INFECTION AND IMMUNITY, June 2002, p. 3227–3233 Vol. 70, No. 6
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.6.3227–3233.2002

Dissemination of the Phage-Associated Novel Superantigen Gene *speL* in Recent Invasive and Noninvasive *Streptococcus pyogenes* M3/T3 Isolates in Japan

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Received 26 December 2001/Returned for modification 18 February 2002/Accepted 21 March 2002

In Japan, more than 10% of streptococcal toxic shock-like syndrome (TSLS) cases have been caused by *Streptococcus pyogenes* M3/T3 isolates since the first reported TSLS case in 1992. Most M3/T3 isolates from TSLS or severe invasive infection cases during 1992 to 2001 and those from noninvasive cases during this period are indistinguishable in pulsed-field gel electropherograms. The longest fragments of these recent isolates were 300 kb in size, whereas those of isolates recovered during or before 1973 were 260 kb in size. These 260- and 300-kb fragments hybridized to each other, suggesting the acquisition of an about 40-kb fragment by the recent isolates. The whole part of the acquired fragment was cloned from the first Japanese TSLS isolate, NIH1, and its nucleotide sequence was determined. The 41,796-bp fragment is temperate phage ϕNIH1.1, containing a new superantigen gene *speL* near its right attachment site. The C-terminal part of the deduced amino acid sequence of *speL* has 48 and 46% similarity with well-characterized erythrogenic toxin SpeC and the most potent superantigen, SmeZ-2, respectively. None of 10 T3 isolates recovered during or before 1973 has *speL*, whereas all of 18 M3/T3 isolates recovered during or after 1992 and, surprisingly, *Streptococcus equi* subsp. *equi* ATCC 9527 do have this gene. Though plaques could not be obtained from ϕNIH1.1, its DNA became detectable from the phage particle fraction upon mitomycin C induction, showing that this phage is not defective. A horizontal transfer of the phage carrying *speL* may explain the observed change in M3/T3 *S. pyogenes* isolates in Japan.

Streptococcus pyogenes is a gram-positive bacterium causing a variety of human diseases ranging from acute pharyngitis and cutaneous infections to severe infections including necrotizing fasciitis and streptococcal toxic shock-like syndrome (TSLS). Since the late of 1980s, marked increases in the incidence and the severity of invasive infections have been reported in developed countries (4, 5, 7, 10, 11, 14, 26, 29). These invasive infections have been mainly caused by M1 and M3 isolates, among others. In Japan, the first definite TSLS case was caused by an M3 isolate in 1992 (27). Huge epidemiological studies for nine recent years (1992 to 2000) in Japan revealed that T3 isolates have accounted for only 3.9% (1,258 out of a total 31,945 isolates) of pharyngitis or pharyngeal colonization cases, whereas 11.0% (10 out of 91) of TSLS cases were caused by this serotype isolates (reference 16 and unpublished observations). Our most recent data obtained by using molecular techniques suggest that recent M3/T3 isolates in Japan have acquired additional DNA fragments and have spread to the population to cause both noninvasive and invasive infections (17). In this study, we have cloned the whole additional DNA fragment from an M3/T3 TSLS isolate and have determined its nucleotide sequence. The fragment is derived from a temperate phage, and an open reading frame (ORF) whose deduced amino acid sequence has high homology to streptococcal superantigens was newly identified near one attachment site of the phage.

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MATERIALS AND METHODS

Bacterial strains. Characteristics of *S. pyogenes* strains used in this study are summarized in Table 1. All strains are M3/T3 except Lewis, whose M is untypeable due to a seven-base insertion in its *emm* gene. The *emm* typing was performed according to the descriptions by Facklam et al. (8). ATCC 10389 (13, 20, 28), SS-265, and Lewis (20) were obtained from American Type Culture Collection, Centers for Disease Control, and Statens Serum Institut, respectively. D58X/11/1 (20) and B930/24/3 (20) were obtained from R. C. Lancefield. All other strains are Japanese clinical isolates deposited in our laboratories. Hereafter in this study, we call *S. pyogenes* strains whose original isolation years are

