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Characterization of NADase-Inactive NAD^+ Glycohydrolase in *Streptococcus pyogenes*

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ABSTRACT

Background: *Streptococcus pyogenes* secretes NAD^+ glycohydrolase (NADase, also known as SPN or Nga). All *S. pyogenes* strains examined to date possess the gene that encodes SPN (*spn*), but some strains produce SPN that lacks detectable NADase activity. Although there is much evidence to support that SPN's NADase activity contributes to virulence, there is very little evidence that NADase-inactive SPN has detectable functions. **Results:** In order to characterize the NADase-inactive SPN, we firstly attempted to clone the NADase-inactive *spn* allele in *Escherichia coli*. Although we obtained recombinants which were shown to have the correct size insert, all had some mutations in the *spn* allele. Therefore, we attempted to change the mutated nucleotides back to the original nucleotides. While a nucleotide mutagenesis (inverse PCR method) easily changed a target nucleotide of control genes back to the original nucleotides, the mutations of NADase-inactive *spn* allele were never successfully converted back to the original nucleotides. Finally the mutant *spn* alleles were sub-cloned into another vector (pLZ12-Km2), which is maintained in both *E. coli* and *S. pyogenes*. The resultant plasmids were subjected to nucleotide mutagenesis using inverse PCR; the resultant mutagenized plasmid DNAs were used to transform both *E. coli* and *S. pyogenes* strains. We observed successful nucleotide substitutions back to the original *spn* nucleotide sequence in *S. pyogenes* transformants, but not in *E. coli* transformants. Thus, the NADase-inactive *spn* allele was successfully cloned in *S. pyogenes*, but not in *E. coli*. However, we could not find an association with NADase-inactive *spn* allele and virulence in a mouse infection model. **Conclusions:** These results suggest that NADase-inactive *spn* allele has some toxic effect to *E. coli*, but not *S. pyogenes*. This effect may due to an as of yet unknown function attributable to NADase-inactive SPN.

Keywords: *Streptococcus pyogenes*; NAD^+ Glycohydrolase; NADase; SPN; Streptococcal; SF370

1. Introduction

Streptococcus pyogenes is a gram-positive bacterium that infects the upper respiratory tract, including the tonsils and pharynx, and is responsible for post-infectious diseases such as rheumatic fever and glomerulonephritis. In addition, *S. pyogenes* causes severe invasive disease including necrotizing fasciitis [1-6]. The molecular mechanisms that the organism utilizes to cause these diseases are not yet elucidated fully. To analyze these mechanisms, it is important to characterize the virulence factors of *S. pyogenes* fully. *S. pyogenes* secretes several distinct proteins such as superantigens, DNases, streptokinase, cysteine proteinase SpeB, C5a peptidase, and streptococcal inhibitor of complement-mediated lysis (Sic) [7,8]. Several of these proteins have been identified as virulence factors and analyzed in detail, and others are still not yet fully characterized. NAD^+ glycohydrolase (NADase, also known as SPN or Nga) is one of the secreted proteins which should be further characterized.

SPN is known as the host attacking enzymatic toxin produced by *S. pyogenes* that shows cytotoxic effects to keratinocytes *in vitro* experiment [9,10]. SPN is also demonstrated toxicity in bacterial cells. To counteract this toxicity, *S. pyogenes* encodes *ifs* gene whose product (IFS) is an endogenous inhibitor of NADase activity and is localized in the bacterial cytoplasmic compartment. Inside the *S. pyogenes* bacterial cell, SPN precursor exists as an inactive complex with IFS [11,12].

Although the SPN had been found long ago [13], initial studies on SPN were hindered by the fact that it was not possible to clone *spn* in *Escherichia coli* which is a commonly used bacterial host for genetic research (due to bacterial death) [11]. In the study conducted by Meehl *et al*. [11], in which *ifs* gene was discovered to exist as a *spn-ifs* operon, they were able to resolve this cloning toxicity issue when the *spn* gene was successfully cloned into a plasmid together with *ifs* (as a *spn-ifs* operon) and subsequently introduced into *E. coli*. The cytotoxicity of

SPN is believed to depend on NADase activity; for example, the hypothesis has been put forth that depletion of cellular NAD^+ through the enzymatic action of SPN induces host cell death [14]. Meanwhile, it has been demonstrated that some strains produce SPN that lack detectable NADase activity [11,15-17]. The presence of an aspartic acid instead of a glycine at amino acid residue 330 (G330D polymorphism) has been associated with loss of SPN NADase activity [18,19]. Additionally, *ifs* genes degrade into psedogenes in strains with the NADase-inactive SPN subtype, suggesting that the subtypes no longer lose the self-toxicity to require the functional IFS [11,19]. In contrast, the study using statistically sufficient number of NADase-inactive strains revealed that the *spn* alleles themselves never degrade into psedogenes [19]. Based on these findings suggesting that SPN has a hidden NADase-independent function [19], we were prompted to re-evaluate the role of NADase-inactive SPN in *S. pyogenes* pathogenesis.

2. Materials and Methods

2.1. Ethic Statement

All animal studies conducted comply with federal and institutional (the Committee on the Ethics of Animal Experiments of the Nagoya City University) guidelines. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Nagoya City University (Permit Number: H23M-07). All efforts were made to minimize suffering.

