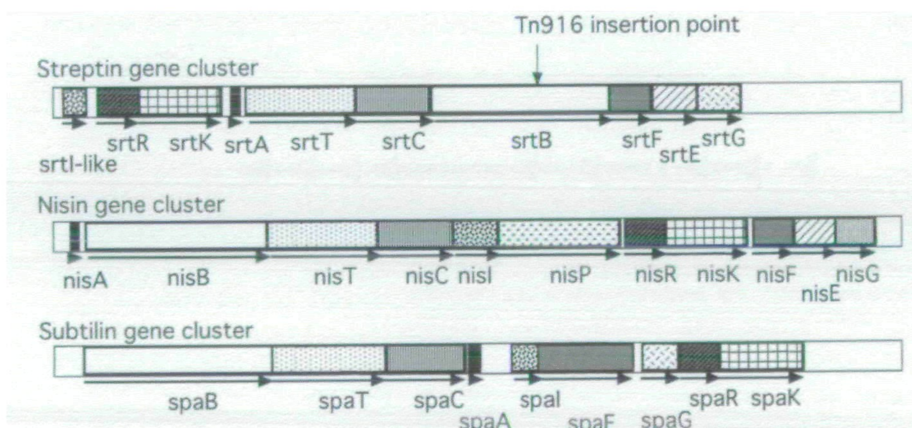

Fig. 3. **Comparison of the streptin (*srt*) gene cluster with those of other Class AI lantibiotics.** The organization of lantibiotic operons and its sequence are based on Siezen *et al.* (*36*), and DNA sequences of accession number therein. srt, streptin; nis, nisin; spa, subtilin. Identical capitals are used for individual genes encoding similar functions. Similar shading indicates sequence conservation. The presumed functions include structural gene (A), modifying factor (B, C), immunity (I), protease (P), regulator (R, K), transport (T), and accessory self-protection (EFG or FG). Structural genes were unified in A, although certain original literatures used S.

TABLE III. **Amino-acid comparison among gene cluster products of *srt*, *nis*, and *spa*.**

Lantibiotic	Acronym	Precursor	Modification		Protease	Regulation		Transport	Transport/immunity			Immunity
		A	B	C	P	R	K	T	F	E	G	I
Streptin	*srt*	46*	988	414	—	228	448	596	229	249	240	105
Nisin	*nis*	57	993	418	682	229	447	600	225	242	214	245
		(48)	(28)	(26)		(60)	(41)	(38)	(45)	(22)	(24)	(7)
Subtilin	*spa*	56	1030	441	—	220	459	614	(—447—)**		203	165
		(46)	(30)	(28)		(41)	(27)	(43)	(55)	(27)	(26)	(8)

*Amino acid length. ( ): percentage of amino acid identity to *srt* gene product. **Single polypeptide (fused).

pared between the wild type and mutant strain. The concentrated culture supernatant of wild type BL(T)^N completely inhibited the growth of Group A *Streptococcus* strain FF22 (Fig. 4). Because strain FF22 is a streptococcin producer, this inhibitory effect is not due to streptococcin. On the other hand, Tn *916*-inserted mutant N22 had no growth-inhibiting effect on Group A *Streptococcus* strain FF22. Upon protease treatment, the culture supernatant of the wild type was also deprived of the inhibitory effect. These data indicate that the anti-bacterial activity in the wild type culture supernatant is due to a protein factor directed by the streptin gene cluster.

The anti-bacterial activity against other indicators was also examined and is included in Table IV. The growth of Group A *Streptococcus* strain W-1, a streptococcin-sensitive strain, and Group C *Streptococcus* strain T-148, a streptococcin-resistant strain, was similarly inhibited by the concentrated culture supernatant of the wild type. The activity of these strains was also lost on Tn *916* insertion or on protease treatment of the culture supernatant of the wild type strain, showing that streptin was responsible for the anti-bacterial activity.

DISCUSSION

Nida and Cleary (*23*) have isolated a streptococcal mutant deficient in SLS activity by Tn *916* introduction *via* filter mating. Since then, several reports have appeared concerning Tn *916*-inserted SLS mutants (*14, 15, 33–35*), and several genes required for manifestation of SLS activity have been identified. These gene factors alone are not sufficient for elucidation of the mechanisms of the synthesis, modification, secretion, and function of SLS. In this paper, we described a new SLS-deficient mutant in which Tn *916* was inserted into a locus distinct from *sag* (*15*). This mutant, N22, of *S. pyogenes* strain BL(T), is completely devoid of SLS activity in the intracellular (ICH-S), cell-bound (CBH), and extracellular fractions (Table II). The fact that ICH-S is absent in N22 suggests a failure of synthesis, modification or maturation, and not of transport of SLS in this mutant.