TABLE 1. Characteristics of *S. pyogenes* used in this study

Strain	M type/ T type	*spe* profile(s)	Mutation(s) in *emm3* from base 81 to base 550[a]	Isolation year	Specimen	Remarks/diagnosis[b]
ATCC 10389	M3/T3	*B*		1937		Richards, D58, puerperal septicemia (13, 20, 28)
D58X/11/1	M3/T3	*B*	328 G to T, 329 A to C			20
SS-265	M3/T3	*B*		Before 1956		
Lewis	Untypable/T3	*ABC*	7-base insertion between 210 A and 211 T; 415 G to A	Before 1958		20
B930/24/3	M3/T3	*AB*	245 G to A	1952	Throat swab	Rheumatic fever (20)
K21	M3/T3	*AB*	415 G to A	1973	Throat swab	Pharyngitis
K22	M3/T3	*AB*	134 G to A, 341 A to G	1973	Throat swab	Pharyngitis
K23	M3/T3	*AB*	134 G to A, 341 A to G	1973	Throat swab	Pharyngitis
K24	M3/T3	*AB*	134 G to A	1973	Throat swab	Pharyngitis
K25	M3/T3	*AB*	134 G to A	1973	Throat swab	Pharyngitis
K31	M3/T3	*AB*	215 A to C, 245 G to A	1994	Throat swab	Pharyngitis
K32	M3/T3	*AB*	215 A to C, 245 G to A	1994	Throat swab	Pharyngitis
K33	M3/T3	*AB*	215 A to C, 245 G to A	1994	Throat swab	Pharyngitis
K34	M3/T3	*AB*	215 A to C, 245 G to A	1994	Throat swab	Pharyngitis
K35	M3/T3	*AB*	215 A to C, 245 G to A	1994	Throat swab	Pharyngitis
NIH1	M3/T3	*AB*	215 A to C, 245 G to A	1992	Fascia	TSLS (27)
NIH3	M3/T3	*AB*	215 A to C, 245 G to A	1993	Blood	Puerperal TSLS
NIH7	M3/T3	*AB*	215 A to C, 245 G to A	1993	Blood	TSLS, LC, HCC
NIH8	M3/T3	*AB*	215 A to C, 245 G to A	1993	Blood	TSLS
NIH9	M3/T3	*AB*	215 A to C, 245 G to A	1994	Blood	TSLS
NIH12	M3/T3	*AB*	215 A to C, 245 G to A, 371 C to T	1994	Blood	Cellulitis
NIH14	M3/T3	*AB*	215 A to C, 245 G to A	1994	Lower limb soft tissue	TSLS
NIH18	M3/T3	*AB*	215 A to C, 245 G to A	1994	Lower limb soft tissue	TSLS
NIH20	M3/T3	*AB*	215 A to C, 245 G to A	1995	Bullous fluid	TSLS
NIH33	M3/T3	*AB*	215 A to C, 245 G to A	1994	Upper limb soft tissue	Necrotizing fasciitis, cutaneous lymphoma
NIH34	M3/T3	*AB*	215 A to C, 245 G to A	1994	Blood	TSLS
NIH152	M3/T3	*AB*	215 A to C, 245 G to A	2001	Lower limb soft tissue	TSLS, LC
NIH158	M3/T3	*AB*	215 A to C, 245 G to A	2001	Bullous fluid	TSLS

[a] The nucleotide location is based on X80168 (25). *emm3* with two mutations, 215 A to C and 245 G to A, was designated *emm3.1*.
[b] TSLS, toxic shock-like syndrome; LC, liver cirrhosis; HCC, hepatocellular carcinoma.

before 1980 "old" and those after 1990 "recent." *Streptococcus equi* subsp. *equi* ATCC 9527, which was isolated from submaxillary abscess of a foal with strangles, was also used.

