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Nucleotide sequence of the streptococcin A-FF22 lantibiotic regulon: model for production of the lantibiotic SA-FF22 by strains of *Streptococcus pyogenes*

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Abstract

Streptococcin A-FF22 (SA-FF22) is a type AII linear lantibiotic produced by *Streptococcus pyogenes* strain FF22. Sequence analysis of an approximate 10 kb region of DNA showed it to contain nine open reading frames arranged in three operons responsible for regulation, biosynthesis and immunity of SA-FF22. This region is organized similarly to the *Lactococcus lactis* lacticin 481 region, however, unlike lacticin 481, a two-component regulatory system is essential for SA-FF22 production. Located immediately downstream of the *scn* region is a putative transposase gene, the presence of which supports earlier data that indicated a mobile nature to this region.

Keywords: Lantibiotic; Bacteriocin; *Streptococcus pyogenes*

1. Introduction

Streptococcin A-FF22 (SA-FF22) is a 26 amino acid lantibiotic produced by certain strains of *Streptococcus pyogenes*, the structural gene of which has been described previously [1]. Lantibiotics, post-translationally modified peptide antibiotics, are produced by a variety of microorganisms including several species of *Bacillus*, *Staphylococcus*, *Lactococcus*, and *Streptococcus* [2]. Emphasis has been placed on understanding the molecular mechanisms of how these unusual antimicrobial peptides are produced [3–9]. Lantibiotics are divided into two groups: type A being the linear type lantibiotic characterized by nisin, and type B being more globular in structure [10]. As the sequence of additional lantibiotics became available, the type A lantibiotics were further divided into three subtypes [7]: type AI characterized by nisin, type AII characterized by streptococcin A-FF22 and lacticin 481, and a third subtype AIII containing lactocin S and cytolysin. The AII subtype, of which SA-FF22 was the first member to be sequenced, differs from the other subgroups in ring structure and characteristic leader peptide cleavage site.

Because of the similarities that exist between lacticin 481 and SA-FF22, and therefore possibly other subtype AII lantibiotics, it is important to ascertain whether similar proteins are involved in the produc-

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tion of these lantibiotics and whether these 'accessory proteins' also share homologies. As analysis of the operons associated with different lantibiotics continues, models explaining the molecular mechanisms of lantibiotic production can be proposed. Here we report on the sequence of the first lantibiotic operon described from *S. pyogenes* and its similarity to the recently reported lacticin 481 gene region [7,11]. A model for the production of SA-FF22 is described based on the genes determined to be present in the *scn* gene clusters and similarities to proteins of other lantibiotic operons.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. pyogenes strains were grown on Todd–Hewitt medium supplemented with 1% (w/v) yeast extract in a 5% CO_2 atmosphere at 37°C. *E. coli* strains were maintained on LB medium supplemented with antibiotics as needed.

2.2. DNA isolation and manipulation

Plasmid DNA was isolated from *E. coli* using standard methods [12]. Chromosomal DNA was isolated from *S. pyogenes* FF22 by the method of Pitcher et al. [13]. Southern hybridization was performed using the Genius non-radioactive system (Boehringer Mannheim).

2.3. λEMBL3 library construction

Chromosomal DNA from *S. pyogenes* FF22 was partially digested with *Sau*3aI and ligated to λEMBL3-*Bam*HI digested arms. The concatameric DNA was packaged and transfected into *E. coli* ER1647. Recombinant phage was isolated using a probe corresponding to the *scn*A-*scn*M region and subsequently purified by three rounds of plaque hybridization. Recombinant phage DNA was subcloned into various vectors for sequencing and further manipulations using standard techniques [12].

2.4. DNA sequencing

DNA sequencing was performed both manually and with an automated sequencing apparatus at the University of Oklahoma Sequencing facility. The sequences were assembled using Sequencer v3.1 software. The sequence data have been submitted to GenBank under accession number AF026542.

2.5. PCR methods

Several regions of the *scn* cluster were cloned directly from FF22 chromosomal DNA by PCR amplification. Amplified DNA was cloned and verified by sequencing. These regions were used in conjunction with Southern hybridization to verify DNA linkage and isolate specific fragments for additional manipulation.