Analysis around the Tn *916* insertion site of N22 led to the detection of an unknown gene cluster involved in the production of a lantibiotic-type bacteriocin, streptin. The streptin precursor gene (*srtA*) exhibits significant homology with *nisA*, encoding precursor of nisin (*28*), and *spaS*, for precursor of subtilin (*30*). The *srtA* gene also shows high homology to genes for precursors of gallidermin (U61158) and epidermin (X62386), but not to those of streptococcin (L11653) and salivaricin (L07740), indicating that streptin is probably a Class AI lantibiotic. This class of lantibiotics requires a series of factors for modification, regulation, and secretion, encoded by genes adjacent to each other. The Class AI lantibiotics including nisin, subtilin, gallidermin and epidermin forms a similar gene cluster (*36, 37*), and there is significant homology among the factors. Based on the homology to the nisin and subtilin genes, 10 members constituting the streptin gene cluster are arranged as shown in Table III, and assigned to modification, regulation, transport, and immunity factors. Exceptionally, there is no significant homology between deduced immunity gene *srtI* and other immunity factor genes, *i.e. nisI* and *spaI* (Table III). Even *nisI* and *spaI* are nonhomologous in their nucleotide sequences. Although these immunity factors are also different in length, the similar hydrophobicity profiles of the deduced gene products suggest that *srtI* encodes an immunity protein. In comparison with the nisin and subtilin gene clusters (*36, 38*), the functions of the *srt* products are inferred to be as follows. SrtK and SrtR are implicated in control of the expression of the streptin gene cluster. SrtT has an ATP binding motif and probably participates in the export of streptin itself or its precursor. SrtC and SrtB may be factors for specific posttranslational modification of precursor peptide. SrtF also has ABC binding motif and, together with SrtE and SrtG, forms ABC transporter system. SrtF may also participate in the immunity to bacteriocin itself, as deduced from the cognate of the nisin and subtilin gene clusters (*39*).

Insertion of Tn *916* into the *srtB* locus in N22 (Fig. 1) is

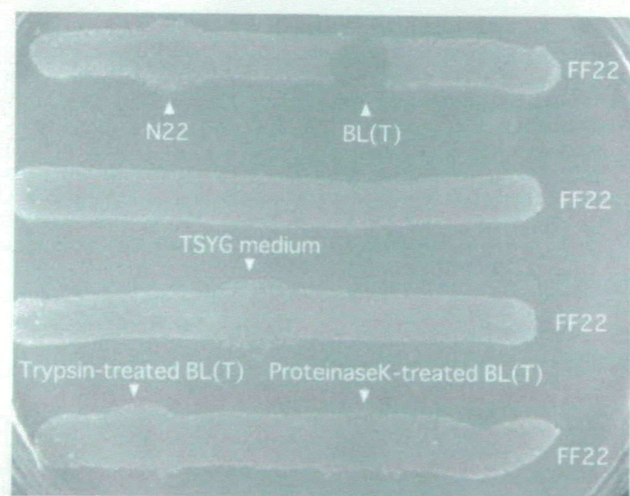

Fig. 4. **The anti-bacterial activity of concentrated culture supernatants against *S. pyogenes* FF22 (*scn*).** Group A *Streptococcus* strain FF22 (abbreviated as FF22) was streaked as the indicator for bacteriocin activity. The position of the spot of the culture supernatant is indicated by an arrow. BL(T) culture supernatants treated with trypsin or proteinase K were also spotted, along with the concentrated TSYG medium.

