

## Identification and Characterization of a New Staphylococcal Enterotoxin-Related Putative Toxin Encoded by Two Kinds of Plasmids

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**We identified and characterized a novel staphylococcal enterotoxin-like putative toxin, which is named SER. Nucleotide sequencing analysis of the *ser* gene revealed that *ser* was most closely related to the *seg* gene. The *ser* gene product, SER, was successfully expressed as a recombinant protein in an *Escherichia coli* expression system, and recombinant SER (rSER) showed significant T-cell stimulation activity. The SER production in *ser*-harboring *Staphylococcus aureus* strains was confirmed by Western blot analysis using anti-rSER antibody. Moreover, *ser* was seen to be encoded by at least two types of plasmids. In particular, one kind of plasmid encoding the *ser* gene has been known as a *sed*- and *sej*-carrying pIB485-related plasmid.**

*Staphylococcus aureus* is an important pathogen in humans and animals because this bacterium produces a wide variety of exotoxins, including staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin 1 (TSST-1) (8, 15). SEs are emetic toxins and are causative agents in staphylococcal food poisoning in humans, although their mechanisms of emetic activity are not fully understood (5). Also, SEs and TSST-1 are superantigens (SAGs), which have the ability to stimulate large populations of T cells that have a particular V $\beta$  element of the T-cell receptor (TCR). This gives rise to symptoms that are characteristic of toxic shock syndrome in humans. Five major serological types, SEA through SEE, have been characterized (5). However, in recent years, many new types of SE (i.e., SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, and SEQ) have been reported (10, 12, 13, 17, 22, 23, 24, 27, 30, 31). In addition, the determination of the complete genome sequences of several *S. aureus* strains has revealed that they maintain many SAG-related genes (staphylococcal exotoxin-like genes *set1* to *set26*) in their genomes (2, 13, 29). Staphylococcal SAGs constitute quite a large family of structurally related proteins.

On the other hand, it has been known that these SAG genes are associated with mobile genetic elements such as pathogenicity islands, prophages, and plasmids. SEB, SEC, SEG, SEI, SEM, SEN, SEO, SEK, SEL, SEQ, and TSST-1 are encoded by pathogenicity islands (2, 12, 13, 14, 30). SEA, SEE, and SEP are encoded by prophages (6, 7, 13), whereas SED and SEJ are encoded by a plasmid known as pIB485 (3, 31). These facts imply that these SAG genes transfer between staphylococcal strains by horizontal transfer. There is a possibility that these

mobile genetic elements play an important role in the evolution of *S. aureus* as a pathogen. To clarify the pathogenicity or virulence of *S. aureus*, it is important to bring to light the full extent of the diversity of staphylococcal SAGs. Here we describe a new staphylococcal superantigen-like putative toxin that is phylogenetically related to SEs, named SER.

In September of 1997, an outbreak of food poisoning occurred at a lunch box shop in the Fukuoka prefecture of Japan. Ten persons ate food prepared by the shop, and four of them developed nausea, vomiting, and diarrhea within 1.5 to 5 h. Many *S. aureus* isolates were obtained from patient feces and swabs of the lunch boxes ( $8.0 \times 10^6$  isolates/lunch box swab). Other food-borne pathogens were not isolated. Four *S. aureus* isolates (Fukuoka 5, Fukuoka 6, Fukuoka 7, and Fukuoka 8) were subjected to PCR analysis for detection of SE genes according to the method of Omoe et al. (21). However, no isolates harbored *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, or *sei* genes. We hypothesized that this outbreak was caused by an unidentified SE and so started a genetic analysis of these staphylococcal isolates. We initially tested whether or not these isolates harbored known enterotoxin-like nucleotide sequences. The bacterial strains and plasmids that were used in this study are listed in Table 1.