2.2. Bacterial Strains

Streptococcus pyogenes strains 1529 and GT01 isolated as causative organisms from invasive diseases patients in Japan possess NADase active SPN [18,20]. *S. pyogenes* (GAS) strain SF370, which is prevalent as the database reference isolate (accessionNC_002737), was provided by the courtesy of J. J. Ferretti [21,22]. Streptococcal strains were cultured in brain heart infusion (E-MC62, EIKEN Chemical Co., Tokyo, Japan) supplemented with 0.3% yeast extract (BD, Sparks, MD, USA) (BHI-Y) broth unless otherwise described.

2.3. Cloning Experiment of Spn_{SF370} Gene

The spn_{SF370} of *S. pyogenes* strain SF370 and the other control DNAs were amplified by PCR with Extaq DNA polymerase (Takara Bio, Ohtsu, Japan) using the corresponding primers listed in **Table 1**. pGEM®-T Easy vector system (Promega, Madison, WI, USA) was used for cloning of the PCR products purified by Gel extraction kit (Qiagen, Hilden, Germany). The high copy-number pGEM®-T Easy vector contains T7 and SP6 RNA poly-

Table 1. Sequences of primers used in this study.

(Cloning primers[a])	
spn_{SF370} (1.5 kbp)	
ngaGT-n1Nhe	5'-GGCTAGCGAACAGATGTGAAGGTTCTG-3'
nga-c4xho	5'-CCTCGAGTTGGCACCTTATACATATTG-3'
$spn\text{-}ifs_{GT01}$ (2.1 kbp)	
Nga-n4Eco	5'-GGAATTCATGAGAAACAAAAAAGTAAC-3'
slo2	5'-ATCATCCGTTTTCTGACCTG-3'
$covRS_{GT01}$ (2.6 kbp)	
csrRn2	5'-CTTTAGAGAATATGGTTACT-3'
csrSc2	5'-GTAATTACATTTTGGACAAC-3'
$covRS_{1529}$ (2.6 kbp)	
csrRn2	5'-CTTTAGAGAATATGGTTACT-3'
csrSc2	5'-GTAATTACATTTTGGACAAC-3'
*spy*1193 (1.0 kbp)	
MG5005-spy1193F	5'-ATGTCCTATCTCATGGTAAGG-3'
MG5005-spy1193R	5'-ACCTATAAAACAGTCAATGAG-3'
$recA_{SF370}$ (0.3 kbp)	
SPYrecA-F	5'-CGAAGTCGTTTGGATGTGCGC-3'
SPYrecA-R	5'-CCTGAACTTTTTGCTCTGCAC-3'
$vicRK_{1529}$ (2.8 kbp)	
Spy528-n1	5'-GTTGATGCAGAAGTAGTGACC-3'
Spy0528-c2	5'-GGCATCAAGCTTACCTAGCA-3'
$vicK_{1529}$ (1.7 kbp)	
Spy528-n5	5'-CGGTTGATGTGACTGTTCG-3'
Spy0528-c2	5'-GGCATCAAGCTTACCTAGCA-3'
$spn\text{-}ifs_{SF370}$ (2.0 kbp)	
ngaGT-n1Nhe	5'-GGCTAGCGAACAGATGTGAAGGTTCTG-3'
nga-c8xho	5'- CCGCTCGAGTTA GACATGTCCTTCATACC-3'
$spn\text{-}ifs_{SF370}$ (2.1 bp)	
ngaGT-n1Nhe	5'-GGCTAGCGAACAGATGTGAAGGTTCTG-3'
IFS-R(EcoRI)	5'-GTTTGTTCGAATTCGCATTAGCAG-3'
$spn\text{-}ifs_{SF370}$ (2.2 kbp)	
ngaGT-n1Nhe	5'-GGCTAGCGAACAGATGTGAAGGTTCTG-3'
slo-c2	5'-ATCATCCGTTTTCTGACCTG-3'
(Inverse PCR primers)	
nga(SF370)-F	5'-AGAAACAAAAAAGTAACATTAG-3'
nga(SF370)-R	5'-CATGTAAACCACCTTATATTA-3'

Continued

nga(SF370)-F2	5'-ATAGTTTACTTAAAAATAATATA-3'
nga(SF370)-R2	5'-ACATTAGCAAATAGTTTTTGTC-3'
vicK-F	5'-ACCCAATTAGCAGTAGAGATGA-3'
vicK-R	5'-TACTTGATTATCAATCCGAGA-3'

[a]Name of insert DNA fragment amplified by PCR (the expected size), used primer name and the sequence were described. The two PCR products of $recA_{SF370}$ and $spy1193_{1529}$ contain a part of the gene, respectively, whereas the others have the hole genes indicated. spn_{SF370} and $recA_{SF370}$ are PCR products from strain *SF*370. *spn-ifs*$_{GT01}$, and $covRS_{GT01}$ are PCR products derived from strain GT01. $spy1193_{1529}$, $covRS_{1529}$, $vicRK_{1529}$, and $vicK_{1529}$ are PCR products derived from strain 1529. *S. pyogenes* strain SF370 encode NADase-inactive SPN, whereas strains 1529 and GT01 encode NA-Dase-active SPN.

merase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase http://www.promega.com/~/media/files/resources/protocols/technical%20manuals/0/pgem-t%20and%20pgem-t%20easy%20vector%20systems%20protocol.pdf?la = en. In the pGEM®-T Easy vector system, recombinant clones are allowed to be directly identified by blue/white color screening on indicator plates.