TABLE IV. **Anti-bacterial activity in concentrated culture supernatants.**

Bacteriocin producer strain	Indicator strain		
	S. pyogenes FF22	Group-A, strain W1	Group-C, strain T-148
S. pyogenes BL(T) (Streptin-producing strain)	+*	+	+
N22 [SLS (−) mutant of BL(T)]	−**	−	−
BL(T) trypsin-treated culture supernatant	−	−	−
BL(T) proteinaseK-treated culture supernatant	−	−	−
S. pyogenes FF22 (Streptococcin-producing strain)	−	+	−

*Inhibited the growth of indicator bacteria. **Did not inhibit the growth of indicator.

expected to spoil the translation of downstream genes (*srtF*, *srtE*, and *srtG*), besides premature termination of *srtB*. The fact that this insertion abolishes the production of active SLS suggests the involvement of some of the four genes (*srt-B*, *-F*, *-E*, and *-G*) in the synthesis, modification, maturation, or secretion of this hemolysin. Because streptin per se is nonhemolytic (data not shown), *srtA*, the structural gene for this bacteriocin, is probably unrelated with the SLS system. *srtF*, *srtE*, and *srtG* are predicted to have a transport function, but this function might not play a pivotal role in active SLS secretion, because even ICH-S and CBH are not detected in N22 (Table II). It is, however, undeniable that these genes might be involved in immunity against SLS. SrtB, a factor for the maturation of active streptin, may participate in the formation of thioether rings. SLS has been reported to be deficient in Val, Leu, Cys, and Arg (*11*), whereas *sagA* has these amino acid codons (*15*). The nucleotide sequence of *sagA* (*14*) is also inconsistent with the result of amino acid incorporation into SLS (*40*). Involvement of SrtB in modification of the SLS precursor might explain these differences partly.

The concentrated culture supernatant of strain BL(T) strongly inhibited the growth of indicator strains, whereas strain N22 completely lost this inhibitory effect (Table IV). Protease treatment of the wild type supernatant abolished the inhibitory effect, indicating that the anti-bacterial activity of the culture supernatant is due to a protein factor or bacteriocin. *S. pyogenes* is known to secrete another lantibiotic, streptococcin. The reported host range of streptococcin (*27*, *32*) differs from that of BL(T). Above all, the BL(T) supernatant inhibited the growth of streptococcin-producing strain FF22, which must have an immunity system against streptococcin. These results demonstrate that the inhibitory protein of BL(T) is not due to streptococcin but to a new bacteriocin, streptin. Mutant strain N22 is devoid of anti-bacterial activity probably owing to impeded maturation or secretion of streptin. Furthermore, insertion of Tn *916* into the streptin gene cluster caused simultaneous loss of the SLS function and streptin activity, possibly by deficiency in factors SrtB, SrtE, SrtF, or SrtG. These factors, originally serving in lantibiotic formation, might be appropriated for SLS production in *S. pyogenes*.

Recently, Nizet *et al.* (*15*) reported the expression of SLS activity in *Lactococcus lactis*, after transfer of the *sag* gene cluster. *sagA* was first identified by Betschel *et al.* (*14*) in a SLS-deficient mutant obtained by Tn *916* insertion. A similar *sag* mutant, N73, was isolated in our filter mating experiment as well, using *S. pyogenes* strain BL(T). In N73, a single Tn *916* is inserted in the promoter region of *sagA*, 9 bp downstream of the insertion point of the mutant reported by Betschel *et al.* (*14*). Both N22 and N73 are SLS-deficient mutants caused by a single Tn *916*, suggesting that some genes in the streptin and *sag* clusters are involved in the formation of active SLS. In *S. pyogenes*, *sag* gene products may be necessary but insufficient for the production of SLS. The *L. lactis* mutant used by Nizet *et al.* lacks the nisin operon (*15*), but it is still possible that other lantibiotics such as lacticin (*41*) may substitute for functions of streptin gene cluster. The hemolytic activity of both extra- and intra-cellular SLS is resistant, whereas the formation of SLS is very sensitive to SH-blocking reagents (*42*). Because the putative peptide encoded in *sagA* is specifically cysteine-rich, this gene is thought to be crucial for SLS production.

All sequences of the streptin gene cluster including the *srtI*-like gene were also detected in the M1 Group A *Streptococcus* sequencing project data at the University of Oklahoma (www.genome.ou.edu/strep.html), with some base diversities. The *sag* gene operon and streptin gene cluster are not closely mapped each other on the M1 Group A *Streptococcus* sequencing project data. The reason why two bacteriocin gene families, for prepropeptide, chemical modification, processing, and export, are involved in SLS formation is at present unknown. Mutagenesis experiments on *covR* (*43*) and CsrR-CsrS (*34*) indicated that *sagA* transcription was under the influence of these factors. The *pel* gene, which is identical with *sagA*, dominated not only SLS but also other surface and secreted proteins (*44*). It seems quite probable that other separate gene factors are necessary for SLS formation. Our preliminary analysis revealed involvement of distinct gene products in the formation of this peculiar hemolysin (manuscript in preparation).

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