PFGE. Pulsed-field gel electrophoresis (PFGE) was performed according to a previous description (32) except that mutanolysin (Sigma, St. Louis, Mo.) was used instead of lysostaphin at a concentration of 100 μg/ml. An electric field of 6 V/cm was switched every 20 s for 12 h and then every 2 s for 10 h to separate *Sma*I-digested genomic DNAs of *S. pyogenes* strains. For estimating phage DNA sizes, 1.2% agarose gel was used and a 2-s switching interval was applied for 10 h. Subsequent transfer of DNAs to nylon filters (Hybond N^+ [Amersham Pharmacia Biotech, Buckinghamshire, England]) and Southern hybridization were performed by using digoxigenin (DIG)-labeled probes in DIG Easy Hyb (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturers' instructions.

Lambda library construction. Genomic DNAs of NIH1 were partially digested by *Sau*3AI (Roche Diagnostics) and coligated with *Bam*HI (Roche Diagnostics)-digested Lambda DASH II DNA (Stratagene, La Jolla, Calif.). XL1-Blue MRA (P2) (Stratagene) was used as a host strain to make a phage library.

PCR. PCRs were performed by using AmpliTaq Gold (Applied Biosystems, Foster City, Calif.) with PCR Gold Buffer. For amplifying *speL*, steps consisting of denaturing at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min were repeated 30 times after initially activating the enzyme at 95°C for 10 min. Primers used were speL-fwrd (5′-GTCATATCATGTTGTATGCAA-3′) and speL-rev (5′-GTTTAAGTGAACATCAAAGTG-3′). The same PCR condition as above was applied for amplifying the downstream junctional region of temperate phage ϕNIH1.1 with primers #5-T7-R1 (5′-AACACTTTTAAGAAAGTCATATCT-3′) and K23rj (5′-CAATTAACAATGAGTACACACGGT-3′). Primers p7-T3-2 (5′-GATAGGCATTAGAAAAAAGGA-3′) and p5-T3-2 (5′-CATCTATTCAAGATAGCCTACTTC-3′) were also used under the same PCR condition as above. Location and direction of each primer are shown (see Fig. 2).

Preparation of phage DNA. NIH1 was grown in 4 ml of Todd Hewitt Broth (Becton Dickinson Microbiology Systems, Sparks, Md.) containing 2 mM $CaCl_2$ at 37°C with gentle shaking. At an early log phase, mitomycin C was added at a final concentration of 200 ng/ml and the cells were further incubated for 3 h at 37°C. The bacterial cells were removed by subsequent centrifugation (9800 × *g*, 10 min) and filtration (0.45 μm). After digesting bacterial DNAs and RNAs by DNase (100 μg/ml) and by RNase (50 μg/ml) at 37°C overnight, phage particles were precipitated in the presence of 500 mM NaCl and 5% polyethylene glycol. Then, DNAs in the particles were extracted by phenol. All of the DNAs prepared from the 4-ml culture were loaded on one lane of PFGE. As a control, a sample without mitomycin C induction was also prepared.

Nucleotide sequencing. The nucleotide sequence of ϕNIH1.1 was determined by automated sequencers ABI PRISM 377 DNA Sequencer (Applied Biosystems) and ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Direct genome sequencing was performed according to a previous description (31) by using primer p7-T3-3 (5′-AGATAAGAGTTTCAATATGACGAG-3′), whose location in NIH1 is shown (see Fig. 2).

Nucleotide sequence accession number. The nucleotide sequence of ϕNIH1.1 has been deposited in GenBank under accession number AY050245.

RESULTS

PFGE analysis of *S. pyogenes* M3/T3 strains. Using PFGE for separating *Sma*I-digested DNA fragments, we compared genomic profiles of three old M3/T3 strains, isolated from patients with pharyngitis or rheumatic fever, with those of recent 15 M3/T3 strains from patients with pharyngitis or invasive infections (Table 1 and Fig. 1a). Interestingly, the overall restriction patterns were very well conserved between the