**Identification of a *seg*-like sequence in *S. aureus* strain Fukuoka 5.** *Hind*III-, *Xba*I-, and *Eco*RI-digested total DNA (2  $\mu$ g) of *S. aureus* strain Fukuoka 5 (food poisoning outbreak-related strain, SE unidentified) was subjected to Southern blot analysis (25) using digoxigenin (DIG)-labeled *seg*, *seh*, and *sei* probes digested from pKOG4, pKOH1, and pKOH6, respectively (21). The membranes were washed, and the signals were detected by chemiluminescence according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The Fukuoka 5 strain contained a nucleotide sequence that hybridized to the *seg* probe, a 6.0-kbp *Hind*III fragment, a 4.0-kbp

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	SE genotype or characteristic	Source or reference
<i>S. aureus</i>		
Isolates from SE-unidentified food poisoning outbreak		
Fukuoka 5	<i>sej ser</i> , carrying pF5	Isolated from swab of lunch box
Fukuoka 6	<i>sej ser</i> , carrying pF6	Isolated from patient feces
Fukuoka 7	<i>sej ser</i> , carrying pF7	Isolated from patient feces
Fukuoka 8	— <sup>a</sup>	Isolated from feces obtained from worker at lunch box shop
<i>sed</i> -harboring strain		
1151-7NG	<i>sed sej ser</i> , carrying p1151	M. S. Bergdoll
196E	<i>sea sed sej ser</i> , carrying p196	M. S. Bergdoll
FRI361	<i>sec2 sed seg sei sej ser</i> , carrying p361	M. S. Bergdoll
11727	<i>sea sed sej ser</i> , carrying p727	Food poisoning
11740	<i>sea sed sej ser</i> , carrying p740	Food poisoning
IVM7	<i>sed seg sei sej ser</i> , carrying p17	Healthy human
IVM16	<i>sed seg sei sej ser</i> , carrying p116	Healthy human
IVM46	<i>sed seg sei sej ser</i> , carrying p146	Healthy human
<i>seg</i> reference strain		
Fukuoka 1	<i>seg sei</i>	21
Plasmids		
pF5	<i>sej ser</i>	This work
pF6	pF5-like plasmid, <i>sej ser</i>	This work
pF7	pF5-like plasmid, <i>sej ser</i>	This work
p1151	pIB485-like plasmid, <i>sed sej ser</i>	This work
p196	pIB485-like plasmid, <i>sed sej ser</i>	This work
p361	pIB485-like plasmid, <i>sed sej ser</i>	This work
p727	pIB485-like plasmid, <i>sed sej ser</i>	This work
p740	pIB485-like plasmid, <i>sed sej ser</i>	This work
p17	pIB485-like plasmid, <i>sed sej ser</i>	This work
p116	pIB485-like plasmid, <i>sed sej ser</i>	This work
p146	pIB485-like plasmid, <i>sed sej ser</i>	This work
<i>E. coli</i>		
Strains		
DH5 $\alpha$		Toyobo
KRX1	DH5 $\alpha$ carrying pKRX1, Ap <sup>r</sup> <i>ser</i>	This work
Plasmids		
pBluescriptSK-	Ap <sup>r</sup>	Stratagene
pGEM3Zf(+)	Ap <sup>r</sup>	Promega
pGEX-6P-1	Ap <sup>r</sup> , GST fusion expression vector	Pharmacia
pKOG4	Ap <sup>r</sup> , pGEM3Zf(+) with <i>seg</i>	21
pKOH1	Ap <sup>r</sup> , pGEM3Zf(+) with <i>seh</i>	21
pKOI6	Ap <sup>r</sup> , pGEM3Zf(+) with <i>sei</i>	21
pKO311	Ap <sup>r</sup> , pBluescript SK- with <i>sej</i> and <i>ser</i>	This work
pKOJ1	Ap <sup>r</sup> , pGEM3Zf(+) with <i>sej</i>	This work
pKOR1	Ap <sup>r</sup> , pGEM3Zf(+) with <i>ser</i>	This work
pKRX1	Ap <sup>r</sup> , pGEX-6P-1 with <i>ser</i>	This work

—<sup>a</sup>, negative for *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, and *ser*.

*Xba*I fragment, and a 3.0-kbp *Eco*RI fragment, under low-stringency conditions (37°C) (Fig. 1). Under high-stringency conditions (65°C), the *seg* probe hybridized to only homologous nucleotide sequence (*seg*) in the Fukuoka 1 strain (positive control; *seg* and *sei* positive) total DNA, and no signal was observed in the Fukuoka 5 strain. The *seh* and *sei* probes did not hybridize to Fukuoka 5 total DNA under low-stringency conditions (data not shown).