2.6. RNA isolation

RNA was isolated from log phase *E. coli* ER1647 cells 30 min after infection with λEMBL3 phage (~5:1 multiplicity of infection). RNA was isolated from FF22 by hot acid-phenol extraction as follows: Cells from 50 ml of an overnight culture grown in THY medium were suspended in 1 ml of lysis solution (8 M guanidine HCl, 0.5% sarkosyl, 0.8% SDS, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol (added just before use)). The cells were transferred to a 2 ml screw capped microfuge tube approximately 1/2 full with 0.1 mm Zirconia/silica beads and lysed by five successive 1 min cycles using a Bead Beater (Biospec Products, Inc., Bartlesville, OK, USA) at 5000 rpm. The samples were cooled on ice for 1 min periods between each cycle. The bead/cell mixture was extracted three times (10 min each at 65°C) with pre-heated acid-phenol followed by extraction using acid-phenol:chloroform:isoamyl alcohol (25:24:1). RNA was precipitated from the aqueous phase by addition of sodium acetate to 0.3 M final concentration and 1 volume ice-cold isopropanol. After overnight storage at −20°C, the RNA was pelleted, washed once with 70% ethanol, and suspended in 200 μl water.

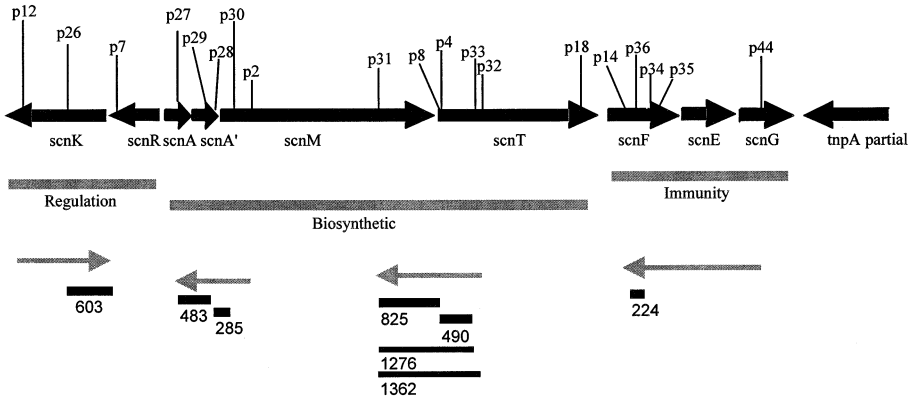

Fig. 1. Diagrammatic representation of the *scn* regulon from *S. pyogenes* FF22. The genes (black arrows) and three operons (regulatory, biosynthetic, and immunity) of the *scn* regulon (grey bars) are shown. Positions of the primers used for the RT-PCR analysis of the *scn* regulon are indicated. The lower part of the diargram indicates the size of the PCR products (boxes) obtained following amplification of cDNA from primers P12, P2, P32 and P44 (arrows).

2.7. Reverse transcriptase-PCR

Reverse transcriptase-PCR (RT-PCR) was performed on RNA samples as previously described [14]. Primers used are shown in Table 1 with positions in operon shown in Fig. 1.

3. Results and discussion

The sequence of the structural gene for the lantibiotic *scn*A has been reported previously [1,15]. We now report the completed sequence (GenBank accession number AF026542) analysis of the entire gene cluster involved with SA-FF22 production. The gene cluster is composed of nine open reading frames (ORFs) arranged in three operonic units (Fig. 1), and is followed by a putative transposase gene. Comparison of the scn ORFs to the non-redundant database using the BLAST algorithms revealed similarity with other known lantibiotic genes.

The SA-FF22 gene cluster is organized similar to other lantibiotic operons, and is comprised of three operons: a regulatory, a biosynthetic, and a putative immunity operon. Transcriptional linkage of the genes within these operons was determined by

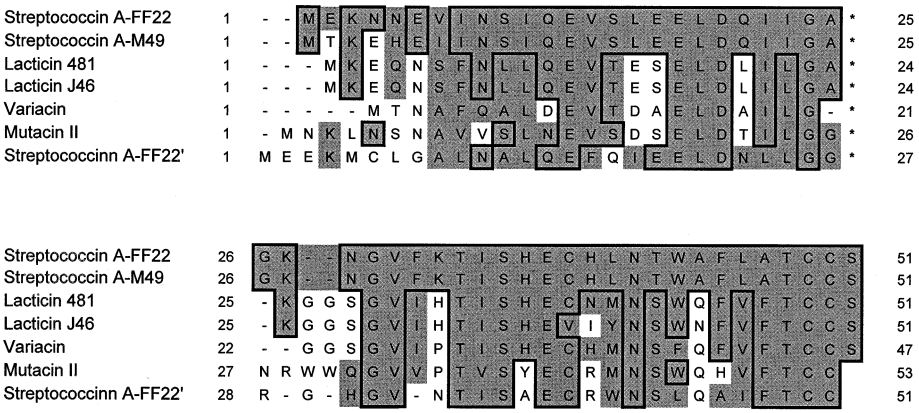

Fig. 2. Multiple sequence alignment of type AII lantibiotics. Amino acids sharing identity (boxed) and similarity (shaded) with SA-FF22 are indicated. The known/proposed pre-lantibiotic cleavage site is represented as a (*) and is aligned in all six sequences.