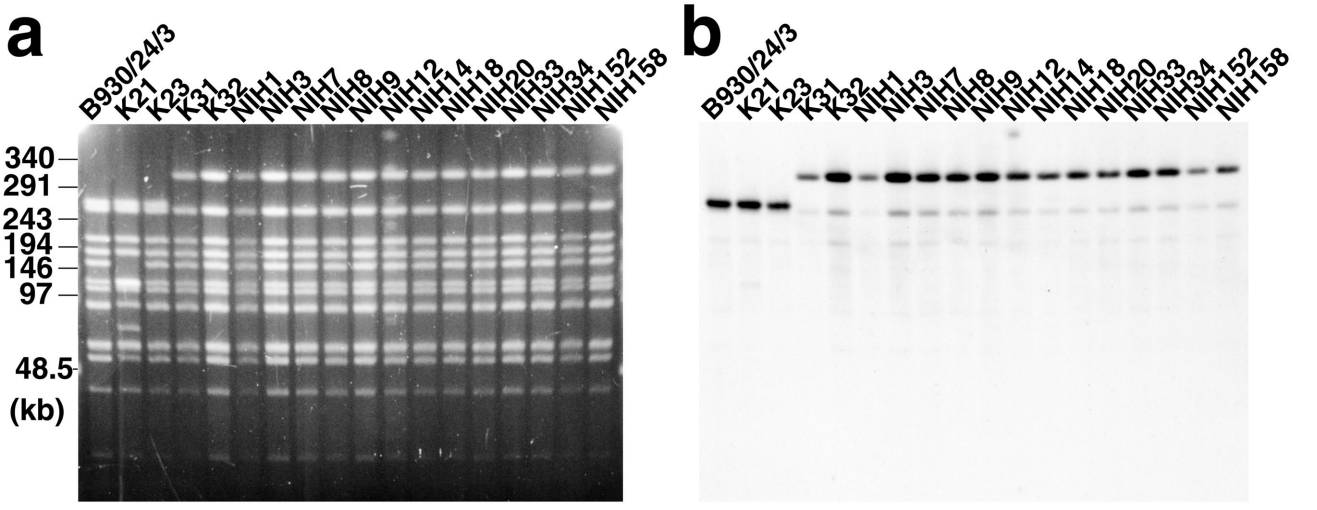

FIG. 1. (a) Ethidium bromide staining of *Sma*I-digested genomic DNAs of M3/T3 *S. pyogenes* strains after PFGE separation. Strain names are on top of each lane. B930/24/3, K21, and K23 are old isolates and the others are recent isolates (see Table 1 and the text). Sizes of lambda concatemers are shown at the left. (b) Southern hybridization results from the DNAs in panel a with a probe prepared from the 260-kb fragment of K23.

old and the recent strains. The most remarkable difference between them was the length of the uppermost bands of each group; the old strains conserved 260-kb fragments, whereas all of the recent strains had lost the 260-kb fragment but had acquired 300-kb fragments instead. Thus, PFGE patterns between the recent strains were indistinguishable, though they were isolated at different locations in Japan and at different times. To examine whether the 300-kb fragments of the recent strains were derived from the 260-kb fragments of the old strains or not, we performed Southern hybridization analysis by using the whole 260-kb fragment of old strain K23 as a probe. As expected, the probe reacted with the 300-kb fragments as well as with the 260-kb fragments (Fig. 1b). When the 300-kb fragment of recent strain NIH1 was used as a probe in turn, both 260- and 300-kb fragments could be detected (data not shown). Based on these observations, we hypothesized that old M3/T3 *S. pyogenes* strain(s) may have acquired an about 40-kb fragment and that the resulting strain(s) had spread in recent years to cause both noninvasive and invasive infections, including TSLS in Japan.