**Cloning and sequencing analysis of the *ser* gene.** To clone the *seg*-like sequence in the Fukuoka 5 strain, a total DNA library was constructed using *Eco*RI-digested Fukuoka 5 DNA and lambda phage vector  $\lambda$ ZAPII (Stratagene, La Jolla, Calif.). The library was screened by plaque hybridization with a DIG-labeled *seg* probe under low-stringency conditions. About 10<sup>4</sup> clones were screened, and three positive clones were obtained. Positive clones were converted to plasmids by in vitro

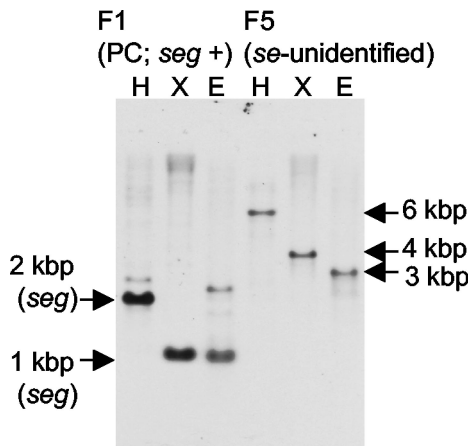


FIG. 1. Southern blot analysis of SE-unidentified *S. aureus* Fukuoka 5 isolate under the low-stringency condition. *Hind*III (H)-, *Xba*I (X)-, and *Eco*RI (E)-digested total DNA of the *S. aureus* Fukuoka 5 strain (F5, food poisoning outbreak-related strain, SE unidentified) and the Fukuoka 1 strain (F1, positive control [PC]; *seg* and *sei* positive) were subjected to Southern hybridization using a *seg* probe.

excision using a Rapid Excision Kit (Stratagene) according to the manufacturer's instructions. To verify the collected clones, these positive plasmids were labeled and subjected to hybridization to *Hind*III-, *Xba*I-, or *Eco*RI-digested Fukuoka 5 total DNA. One clone, pKO311, hybridized to a 6.0-kbp *Hind*III fragment, a 4.0-kbp *Xba*I fragment, and a 3.0-kbp *Eco*RI fragment of Fukuoka 5 total DNA. Thus, we concluded that pKO311 was a true positive clone. Nucleotide sequences of pKO311 were obtained for both strands by primer walking using an automatic DNA sequencer ABI 310 (Perkin-Elmer Applied Biosystems, Foster City, Calif.). pKO311 contained 2,751 bp of the insert. This insert contained two open reading frames (ORFs), and these ORFs could be transcribed in opposite directions. A search of BLAST at the DDBJ showed that one ORF was identical to a previously reported SE gene, *sej*. However, another ORF showed only a 65.9% homology with the *seg* gene. We concluded that this ORF is a new putative SE gene and designated it *ser* (Fig. 2). Figure 3 shows the nucleotide and deduced amino acid sequences for *ser*. The *ser* ORF encoded a polypeptide 259 amino acids in length. In the putative regulatory region of *ser*, we identified a potential promoter sequence and potential ribosomal binding site using the computer program Genetyx-Mac version 8.0 (Genetyx, To-

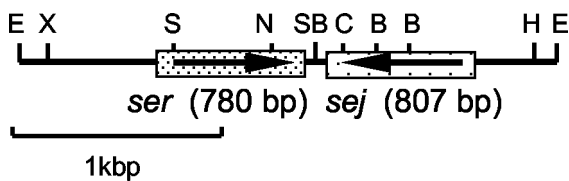


FIG. 2. Diagram of cloned DNA containing SE genes. Subclone pKO311 contained 2,751 bp of insert. This insert contained two kinds of SE genes, *sej* and *ser*. The directions of transcription of the genes are shown by the arrows. The letters refer to sites cut by the restriction endonucleases: B, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hind*III; N, *Nde*I; S, *Sna*I; SB, *Sna*BI; X, *Xba*I.