For spn_{SF370}-cloning, we obtained three recombinants that have the correct size insert in the corresponding plasmids (named pGEM-spn_{SF370}26, pGEM-spn_{SF370}32, and pGEM-spn_{SF370}13; see Result section for additional detail on the creation of these plasmids).

2.4. Nucleotide Substitution by Inverse PCR

Primestar Taq DNA polymerase (Takara) was used for the inverse PCR described previously [18]. Primers used are listed in **Table 1**. PCR product was self-ligated and used to transform *E. coli* strain DH5α.

2.5. Construction of pLZ12-Km2 Derivatives

The inserts of pGEM-spn_{SF370}26, pGEM-spn_{SF370}32, and pGEM-spn_{SF370}13 were digested with *Eco*RI, and subcloned into pLZ12-Km2 to yield pLZ-spn_{SF370}26, pLZ-spn_{SF370}32, and pLZ-spn_{SF370}13, respectively.

2.6. Creation of *Spn* Mutant of Strain SF370

E. coli JM109 was used to propagate plasmid constructions. Non-polar inactivated mutant of *spn* was constructed via double-crossover allelic replacement in the chromosome of *S. pyogenes* SF370. To construct the plasmid for the *spn* knockout mutant, the 5' end of *spn* (fragment 1) was amplified with oligonucleotide primers ngaGT-n1 (5'-GGCTAGCGAACAGATGTGAAGGTTCTG-3') with an *Nhe*I restriction site and ngaGT-c1 (5'-TCCCCCGGGTTTCTCATGTAAACCACCT-3') with an *Sma*I restriction site, and the 3' end of *spn* (fragment 2) was amplified with ngaGT-n2 (5'-TCCCCCGGGATAGGAAGTAACAATATGT-3') with an *Sma*I restriction site and ngaGT-c2 (5'-GGACTAGTATGTTAGCTTTCAATTGGGT-3') with an *Spe*I restriction site. Oligonucleotides ngaGT-n1, ngaGT-c1, ngaGT-n2 and ngaGT-c2 contained a restriction site for *Nhe*I, *Sma*I, *Sma*I and *Spe*I, respectively, (shown in underline in the primer sequence). Fragment 2 was digested with *Sma*I and *Spe*I for insertion into multi-cloning site 2 of the pFW12 plasmid [23]. The resulting plasmid was then digested with *Nhe*I and *Sma*I, and both the spc2 DNA fragment containing *aad*9 (promoter less spectinomycin resistant gene), which was obtained from a *Sma*I digested fragment of pSL60-2 [24], and the *Nhe*I-*Sma*I-digested fragment 1 were inserted. This plasmid, pFW12::(*spn*::*aad*9), was a suicide vector for *S. pyogenes*. For the preparation of competent cells, strain SF370 was harvested at early- to mid-log phase (OD_{660} = 0.4 to 0.5) and washed twice with 0.5 M sucrose buffer. The constructed suicide vector pFW12::(*spn*::*aad*9) was used to transform strain SF370 by electroporation. The conditions of electroporation were 1.25 kV/mm, 25 μF capacitance and 200 Ω resistance, using Gene Pulser II (Bio-Rad, Hercules, CA, USA). After incubation at 37˚C for 3 h, competent cells were spread onto BHI agar plates containing 0.3% yeast extract and spectinomycin (final concentration 100 μg/ml). Selected colonies on the plates were cultured. Cultured bacteria were washed once with saline, resuspended in 10 mM Tris, 1 mM EDTA and boiled for 10 min. Genomic DNA was obtained from the supernatant of boiled bacteria. The double-crossover replacement was analyzed using genomic DNA by PCR and successful double-crossover replacement was further confirmed by DNA sequencing.

2.7. Mouse Model of Invasive Skin Tissue Infection

The ability of *S. pyogenes* to cause local skin lesions and necrosis in mice after skin inoculation was assessed using a procedure similar to that described elsewhere [25]. In brief, 3-week-old female ICR mice (10 - 12 g) were anesthetized with sevoflurane, and the skin of the left flank was bared by separating hair with alcohol swab, unless otherwise indicated. Bacteria (0.2 ml; 2×10^7 cfu per mouse) grown in BHI-Y were injected with a 27-gauge needle just under the surface of the skin so that a superficial bleb was raised immediately below the skin surface. The number of colony-forming units injected was verified for each experiment by plating bacteria on BHI-Y or sheep blood agar plates (with or without kanamycin) and counting colony-forming units. Lesion sizes (length × width) were measured, with the length determined as the longest dimension of the lesion at day

3 or at the death time point.