Cloning of 40-kb fragment. We selected NIH1 as a representative TSLS strain of recent years (Table 1). For cloning the additional 40-kb fragment from this strain, a genomic lambda library was constructed. The 260-kb fragment of K23 was used as the first probe for screening the library, and then the 300-kb fragment of NIH1 was used as the second probe for screening the same library to obtain clones corresponding to the additional fragment of NIH1. A total of 12 phage clones, which were negative for the 260-kb-fragment probe but positive for the 300-kb-fragment probe, could be obtained. By restriction mapping and preliminary sequencing analysis of each insert, a single contig as long as 40 kb could be constructed from 6 of the 12 clones (Fig. 2). None of the remaining six clones belonged to this contig despite some of them having sequences similar to those of the contig. At one end of the contig was located the methionine sufoxide reductase gene *msrA*, whose deduced amino acid sequence has 98% similarity with that of the published genome sequence of M1 *S. pyogenes* strain SF370 (9). Next to *msrA*, phage-associated integrase-like sequence *int* was found on the strand opposite to *msrA* in NIH1. Because no integrase-like sequence could be found downstream of *msrA* in SF370, we thought that one junction of the additional fragment of NIH1 would be between *msrA* and *int*. To confirm this, a genomic sequence downstream of *msrA* in K23 was directly determined by using primer p7-T3-3, which was made from a downstream sequence of *msrA* (Fig. 2). Indeed, K23 does have a different sequence from *int* (data not shown). As for the other end part of the contig, however, we could not identify the anticipated sequence corresponding to that downstream of *msrA* in K23. Attempts to amplify a genomic region of K23 with primers, p7-T3-2 and p5-T3-2, which were synthesized from sequences at both ends of the contig and directed inwards (Fig. 2), also failed (data not shown). These results showed that the contig does not cover the whole region of the additional fragment hitherto. Instead, primers #5-T7-R1 and K23rj, the former of which was located at the downstream end of the contig and directed downwards and the latter of which was synthesized from a downstream sequence to *msrA* in K23 and directed upwards (Fig. 2), successfully reacted with the NIH1 genomic DNA to produce a 2.3-kb PCR product (data not shown). With the lambda contig plus this PCR product, a 42-kb section of the genomic region, which exists in NIH1 but not in K23, could be cloned. Consecutive sequencing experiments revealed lambdoid temperate phage characteristics of this region: a 41,796-bp sequence starting from 17-bp *attL* (5′-TCTGATATAATATAAGA-3′) and ending with 17-bp *attR* (5′-TCTGATATAATAAAAGA-3′), *int* located just downstream of *attL*, and putative regulatory genes for transcriptional regulations about 3 kb downstream of *attL*; also, ORFs to the left and right of the regulatory genes were located on the opposite strand. Based on these observations, we named this sequence ϕNIH1.1. Like other streptococcal temperate phages (15), ϕNIH1.1 has a putative hyaluronidase gene (Fig. 2). Interestingly, at a 3′ part of ϕNIH1.1, there is an ORF