RT-PCR amplification of the junctions between each ORF using RNA isolated from both FF22 and *E. coli* transfected with recombinant λEMBL3 phage. The regulatory operon consists of two ORFs (*scn*R and *scn*K) transcribed together, but divergently from the *scn* biosynthesis regulon. *scn*R and *scn*K form a 'typical' two-component regulatory system with similarity to other known response regulatory systems, and consists of the response regulator (ScnR) and a histidine kinase type sensor protein (ScnK). Inactivation studies showed that these two genes play an important role in the production of SA-FF22; disruption of either gene resulted in loss of SA-FF22 production. To date, no regulatory genes have been reported associated with the lacticin 481 operon, suggesting that in the group A streptococci lantibiotic production may be more tightly regulated than in *Lactococcus lactis*.

The biosynthetic operon is comprised of *scn*A, *scn*A′, *scn*M and *scn*T (Fig. 1). The SA-FF22 prelantibiotic (*scn*A product) shares a high degree of similarity with the lactococcal lantibiotics 481 and J46, as well as the lantibiotics variacin and mutacin II, produced by *Micrococcus varians* and *Streptococcus mutans*, respectively (Fig. 2). These lantibiotics all share similarity at the peptidase cleavage site (Gly^{-2}-Ala^{-1}-X^{+1}) as well as being lantibiotic peptides which terminate in the sequence Cys-Cys-Ser. Downstream from *scn*A is an ORF, designated *scn*A′, that encodes a deduced peptide with similarity to ScnA and the lantibiotics which share similarity with ScnA. This peptide differs from the SA-FF22 type lantibiotics by (1) possessing a proposed peptidase cleavage site of Gly^{-2}-Gly^{-1}-X^{+1} (similar to salivaricin A and non-lanthionine-containing bacter-

Table 1
Primers used in RT-PCR amplification of the scn regulon

Primer	Sequence
p12	ATTTTAATGTCGCAACCAGA
p26	ATAAATGTAAAGAAATAGGCAGAA
$p7_c$	TAACTCCATCTAAGCCAAAT
$p2_c$	CCAGTTATTTTGATTTTGAC
p27	AAGTAATCAACTCTATTCAAGAAG
$p28_c$	AAATTGCCTGTAAACTATTCCAAC
p29	CTGAATGTCGTTGGAATAGTTTAC
$p30_c$	TTCATAGCGTGACTCTGGAGAATC
$p32_c$	CCTAAAAGGAATAGGCTGATAC
p31	TTTTCTATCAAGTTTTCCTAATGA
p8	TGCAAAACAATGAGGAGGAT
$p4_c$	TAACAAGCTAAGAGACAATC
$p33_c$	AATAAACAGGAGTGACAACA
$p44_c$	GCTAACAAGAACTTACTAAGAAGC
p18	TTTTGGGACGAGGCTTTCAG
p14	GACTTTGGCATTGGTTGGTT
$p36_c$	TTTTCGGTTATTTTCCTCTGCTAA
p35	GGGCTAACAGTATTTATCTCAAGT
$p34_c$	AATACTGTTAGCCCTTTCTCCT

The positions of the primers in the regulon are indicated in Fig. 1. Primers on the complimentary strand are designated with a '$_c$'.