Bacteria were recovered from the mice which survived until day 8. For mouse blood samples: 100 µl of blood from the heart was spread on a sheep blood agar plate. For the spleen samples: Spleen was homogenized and suspended with 100 µl of PBS and spread on a sheep blood agar plate. *β*-hemolysis positive colonies were calculated. Two colonies per plate were randomly selected and inoculated into BHI-Y broth supplemented with or without 50 µg/ml spectinomycin for microscopic analysis to confirm coccus morphology and chain arrangements characteristic of Streptoccoal species.

All animal procedures were approved by the Institutional Animal Care and Use Committee at Nagoya City University.

2.8. Statistical Analysis

Data collected for virulence to mouse (survival days) were assessed using a log-rank comparison described previously [20]. R software was used for statistical analysis http://bioinf.wehi.edu.au/software/russell/logrank/. *P*-value ≤ 0.05 was considered significant.

3. Results

3.1. Cloning of *Spn*$_{SF370}$ Gene into a pGEM®-T Easy Vector

S. pyogenes strain SF370 is a representative among NADase negative strains. In order to evaluate the role of NADase-inactive SPN, we firstly attempted to clone the *spn*$_{SF370}$ gene of the strain SF370 into a pGEM®-T Easy vector in *E. coli* strain DH5*α* without support of the *ifs*$_{SF370}$ gene. The vector and the strain DH5*α* are compatible with the blue/white color screening for recombinants. Typically, we see about half of the transformants showing white color under our experimental condition when a DNA insert without toxicity to *E. coli* is used. We show data from two representative experiments in **Table 2** (40.9% and 44.4% white colonies for *recA*$_{SF370}$ and *spy*1193$_{1529}$, respectively; these genes have been cloned for our other studies around the same time as this study). In contrast, the four cloning experiments using the insert encoding *spn*$_{SF370}$ showed only 8.7%, 27.6%, 31.8% and 0.8% white colonies (**Table 2**). Eighty-five of the white colonies derived from the *spn*$_{SF370}$-insert were randomly selected for further plasmid analysis (named as pGEM-*spn*$_{SF370}$1 to 85), and only three colonies (4%) possessed the correct size insert (1.5 kbp) in the corresponding plasmids (pGEM-*spn*$_{SF370}$26, pGEM-*spn*$_{SF370}$32, and pGEM-*spn*$_{SF370}$13; see **Table 3** and **Figure S1**). In contrast, in the experiments using control inserts, more than 50% of white colonies had the correct size inserts: 94%, 67%, 100%, 100%, 86%, 57% and 88% for *spn-ifs*$_{GT01}$, *recA*$_{SF370}$, *spy*1193$_{1529}$, *covRS*$_{GT01}$, *covRS*$_{1529}$, *vicRK*$_{1529}$ and *vicK*$_{1529}$, respectively (**Table 3**).

For the *spn*$_{SF370}$, the insert of the three plasmids (pGEM-*spn*$_{SF370}$26, pGEM-*spn*$_{SF370}$32, and pGEM-*spn*$_{SF370}$13) were sequenced, and the following mutations were found: substitution of the start codon ATG to ACG in pGEM-*spn*$_{SF370}$26, the second codon AGA (Arg) to TGA (stop) in pGEM-*spn*$_{SF370}$32, and an adenine nucleotide was substituted to a guanine (G) at nucleotide 34 upstream from the adenine (A) of the start codon in pGEM-*spn*$_{SF370}$13, respectively (see most right column in **Table 3**). We propose these mutations may have been introduced for the following reasons: 1) A spontaneous mutation is often inserted in DNA fragment amplified by PCR or 2) *spn*$_{SF370}$ gene is toxic to *E. coli* cells, so mutations to make the gene inactive were given for a natural survival advantage. In order to examine those possibilities,

Table 2. The numbers of blue/white colonies.

PCR product[a]		W[b]	B[b]	W + B[b]	w/w + B%[c]
spn$_{SF370}$	exp. 1	21	220	241	8.7
	exp. 2	107	280	387	27.6
	exp. 3	1070	2300	3370	31.8
	exp. 4	21	2725	2746	0.8
	Total[d]	1219	5525	6744	18.1
recA$_{SF370}$	exp.1	2523	3650	6173	40.9
*spy*1193$_{1529}$	exp.1	1602	2004	3606	44.4
vicRK$_{1529}$	exp.1	31	4400	4431	0.7
vicK$_{1529}$	exp.1	106	3200	3306	3.2

[a]PCR products of *recA*$_{SF370}$ and *spy*1193$_{1529}$ contain a part of the gene, respectively, whereas the others have the hole genes indicated. [b]The numbers of white (W), blue (B), and the total (W + B) colonies. [c]% white colonies. [d]sum of the colony numbers from 4 experiments.

Table 3. Cloning of the insert containing *spn*$_{SF370}$ gene into pGEM-T easy vector.