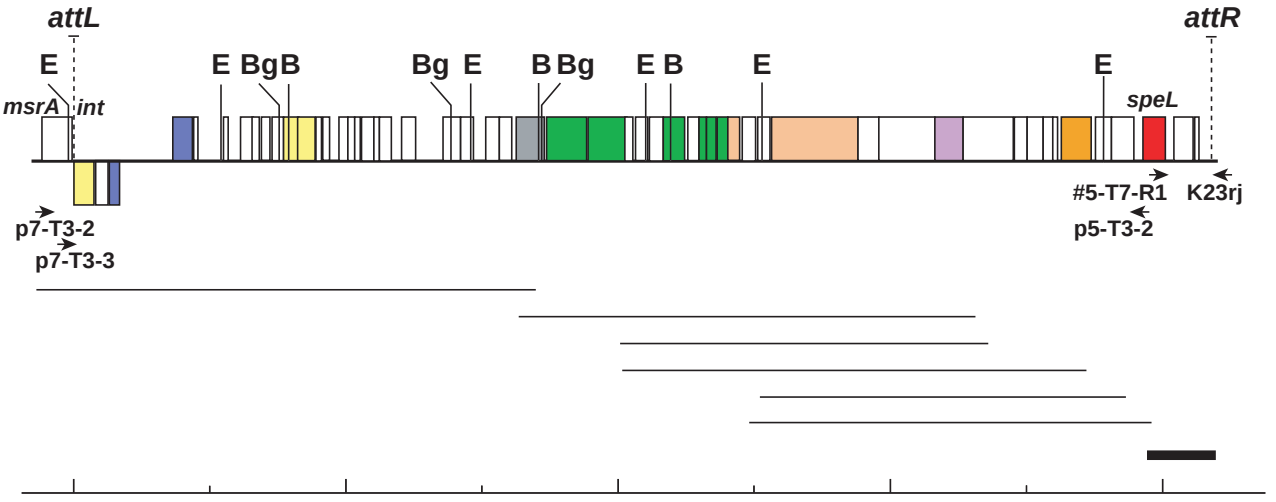

FIG. 2. ORFs and restriction enzyme sites of ϕNIH1.1. White and colored boxes show ORFs; boxes above the horizontal line show ORFs directed downward or rightward and those below the line show ORFs directed upward or leftward. Some gene names are on the corresponding boxes. Names of locations of the attachment sites of the phage and of primers for PCRs or for direct sequencing are also given. Thin lines under the map show inserts of lambda phage clones, and the thick line shows a PCR fragment amplified with primers #5-T7-R1 and K23rj. A scale is indicated at the bottom of the map with vertical lines plotted for every 5 kb. Abbreviations of restriction enzymes: E, *Eco*RI; Bg, *Bgl*II; B, *Bam*HI. The color in each box shows a predicted function as follows: yellow, DNA-binding proteins; blue, gene regulator proteins; gray, terminase; green, capside proteins; flesh, tail proteins; purple, hyaluronidase; orange, cell-wall hydrolase; and red, a superantigen protein.

whose amino acid sequence has 48 and 46% similarity with the C-terminal part of erythrogenic toxin SpeC and with that of the most potent superantigen, SmeZ-2 (24), respectively. Figure 3 shows multiple amino acid sequence alignments of streptococcal superantigens with this ORF. The C-terminal half of the ORF shares the well-conserved amino acids with the other superantigens; from this part, three zinc-binding ligands, histidine or aspartic acid, are provided, and the fourth is from the β-chain of the major histocompatibility class II (MHC-II) molecule (1, 24). Sequence homologies and experimental superantigenic characteristics (see Discussion) of the ORF led us to name this reading frame *speL*.

Distribution of *speL*. We examined the distribution of *speL* among old and recent *S. pyogenes* strains by PCR with primers speL-fwrd and speL-rev. None of the 10 old M3/T3 strains have this gene whereas all of the 18 recent M3/T3 stains were

FIG. 3. Multiple sequence alignment of amino acid sequences of well-characterized streptococcal superantigens and SpeL by the PileUp program on the GCG package. Regions of identical and similar sequences are shown with black and gray boxes, respectively. The asterisks on top of the sequence alignments show sequences conserved in all proteins. The arrowheads indicate positions of histidine or aspartic acid residues essential for zinc binding.