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541
CGTTTCAGCATTTTAAACATTGCCTAATTTTATAATCTTTATTATGTTATATTAATAA
AAAAGAGGTGTATTCATGTTAAATAAAATCTTACTTTTATTGTTCTCTGTTACTTTTAT
M L N K I L L L L F S V T F M
TTGTTATTTTCTCTTTACATTGAGTAAAGTCTAAACAGATCCAAGGCTGGAGAATTG
L L F F S L H S V S A K P D P R P G E L
AATAGAGTTAGTGATTATAAAAAACAAGGTAAGTACTATGGGAATGTTGAATCCTTAT
N R V S D Y K K N K G T M G N V E S L Y
AAGGATAAAGCGGTAATAGCAGAAAATGTTAAAATACTAGACAATCTTAGGACATGAT
K D K A V I A E N V K N T R Q F L G H D
TTGATTTTCTTATTCTTATTGAGTAAAGGTAAGGTAAGGTAAGGTAAGGTAAGGTAAGG
L I F P I P Y S E Y K E V K S E F I N K
AAAACAGCTGATAAATTTAAAGACAAACGGTTAGATGTGTTTGAATACCTATTTTAT
K T A D K F K D K R L D V F G I P Y F Y
ACATGTTTAGTACTAAGAATGAATCAAGAGAAGAATTTATTTTGGAGTTTGCATA
T C L V P K N E S R E E F I F D G V C I
TACGGGGCGTTACAATGCACTCCACAGCTGATAGTATATCCAAAATAATAATTTGTC
Y G G V T M H S T A D S I S K N I I V P
GTTACTGATAGATAAACAACAGTCTCTTTTACAATGATACAATAAAAAACCGGTT
V T V D N K Q Q F S F T I S T N K K T V
ACGGTACAAGAGCTAGATTATAAGTAAAGGAAATGGTTGACTACAATAAAAAATTATAT
T V Q E L D Y K V R N W L T T N N K K L Y
GAATTTGATGGGTGGCATATGAGACGGGTATATCAAAATTTGTAAGATAAAGAT
E F D G S A Y E T G Y I K F I E Q N K D
AGTTTTGGTACGATTTGTTCTCTAAAAAGGATTTGGTCCCTTTATACCTTATAAATTT
S F W Y D L F P K K D L V P F I P Y K F
GTAATAATTTATGAGATAAATAAACTATAGATGCTTCGAGTGTAAAGATAAAGTTTAC
V N I Y G D N K T I D A S S V K I E V H
CTGACTACAATGTGATTCATACGGTTGACTTTTGATAGATGACTACGTATATGATTT
L T T M *
    
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FIG. 3. Nucleotide sequence of *ser*. Nucleotide sequence corresponding to positions 541 to 1440 of 2.8-kbp inset of pKO311 is shown. The putative ribosomal binding site sequence is in boldface. The putative promoter sequences are indicated by double underline. The deduced amino acid sequence with a putative signal peptide (underlined) and its stop codon (asterisk) are indicated below the nucleotide sequence.

kyo, Japan). The N-terminal signal peptide sequence of SER was predicted using the online signal peptide prediction software SignalP (<http://www.cbs.dtu.dk/services/SignalP>) (20). The mature form of SER was predicted to have a molecular weight of 27,049.

**Expression of recombinant SER in *E. coli* and T-cell-stimulating activity of recombinant SER.** In order to construct the recombinant SER (rSER) expression plasmids, PCR primers were designed to amplify the gene fragment corresponding to the mature form of SER (SERS1, the sequence includes a 5' *Bam*HI site, 5'-CCCCGGATCCAAACCAGATCCAAGGCC TGGAG-3'; and SERS2, the sequence includes a 5' *Eco*RI site, 5'-CCCCGAATTCTCACATTGTAGTCAGGTGAACCTT-3'). The *ser* gene was amplified by PCR using Pyrobest DNA polymerase (Takara, Kyoto, Japan), and the *ser* fragment was then subcloned into pGEM3Zf(+) (Promega, Madison, Wis.) and designated pKOR1. The nucleotide sequence of the *ser* gene in pKOR1 was verified by an ABI 310 automatic DNA sequencer (Perkin-Elmer Applied Biosystems). The *ser* fragment was then digested from pKOR1 with *Bam*HI and *Eco*RI and was subcloned into the pGEX-6P-1 (Amersham Pharmacia Biotech, Piscataway, N.J.) glutathione *S*-transferase (GST) fusion expression vector. The resulting plasmids containing *ser* were named pKRX1. Expression, purification of GST-fused rSER, and the cleavage and removal of the GST tag from rSER were performed according to the method of Omoe et al. (21). The resulting mature rSER has five additional amino acid