Insert DNA[a]	Insert + (%)	P[b]	No mutation[c]
spn$_{SF370}$	3/85 (4)	NA	0/3
spn-ifs$_{GT01}$	17/18 (94)	<0.01	2/8
recA$_{SF370}$	2/3 (67)	<0.01	2/2
*spy*1193$_{1529}$	2/2 (100)	<0.01	N.D.
covRS$_{GT01}$	4/4 (100)	<0.01	1/4
covRS$_{1529}$	6/7 (86)	<0.01	1/6
vicRK$_{1529}$	4/7 (57)	<0.01	0/4
vicK$_{1529}$	7/8 (88)	<0.01	0/7

[a]As a control for *spn*$_{SF370}$, seven examples *spn-ifs*$_{GT01}$, *recA*$_{SF370}$, *spy*1193$_{1529}$, *covRS*$_{GT01}$, *covRS*$_{1529}$, *vicRK*$_{1529}$, and *vicK*$_{1529}$ were shown. See **Table 1** about information for the insert DNAs. [b]Z-test was used in order to compare with *spn*$_{SF370}$. [c]Number of insert without any mutation/number of sequenced insert DNA.

we attempted to change the mutated nucleotides back to the original nucleotides using the inverse PCR method described previously [18]. We performed inverse PCR with primers nga(SF370)-F and nga(SF370)-R (**Tables 1** and **4**) constructed to substitute the mutated second codon of the spn_{SF370} on pGEM-spn_{SF370}32 to the original

Table 4. Physical maps of primers used for inverse PCR were shown.

***spn*$_{SF370}$**	
WT[a]	AATAATATAAGGTGGTTTACA**T**G **A**GAAACAAAAAAGTAACATTAG
	Primer: nga(SF370)-F →
No. 32[b]	AATAATATAAGGTGGTTTACATG **T**GAAACAAAAAAGTAACATTAG
	← Primer: nga(SF370)-R
32 - 1	AATAATATAAGGTGGTTTACATG
32 - 2	AATAATATAAGGTGGTTTACAT*A* AGAAACAAAAAAGTAACATTAG
32 - 4	AATAATATAAGGTGGTTTACATG GAAACAAAAAAGTAACATTAG
32 - 8	AATAATATAAGGTGGTTTACAT **T**GAAACAAAAAAGTAACATTAG
32 - 9	AATAATATAAGGTGGTTTACATG GAAACAAAAAAGTAACATTAG
32 - 12	AATAATATAAGGTGGTTTACATG GAAACAAAAAAGTAACATTAG
	Primer: nga(SF370)-F →
No. 26[c]	AATAATATAAGGTGGTTTAC**A**CG AGAAACAAAAAAGTAACATTAG
	← Primer: nga(SF370)-R
26 - 1	AATAATATAAGGTGGTTTACA**T**G GAAACAAAAAAGTAACATTAG
26 - 2	AATAATATAAGGTGGTTTACA**T** AGAAACAAAAAAGTAACATTAG
WT[d]	GACAAAAACTATTTGCTAATGT **A**TAGTTTACTTAAAAATAATATAAG
	Primer: nga(SF370)-F2 →
No. 13[e]	GACAAAAACTATTTGCTAATGT **G**TAGTTTACTTAAAAATAATATAAG
	← Primer: nga(SF370)-R2
13 - 1	GACAAAAACTATTTGCTAA **A**TAGTTTACTTAAAAATAATATAAG
13 - 3	GACAAAAACTATTTGCTAAT AGTTTACTTAAAAATAATATAAG
13 - 4	GACAAAAACTATTTGCTAATGT **G**TAGTTTACTTAAAAATAATATAAG
13 - 5	GACAAAAACTATTTGCTAATGT *C*TAGTTTACTTAAAAATAATATAAG
***vicRK*$_{1529}$**	
WT[f]	GTCTCGGATTGATAATCAAGTA A**C**CCAATTAGCAGTAGAGATGAC
	Primer: vicK-F →
No. 11[g]	GTCTCGGATTGATAATCAAGTA **G**CCCAATTAGCAGTAGAGATGAC
	← Primer: vicK-R
11 - 1	GTCTCGGATTGATAATCAAGTA CCCAATTAGCAGTAGAGATGAC
11 - 2	GTCTCGGATTGATAATCAAGTA **A**CCCAATTAGCAGTAGAGATGAC
	Primer: vicK-F →
No. 28[h]	GTCTCGGATTGATAATCAAGTA **G**CCCAATTAGCAGTAGAGATGAC
	← Primer: vicK-R
28 - 1	GTCTCGGATTGATAATCAAGTA **A**CCCAATTAGCAGTAGAGATGAC
28 - 2	GTCTCGGATTGATAATCAAGTA **A**CCCAATTAGCAGTAGAGATGAC

The mutated nucleotides were attempted to change back to the original nucleotides. Primers used for the inverse PCR were shown by arrows. The primer' nucleotide sequences were also shown in **Table 1**. Unsuccessful substitutions were shown in italic type. [a]Original nucleotide sequence of the junction site. The adenine and thymine nucleotides which were substituted in No. 32 and No. 26, respectively, were shown as bold **"A"** and **"T"**. The start (ATG) and second (AGA) codons were underlined. [b]The mutated thymine nucleotide of the spn_{SF370} on pGEM-spn_{SF370}32 was shown as bold **"T"**. [c]The mutated cytosine nucleotide of the spn_{SF370} on pGEM-spn_{SF370}26 was shown as bold **"C"**. [d]Original nucleotide sequence of the junction site. The adenine (**A**) nucleotide, which was substituted in No. 13, was shown in bold type. [e]The mutated guanine nucleotide on the pGEM-spn_{SF370}13 was shown as bold **"G"**. [f]Original sequence of the junction site. The adenine nucleotide, which was substituted in No. 11 and No. 28, was shown as bold **"A"**. The 267th codon (ACC) was underlined. [g]The mutated guanine nucleotide of the $vicK_{1529}$ on pGEM-$vicRK_{1529}$11 was shown as bold **"G"**. [h]The mutated guanine nucleotide of the $vicK_{1529}$ on pGEM-$vicK_{1529}$28 was shown as bold **"G"**.