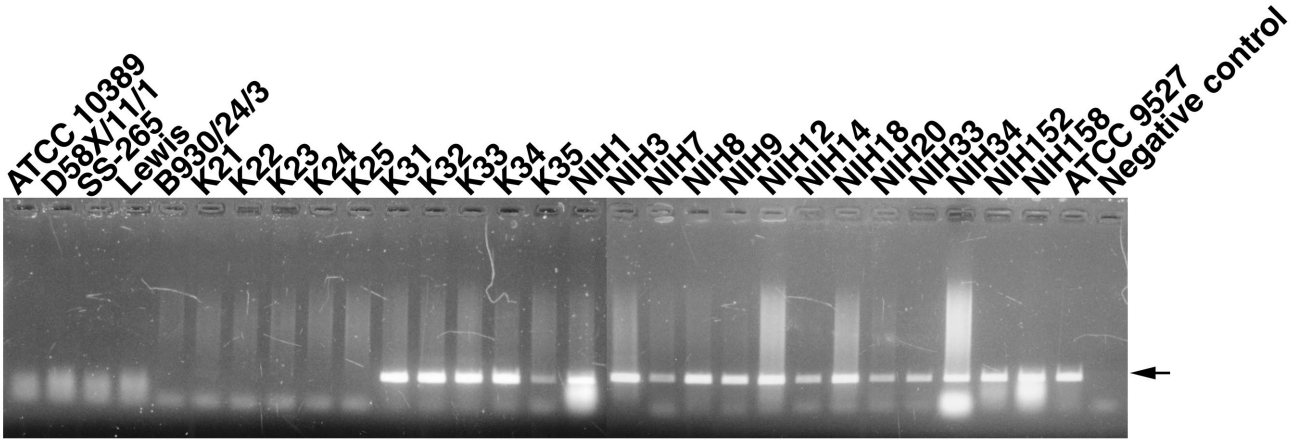

FIG. 4. Amplification result of *speL* by PCR. Strain name is indicated on each lane. Lanes ATCC 10389 to K25, old strains; lanes K31 to NIH158, recent strains (see Table 1 and the text). ATCC 9527 is a strain of *Streptococcus equi* subsp. *equi*. The position of the specific *speL* PCR product is indicated by an arrow.

positive irrespective of their clinical origins (Table 1 and Fig. 4). With regard to other serotypes of recent invasive isolates, however, only two of three M4/T4 isolates and one of two M22/T22 isolates were positive for the *speL* PCR; none of six M1/T1 isolates, of one M11/T11 isolate, of three M12/T12 isolates, of three *emm28*/T28 isolates, or of two *emm89*/TB3264 isolates yielded positive results for this gene. *S. equi* subsp. *equi* ATCC 9527, another beta-hemolytic streptococci having Lancefield's group C antigen, is positive for the *speL* PCR.

Induction of ϕNIH1.1. We examined the inducibility of ϕNIH1.1 by using mitomycin C. In spite of multiple attempts, we could not obtain plaques with a variety of old indicator strains shown in Table 1. Instead, when induced, a 40- to 42-kb DNA fragment became detectable from the phage particle fraction by ethidium bromide staining (Fig. 5, lane 2). And, this band reacted with a *speL* probe (lane 4), which was prepared from the PCR product from NIH1 shown in Fig. 4. On lane 3, a faint hybridization signal was also detected from the phage particle fraction owing to leakage, i.e., a low grade of induction without inducer, of ϕNIH1.1 (lane 3).

DISCUSSION

Our previous study showed that recent M3/T3 *S. pyogenes* isolates in Japan have acquired new fragments (17), which were suggested to be remnants of temperate phages by Desiere et al. (6). This study, however, reveals that the acquired fragment of NIH1 corresponds to the whole, not a remnant, of temperate phage ϕNIH1.1 comprising 41,796-bp nucleotides. The profiles of the erythrogenic toxin genes *speA* and *speC*, the sequence data of the *emm* genes (Table 1), and the PFGE data (Fig. 1) strongly suggest that recent M3/T3 isolates in Japan have originated from the same ancestor.

The acquired sequence has characteristics of lambdoid temperate phages in low-GC gram-positive organisms (6). It contains a putative hyaluronidase gene and the superantigen gene *speL*. Several streptococcal superantigens, SmeZ, SmeZ-2, SpeC, SpeJ and SpeG, make a superantigen subfamily (24). In addition to these members, SpeH and SpeI were already shown to possess superantigenic activities with different potentials toward different T-cell subsets (19, 21, 23, 24). Besides, SpeL does have superantigenic properties: a recombinant fusion protein between glutathione *S*-transferase and SpeL stimulates T cells with particular Vβs with a dependence on MHC-II molecules (Imanishi et al., unpublished data).

The notion that SpeL may play roles for severe streptococcal

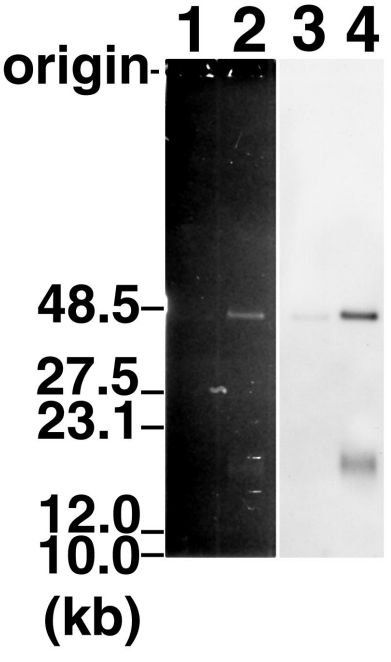

FIG. 5. Ethidium bromide-stained gel containing DNAs extracted from phage particle fraction (lanes 1 and 2), and their Southern hybridization results with a *speL* probe (lanes 3 and 4). Samples on lanes 1 and 3 were obtained without mitomycin C induction, whereas those on lanes 2 and 4 were with induction. DNAs were separated by PFGE with 2-s switching intervals. Sizes of DNA markers are indicated to the left.