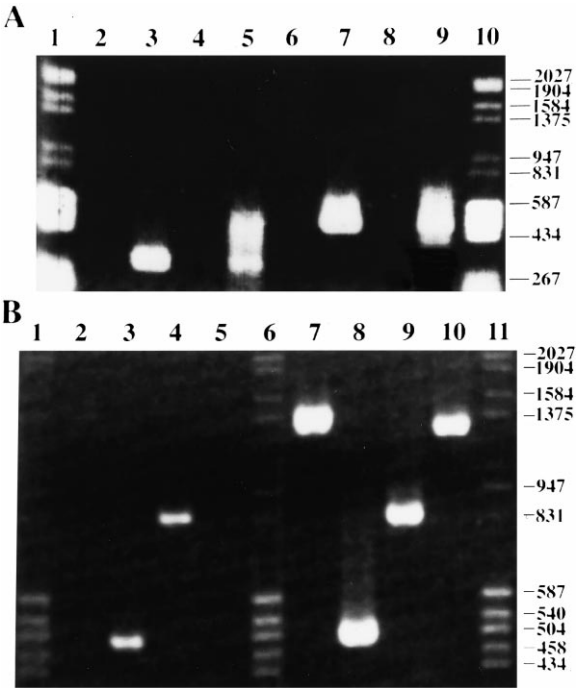

Fig. 3. RT-PCR analysis of the *scn* biosynthetic operon using RNA isolated from *E. coli* transfected with recombinant λEMBL3. A: cDNA was generated using primer p2 and amplified using primers p29×p30 (lanes 2–5) and primers p27×p28 (lanes 6–9). Lanes 2 and 6: control reactions containing no template (reagent control); lanes 3 and 7: positive controls (amplified FF22 DNA); lanes 4 and 8: cDNA generated in the absence of reverse transcriptase (template controls); and lanes 5 and 9: RT-PCR products. B: cDNA was generated using primer p32 and amplified using: p31×p32 (lane 2), p33×p8 (lane 3), p31×p4 (lane 4) p31×p33 (lane 5). Lanes 7–10 contained the corresponding product amplified from FF22 DNA. Control reactions described above revealed no amplification products. Small amounts of amplified product was obtained from p31×p32 (lane 2) and p31×p33 (lane 5) (not visible in photo). RNA isolated directly from *S. pyogenes* FF22 gave identical results although with less consistency and lower product yield.

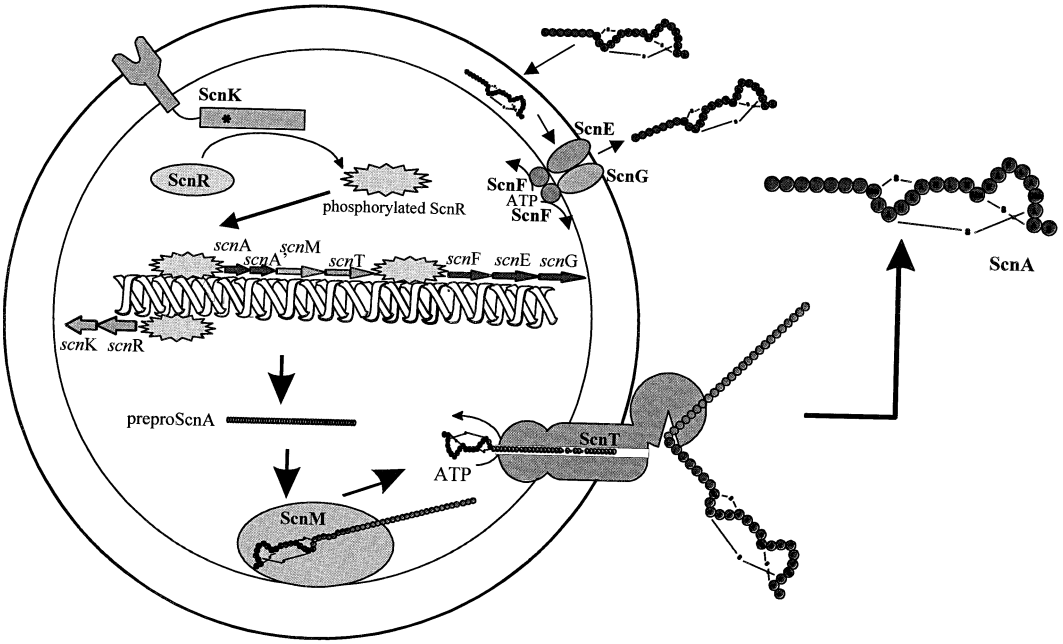

Fig. 4. Schematic model for the production of streptococcin A-FF22 (ScnA). Production of ScnA requires three operonic units: The biosynthetic operon consisting of *scn*A (ScnA), *scn*A′ (unknown function), *scn*M (modifying enzyme), *scn*T (transport and leader peptide cleavage), the immunity operon containing *scn*F, *scn*E and *scn*G (ABC transport system), and the regulatory operon consisting of *scn*R (response regulator) and *scn*K (histidine kinase). See text for in depth description of proposed model.

iocins) and (2) terminating in Cys-Cys, also present in salivaricin A, as well as gallidermin and epidermin. RT-PCR using primers specific to *scn*A and *scn*M revealed that the entire region is transcribed as part of the biosynthetic operon (Fig. 3), however, to date translation of the ScnA′ peptide has not been demonstrated.