codon AGA (R). The amplification product was self-ligated and used to transform *E. coli* strain DH5*α*. Plasmids were prepared from randomly selected 14 transformants (named as 32 - 1 to 32 - 14). Five plasmids (32 - 2, 32 - 4, 32 - 8, 32 - 9 and 32 - 12) appeared to possess the correct size insert, whereas the other nine (32 - 1, 32 - 3, 32 - 5, 32 - 6, 32 - 7, 32 - 10, 32 - 11, 32 - 13, and 32 - 14) had the smaller size inserts based on the result seen during agarose gel electrophoresis (data not shown). In addition to the inserts of the five passed plasmids (32 - 2, 32 - 4, 32 - 8, 32 - 9 and 32 - 12), one of the dropped-out plasmids (32 - 1) which was added as a representative (internal) negative-control were sequenced. As shown in **Table 4**, the 32 - 1 had a large deletion and the other five contained a nucleotide mutation or deletion at the junction site. Additionally for pGEM-spn_{SF370}26, and pGEM-spn_{SF370}13, we attempted to change the mutated nucleotides back to the original nucleotides by using same method with primers nga (SF370)-F and nga (SF370)-R, or primers nga(SF370)-F2 and nga(SF370)-R2 (**Table 4**). Two of the seven pGEM-*spn*SF_{370}26 derivative plasmids (26 - 1and 26 - 2) prepared from randomly selected transformants (named as 26 - 1 to 26 - 7) appeared to possess the correct size insert. However, both plasmids had a nucleotide deletion at the junction site (**Table 4**). For pGEM-spn_{SF370}13, four (13 - 1, 13 - 2, 13 - 4, and 13 - 5) of seven plasmids prepared from randomly selected transformants appeared to possess the correct size insert. However, one plasmid (13 - 4) was same as the original pGEM-spn_{SF370}13 and the other three possessed a nucleotides deletion or a nucleotide mutation at the junction site (**Table 4**). We have observed successful substitution of a corresponding nucleotide for more than 50% of the transformants checked in other recent experiments. Two representative examples are shown below. As described above (see **Table 3**), the four and the seven types of pGEM-T easy derivatives having $vicRK_{1529}$ and $vicK_{1529}$ have been previously constructed, respectively (named as pGEM-$vicRK_{1529}$11, pGEM-$vicRK_{1529}$12, pGEM-$vicRK_{1529}$13, pGEM-$vicRK_{1529}$15 and pGEM-$vicK_{1529}$22, pGEM-$vicK_{1529}$23, pGEM-$vicK_{1529}$24, pGEM-$vicK_{1529}$25, pGEM-$vicK_{1529}$26, pGEM-$vicK_{1529}$27, pGEM-$vicK_{1529}$28: each plasmid has a mutation(s) in somewhere of $vicRK_{1529}$ or $vicK_{1529}$). Among the plasmids, pGEM-$vicRK_{1529}$11 and pGEM-$vicK_{1529}$28 which have both a mutation changing the codon 276 of $vicK_{1529}$ gene from a ACC (encoding "T") to a GCC (encoding "A") were used for the control experiments performed with primers vicK-F and vicK-R (**Table 4**). We observed a successful substitution of the corresponding nucleotide in one of two transformants (11 - 1 and 11 - 2) analyzed for pGEM-$vicRK_{1529}$11, two of two transformants (28 - 1 and 28 - 2) in pGEM-$vicK_{1529}$28 (**Tables 4** and **5**). In the case of spn_{SF370} gene, we never observed the expected nucleotide change when using any of three plasmids as template described above (**Tables 4** and **5**). These results suggest that spn_{SF370} gene is toxic to *E. coli* cells.

In strain SF370, the ifs_{SF370} allele has a nonsense mutation in the codon for leucine 24 to produce a truncated open reading frame [11]. In order to determine whether the truncated ifs_{SF370} open reading frame can successfully inhibit the toxicity of spn_{SF370} gene in *E. coli* cells, we attempted to clone a *spn-*ifs_{SF370} operon into the pGEM®-T Easy vector. The original forward primer ngaGT-n1Nhe previously used for cloning of spn_{SF370} gene and three altered reverse primers (nga-c8xho, IFS-R(EcoRI), and slo-c2) to include *ifs* were tested (**Table 1**). However, we did not obtain any recombinants having the expected insert in the size (data not shown).