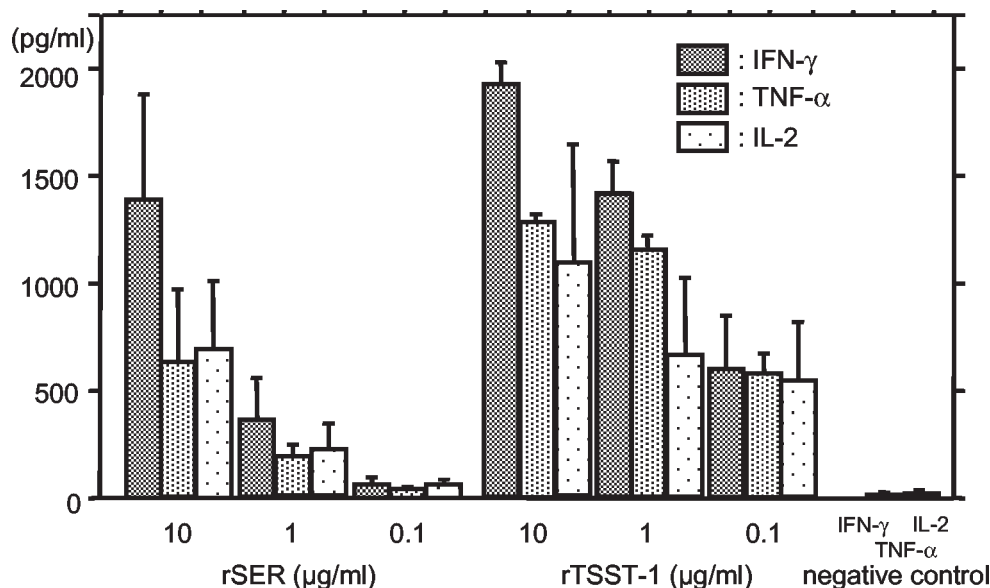


FIG. 4. Production of IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 by murine splenocytes in response to stimulation with rSER. Mouse splenocytes isolated from C57BL/6 mice were stimulated by various concentrations of rSER and rTSST-1 (positive control). Production of IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 was measured by sandwich ELISA. Negative control cells were stimulated with phosphate-buffered saline rather than SAGs.

residues, GPLGS, at the N terminus. T-cell-stimulating activity of purified rSER was assessed by induction of gamma interferon (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), and interleukin 2 (IL-2) production in murine splenocytes. Splenocytes isolated from specific-pathogen-free female C57BL/6 CrSic mice (CLEA, Kanagawa, Japan) were stimulated by several concentrations of rSER or recombinant TSST-1 (rTSST-1). The cells were incubated at 37°C for 72 h in a humidified 5% CO<sub>2</sub> atmosphere. Culture supernatants were harvested for use in IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 assays. Production levels of IFN- $\gamma$  and TNF- $\alpha$  were determined by means of sandwich enzyme-linked immunosorbent assays (ELISA) (18, 19). The IL-2 concentration was determined by using the mouse IL-2 ELISA kit (Biosource International, Camarillo, Calif.) according to the manufacturer's instructions. rSER induced significant amounts of IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 in stimulated murine splenocyte culture supernatants (Fig. 4). This result suggests that SER would act as a superantigen and stimulate T cells via TCR V $\beta$  and major histocompatibility complex class II binding. Further analyses of the molecular basis of T-cell stimulation activity of SER, such as the induction of selective expansion of T cells bearing particular TCR V $\beta$  regions and the requirement for major histocompatibility complex class II molecules to stimulate T cells, is needed. In addition, the rSER expressed by our system induces cytokine production in murine splenocytes, although rSER has an additional five amino acid residues derived from the expression vector sequence at the N terminus. Many biologically active SEs have been successfully expressed in a similar manner previously (11, 21). It seems that the additional five amino acid residues do not have a significant effect on the T-cell-stimulating activity of SEs.