ScnM and ScnT are the proposed processing and transport proteins involved with the maturation of the pre-ScnA lantibiotic molecule. ScnM has similarity to LctM from *L. lactis* [7], MutM from *S. mutans* [16], and CylM from *Enterococcus faecalis* [9]. Computer analysis indicates that ScnM has a single potential transmembrane region at the extreme C terminus, suggesting it may be anchored in the membrane. ScnT shares similarity with ATP-dependent transport proteins and has seven potential transmembrane segments. In addition, ScnT contains the motif signature for ABC transport proteins as well as an ATP/GTP-binding site motif A (P-loop). In lacticin 481, LctT is proposed to be a dual functioning dimer, used in export of the modified prepeptide and cleavage of the leader peptide region to give mature active lantibiotic [7]. A similar function is thought to reside with ScnT since the *scn* operon does not appear to encode a separate protease which could cleave the leader peptide. RT-PCR amplification of the junction between *scn*M and *scn*T shows transcriptional linkage of these two genes, thereby linking *scn*A, A′, M and T as a single transcription unit.

The proposed immunity operon consists of three genes (*scn*F, G and E) which are transcriptionally linked, as determined by RT-PCR; no linkage was observed between *scn*T and *scn*F. This operon appears to comprise another ABC transport system with similarity to the immunity operons of lacticin 481 [7], and other lantibiotics [5,17]. These genes are reported to encode proteins which associate to form a transport complex that prevents the formation of pores in the cytoplasmic membrane by keeping the number of lantibiotic molecules that could come in contact with the cytoplasmic membrane low [6]. The deduced proteins of the immunity operon (ScnE and ScnG) are predicted to contain multiple membrane spanning domains. Unlike the *lct* immunity cluster which appears to confer immunity to strains of *L.*

lactis when acquired, placing the *scn* immunity cluster into an SA-FF22 sensitive strain of *S. pyogenes* did not confer resistance (data not shown). This lack of resistance may be due to a requirement for the ScnR/ScnK components of the lantibiotic operon to promote expression of the immunity genes. Resistance to the action of subtilin by expression of the immunity genes is also under the control of a two-component regulatory system [3]. The action of SA-FF22 on the producer strain [18] may be due to production of an over-whelming level of SA-FF22 such that the transport system cannot prevent pore formation.

Located approximately 100 bp down of *scn*G, and divergently transcribed, is *tnp*A. *tnp*A is predicted to encode a transposase gene, based on homology of 66% identity and 81% similarity with IS-LL6 of plasmid pWV04 from *L. lactis* (GB:U23813). The organization and location of the *tnp*A gene suggest that the *scn* regulon may be present on a mobile element. These data are also supported by earlier observations [19] that suggested the *scn* (SA-FF22) lantibiotic gene was on some type of mobile genetic element. The presence of a sequence with similarities to a lactococcal transposon suggests the possibility that transfer of genes (operons and regulons) occurred between lactococci and streptococci.

Comparison of the partial transposase gene with data present in the *S. pyogenes* genome database (http://www.genome.ou.edu) revealed the presence of the *tnp*A gene (98% identity at nucleotide level) in another streptococcal strain (SF370). A region 4 kb distal to *tnp*A appears to be essentially identical in both SF370 and FF22. The 1 kb region immediately upstream of *tnp*A in SF370 (the region occupied by the *scn* regulon in FF22) is completely different, lacking any ORFs with significant homology to sequences deposited in the various databases.

Based on the results of the current study and similarity to other lantibiotic proteins, we propose the following model for the synthesis of SA-FF22 (Fig. 4). ScnK detects some as yet unidentified environmental signal resulting in an autophosphorylation. The phosphoryl group is then transferred to ScnR, activating the protein. Activated ScnR is able to bind to the appropriate promoters, initiating transcription of the operonic units. ScnA is produced as a prepropeptide which is modified by ScnM. The modified propeptide is transported out of the cell by ScnT, which also cleaves the leader peptide resulting in production of active SA-FF22. Immunity to active SA-FF22 occurs by the action of a multimeric ABC transport complex (ScnF, ScnE and ScnG) which actively prevents the lantibiotic from reaching levels which can produce pores in the cytoplasmic membrane.

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