3.2. Cloning Spn_{SF370} Gene into pLZ12-Km2 Vector

We attempted to clone spn_{SF370} by using plasmid pLZ12-Km2 instead of pGEM®-T Easy. pLZ12-Km2 which has a rolling circle type of replication can be successfully maintained in both *E. coli* and *S. pyogenes* [26]. Firstly, each insert DNA of pGEM-spn_{SF370}26, pGEM-spn_{SF370}32, and pGEM-spn_{SF370}13 was subcloned into pLZ12-Km2 (named as pLZ-spn_{SF370}26, pLZ-spn_{SF370}32, and pLZ-spn_{SF370}13 respectively). By using these plasmids as template for inverse PCR, we attempted to change the mutated nucleotides back to original nucleotides. Ligated DNA was introduced into *E. coli* strain DH5*α* and *S. pyogenes* strain 1529. We obtained a handful of and many transformants in *E. coli* and *S. pyogenes*, respectively. Therefore, all *E. coli* transformants obtained were investigated, while only a subset of transformants in *S. pyogenes* were further investigated. We did not observe successful substitution in any of the *E. coli* transformants

Table 5. Substitution of the mutated nucleotides back to the original one.

Template[a]	Recovered[b]	
	E. coli	*S. pyogenes*
pGEM-spn_{SF370}32	0/14 (0%)	N/A
pGEM-spn_{SF370}26	0/7 (0%)	N/A
pGEM-spn_{SF370}13	0/7 (0%)	N/A
pGEM-$vicRK_{1529}$11	1/2 (50%)	N/A
pGEM-$vicK_{1529}$28	2/2 (100%)	N/A
pLZ-spn_{SF370}32	0/2 (0%)	1/2 (50%)
pLZ-spn_{SF370}26	0/5 (0%)	5/5 (100%)
pLZ-spn_{SF370}13	0/5 (0%)	1/5 (20%)

[a]Plasmids used for inverse PCR. [b]Number of plasmid having the successful substitution/number of the analyzed plasmid (%). N/A: not applicable.

regardless of the plasmid templates used (**Table 5**). We did observe successful substitution in the *S. pyognenes* transformants for all three of the plasmid templates used (**Table 5**). Finally, the spn_{SF370} gene was successfully cloned only when *S. pyogenes* strain 1529 was used as host. These results suggest that the insert DNA encoding spn_{SF370} has some toxic effect to *E. coli*, but not *S. pyogenes*.

3.3. Construction and Analysis of SF370Δspn

The toxicity of spn_{SF370} could be related with Riddle *et al.*'s claim that SPN has a NADase-independent function. In order to further explore this hypothesis, spn_{SF370} gene was replaced with an antibiotics marker and the resulting strain SF370Δspn was used to infect mice.

When the SF370Δspn was inoculated in mouse skin, the mortality (36%) of the infected mice was not significantly different from the infection with the parental strain SF370 (14%) ($p = 0.214$, see **Table 6**). In addition, there were not significant differences in the lesion sizes of mouse skin infected with either SF370Δspn or strain SF370 (data not shown). Furthermore, bacteria were recovered from blood and spleen of the surviving mice on day 8 (**Table 7**). The bacterial number recovered from the mice infected with SF370Δspn was not reduced compared with the case infected with the parental strain SF370.

4. Discussion

Riddle *et al*. suggested that SPN has both NADase-dependent and NADase-independent function [19]. Cloning of a target gene is the first step in studying the function of genes in many biological researches. Therefore, in order to explore what is the NADase-independent function, we attempted to clone the spn_{SF370} gene encoding the NADase-inactive SPN in *Escherichia coli.* We initially expected that cloning this gene into *E. coli* would be simple, because it was believed that the toxicity of SPN for bacterial cells is associated with the NADase activity [11,19]. But in actuality, we were not able to clone the gene in *E. coli*, suggesting that spn_{SF370} gene has some NADase-independent toxicities to *E. coli* cells compared with several controls used in this study. Therefore, we were forced to explore another strategy to achieve successful cloning of the gene. We tested ifs_{SF370} gene as the first strategy, because *spn* alleles encoding NADase-active SPN subtype have ever been cloned by aid of *ifs* gene in *E. coli* [11,12,20]. Consequently, $spn\text{-}ifs_{SF370}$ also was not able to be cloned in *E. coli.* This result could be explained by the fact that ifs_{SF370} has been previously shown to degrade into a peudogene [19]. Additionally, we took into account that IFS does not necessarily provide a perfect suppression of the self-toxicity of the NADase-active SPN as described bellow. We had attempted to clone spn_{GT01} gene encoding NADase-active SPN by aid of ifs_{GT01} gene in *E. coli* in the previous study [20]. For this experiment, four different forward primers were used to amplify the $spn\text{-}ifs_{GT01}$ genes with the reverse primer slo2 (**Figure S2**). While the first forward

Table 6. Virulence (Mortality) to mouse of SF370Δspn.

	Mortality (death/trial)
SF370 wt	14% (2/14)
SF370Δspn	36% (5/14)

Mortality was determined on Day 8 ($P = 0.214$ for comparison of survival days).

Table 7. Bacterial number (CFU) recovered from the survived mice.