#### mRNA transcription of *ser* and *sej* in strain Fukuoka 5.

Total RNAs isolated from *S. aureus* Fukuoka 5 at 6 and 9 h after inoculation were subjected to Northern hybridization

analysis according to the method of Sambrook et al. (25). To obtain the *sej* probe, PCR primers were designed to amplify the gene fragment corresponding to the mature form of SEJ (SEJS1, the sequence includes a 5' *Bam*HI site, 5'-CCCCGGATCCGATAGCAAAAATGAAAC-3'; SEJAS2, includes a 5' *Eco*RI site, 5'-CCCCGAATTCCTAAACCAAGGTAGACTTATTA-3'). The *sej* gene was amplified and subcloned into pGEM3Zf(+). The resulting plasmid was designated pKOJ1. DIG-labeled antisense RNA probes were synthesized by using a DIG RNA labeling kit (Roche Diagnostics) employing pKOR1 (*ser* probe) and pKOJ1 (*sej* probe). Northern analysis using an *ser* antisense RNA probe confirmed 1.3- and 0.8-kbp transcripts. As well, an *sej* antisense probe hybridized 1.3- and 0.6-kbp transcripts (data not shown). The *ser* and *sej* mRNA might be transcribed from multiple promoters.

**Preparation of polyclonal antibody and production of SER in *S. aureus* Fukuoka strains.** Anti-rSER serum was prepared by immunizing rabbits with purified rSER according to the method described by Shinagawa et al. (26). Titers of antiserum were monitored by means of ELISA. Monospecific rabbit anti-rSER antibody was affinity purified from hyperimmune serum by using an rSER-coupled Sepharose column. The anti-rSER antibody recognized only rSER, and no cross-reactivity with other SEs (SEA through SEE and SEG through SEI) was observed by Western blot analysis (data not shown). Using the anti-rSER antibody, we examined the SER productivity of *S. aureus* isolates from the food poisoning outbreak in Fukuoka. Culture supernatants of *S. aureus* strains were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Bio-Rad, Richmond, Calif.) according to the method described by Towbin et al. (28). The reactive signals were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. These food poisoning-related isolates, strains Fukuoka 5, Fukuoka 6,



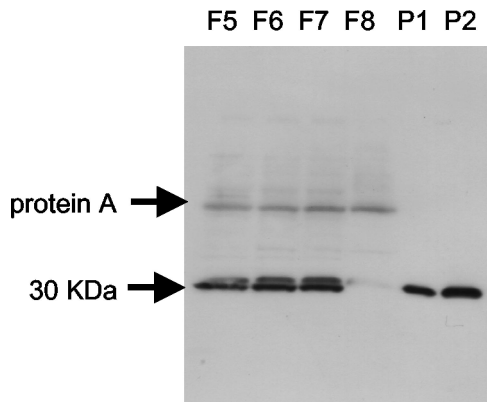


FIG. 5. Confirmation of secretion of SER by *S. aureus* isolates by Western blot analysis. Using anti-rSER antibody, we examined SER productivity of *S. aureus* isolates from a food poisoning outbreak in Fukuoka. Culture supernatants from Fukuoka 5 (F5), Fukuoka 6 (F6), Fukuoka 7 (F7), and Fukuoka 8 (F8) were subjected to Western blot analysis. An approximately 30-kDa signal was detected by anti-rSER antibody. P1, 25 ng of rSER per well; P2, 50 ng of rSER per well.

and Fukuoka 7, produced and secreted significant amounts of SER into the culture supernatants (Fig. 5). Although the sizes of native and recombinant SER seem somewhat higher than the predicted molecular mass (27 kDa), it is not uncommon for SEs to show a higher molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis than the amino acid

sequence would suggest (22). One isolate, Fukuoka 8, did not produce SER. The lack of the *ser* gene in *S. aureus* strain Fukuoka 8 was confirmed by Southern hybridization (data not shown). The SE-unidentified food poisoning outbreak-related Fukuoka strains, which harbor *ser* and *sej* genes, were able to produce significant amounts of staphylococcal enterotoxin-like putative toxin, SER. There is a possibility that SER was the cause, at least in part, of this food poisoning outbreak, although we could not deny the possible involvement of another SE, SEJ. In this study, we have not assessed the emetic activity of SER by using monkeys. Monkeys have typically been the primary animal models used to assess the emetic activity of SEs (4), although the use of monkeys is severely restricted by their high cost and limited availability. Orwin et al. (23) recently reported that a novel SE-like SA<sub>g</sub>, named SEQ, lacked emetic activity in young pigtail monkeys. They suggested that it might be inappropriate to refer to SEQ as an enterotoxin, because SEs are defined by their capabilities to cause emesis after being orally administered to monkeys. Newly reported SEs, such as SEJ, SEK, SEL, SEM, SEN, SEO, and SEP, were so designated based on their sequence similarity with classical SEs, and they were not assessed as having emetic activity in monkeys. At present, we have only circumstantial evidence that SER is an emetic toxin. In the future, quantitative elucidation of the emetic activity of SERs and other novel SEs should be examined using monkeys in order to accurately elucidate the capability to cause food poisoning and to assess the risk related to newly reported SEs.