infections is an attractive hypothesis, but the distribution of *speL* is almost specific to M3/T3 isolates, and no M1/T1 TSLS isolate in Japan examined has this gene. Another hypothesis is that regulatory gene(s) in ϕNIH1.1 would alter expression patterns of multiple toxins and superantigens, whose genes had been present in genomes of old M3/T3 strains before acquisition of the phage sequence. M1 *S. pyogenes* strain SF370, whose genome sequence has been determined by Ferretti et al. (9), has three complete temperate phage sequences and five remnant phage sequences (5) accompanied by multiple toxin, superantigen, or mitogenic-factor genes near the attachment sites of each phage sequence. The locations of these toxin genes in temperate phages are also true for the *speA* in phages T12, ϕ270, and ϕ49 (33) and the *speC* in phage CS112 (12, 18). Broudy et al. have reported that SpeC is induced along with phage CS112 induction by factors released from human cells (2), suggesting the *speC* is transcribed from internal promoter(s) of phage CS112. Similarly, other phage-associated virulence genes are likely to be controlled by one or more regulators within these phage sequences. In the case of the recent Japanese M3/T3 isolates, along with induction of ϕNIH1.1, multiple virulence genes would be activated simultaneously to cause various severe and sometimes fatal symptoms. Acquisition of large genomic fragments was also observed in recent M1 (3, 22), M12 (T. Murase, personal communication), and M22 (our unpublished observation) isolates, including those of TSLS origin. Thirdly, additional change(s) in M3/T3 genomes not detectable by PFGE may be essential for causing TSLS or severe infection. Tanaka observed the sudden change in PFGE patterns of Japanese T3 isolates between 1984 and 1985 (30); the pattern after the change corresponds to that observed in our recent M3/T3 isolates. In another report (22), similar changes in PFGE patterns of Japanese M3/T3 isolates seem to have occurred more gradually, but actually, the period was between 1983 and 1985 or earlier (T. Murase, personal communication). Since then, almost 10 years had passed until the first TSLS case was presented in Japan in 1992 (27). The *speL* gene or other gene(s) in ϕNIH1.1 must have conferred the advantage on the M3/T3 *S. pyogenes* clones for surviving and disseminating in human populations in the recent antibiotic era, but the event or events that occurred during this lag period remain veiled.

We could not obtain plaques from ϕNIH1.1, but the increased hybridization signal from mitomycin C-induced phage DNA with the *speL* probe proves that the phage is not defective. In agreement with our observation, Broudy et al. reported that the *speC*-carrying phage is detectable by electron microscopy but does not form plaques on a wide range of indicators (2). Goshorn also reported similar results (12). Interestingly, a contig in the ongoing genome sequencing project of *S. equi* subsp. *equi* in the Sanger Center contains a phage-associated nucleotide sequence whose deduced amino acid sequence has 98% similarity to SpeL. Another *S. equi* subsp. *equi* strain ATCC 9527 is also positive for PCR detecting *speL* (Fig. 5). Because this strain was isolated early in the 20th century, a horizontal transfer of the phage-associated *speL* from *S. equi* subsp. *equi* to *S. pyogenes* in 1980s may explain the observed change in M3/T3 *S. pyogenes* isolates in Japan. Analysis of protein profiles affected by phage gene products and the inter- and intraspecies transducing experiments will provide clues to solving the enigmatic changes in *S. pyogenes* infections in recent years.

ACKNOWLEDGMENTS

We thank S. Matsunaga for excellent technical assistance and T. Murase and T. Miyoshi-Akiyama for helpful discussions and critical readings of the manuscript.

This study was partially supported by a grant from the Ministry of Health, Labour and Welfare (H12-Shinkou-27).

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Editor: E. I. Tuomanen