	Blood[a]	Spleen[b]
wt-1	9	$>10^3$
wt-2	0	0
wt-3	0	0
wt-4	0	0
wt-5	0	5
wt-6	0	0
wt-7	0	$>10^3$
wt-8	0	1
wt-9	0	0
wt-10	0	0
wt-11	0	4
wt-12	0	0
Δspn-1	0	$>10^3$
Δspn-2	0	$>10^3$
Δspn-3	0	$>10^3$
Δspn-4	0	52
Δspn-5	44	930
Δspn-6	0	0
Δspn-7	0	$>10^3$
Δspn-8	0	15
Δspn-9	0	9

The mice, which survived until day 8 in Table 6, were used. [a]100 µl of blood from heart was spread on a sheep blood agar plate. [b]Spleen was homogenized and suspended with 100 µl of PBS. All the PBS (100 µl) was spread on a sheep blood agar plate. Two colonies per plate were randomly selected, and we observed coccus morphology and chain arrangements characteristic of Streptoccoal species by using a microscope. In addition, the colonies derived from the mice challenged with SF370Δspn were spectinomycin-resistant, whereas the colonies from the mice challenged with the parental strain SF370 were sensitive to spectinomycin.

primer (Nga-n4Eco) does not contain any upstream DNA sequences encoding a potential ribosome-binding site, the other three would contain longer upstream DNA sequences (118, 185 and 287 bp respectively as shown in the **Figure S2**). For the latter three primers, we did not obtain any transformants containing the prospective plasmids. Using the Nga-n4Eco we obtained the resulting 13 transformants having the plasmids (pNGIe1 to 13, respectively) in which only the coding regions of spn_{GT01} were cloned. In addition, all the spn_{GT01} genes (of pNGIe1 to 13) were oriented in the opposite direction as the *lacUV*5 promoter on the pGEM®-T Easy vector. These selections for the upstream DNA sequences length and the orientation of the cloned spn_{GT01} may decrease the amount of NADase produced, because it have been already shown that at least addition of 16 bp and 26 bp upstream DNA sequences to spn_{GT01} resulted in the increased production of NADase activity in our previous study [20]. In that study, therefore, we hypothesized that plasmids producing NADase at lower level were selected for due to the potential toxicity of over produced NADase to bacterial cell. However we were not able to explain the reason why ifs_{GT01} gene did not sufficiently suppress the potential toxicity. Now, we propose a hypothesis that IFS_{GT01} could not inhibit the potential NADase-independent (self-) toxicity of SPN_{GT01} because IFS_{GT01} was inhibitor of NADase activity.

It is possible that *S. pyogenes* has some mechanism to manage the NADase-independent toxic properties of SPN as well as IFS for the NADase-dependent toxic property. Therefore, we attempted to use *S. pyogenes* as a host for cloning of spn_{SF370} gene. For this experiment, we used the *E. coli-Streptococcus* shuttle vector pLZ12-Km2 which copy number would be intermediate (personal communication with Dr. June R. Scott). The spn_{SF370} gene was successfully cloned in *S. pyogenes*, but not in *E. coli*. These results suggested that *S. pyogenes* has a mechanism for management of NADase-independent toxic properties of SPN that is lacking in *E. coli*.

The toxicity of spn_{SF370} could be related with Riddle *et al*.'s claim that SPN has a NADase-independent function. In order to further investigate the hypothetical function, we used the experimental mouse infection model. Based on our findings, we could not find any direct evidence for the hypothetical function. This may be related with the limitation of this experimental model, since humans are the only natural host for *S. pyogenes*. However, there was an unexpected result. It seemed that spn_{SF370} mutants survived better in the spleen (**Table 7**). About this, we have only one positive idea. Strain SF370 is not hyper virulent, compared with clinical isolates from severe invasive disease. NADase inactive SPN_{SF370} might contribute to the low virulence of the SF370. Hyper virulence is not only strategy in order to survive in host, because non-pathogenic, but not enterohemorrhagic, *E. coli* is living persistently in all human gut.

5. Conclusion

We have presented further supportive evidence that SPN has a NADase-independent function.

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Supplement

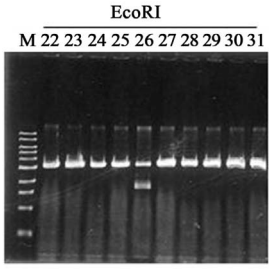

Figure S1. Cloning of *Spn*$_{SF370}$ gene into a pGEM®-T Easy Vector. Representative plasmids (pGEM-*spn*$_{SF370}$22 to 31) were shown. The pGEM-*spn*$_{SF370}$26 (lane number 26) possessed the correct size insert (1.5 kbp).

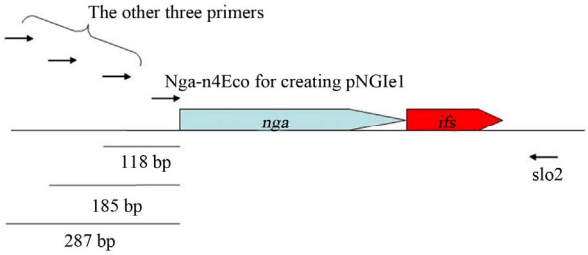

Figure S2. Physical map of *spn-ifs*$_{GT01}$ genes. Used primers were shown as arrows.