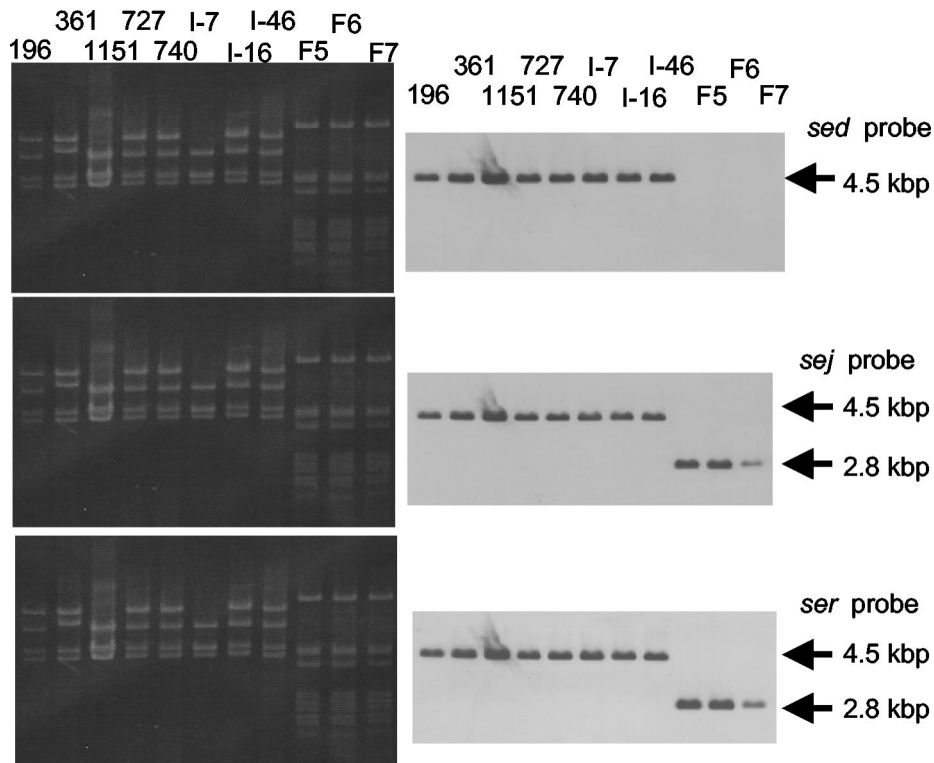


FIG. 6. *ser* and *sej* genes are carried by two kinds of staphylococcal plasmid. The pF5-like plasmids and pIB485-like plasmids were purified from *S. aureus* laboratory strains, food poisoning outbreak isolates, and healthy human isolates. These plasmids were digested by *EcoRI* and then subjected to Southern blot analysis. Lanes: 196E, p196E; 361, p361; 1151, p1151; 727, p727; 740, p740; I-7, pI7; I-16, pI16; I-46, pI46; F5, pF5; F6, pF6; and F7, pF7.

*ser* and *sej* genes exist on two kinds of plasmids. A search of FASTA showed that the 5' flanking sequence of the *ser* gene has high homology with several staphylococcal plasmids, such as pN315B (GenBank, EMBL, and DDBJ accession number AP003139) or pMW2 (GenBank, EMBL, and DDBJ accession number AP004832). As well, a FASTA search using the *sej* flanking sequences of 2.8 kbp of the *EcoRI* fragment of pKO311 showed high homology with pIB485 (GenBank, EMBL, and DDBJ accession number AF053140), which contains the *sed* and *sej* genes (31). Thus, we purified plasmids from the Fukuoka 5, Fukuoka 6, and Fukuoka 7 strains and named them pF5, pF6, and pF7, respectively. Then, several pIB485-like plasmids were purified from *S. aureus* laboratory strains, food poisoning outbreak isolates, and healthy human isolates. These Fukuoka outbreak-related plasmids and pIB485-like plasmids were analyzed by Southern hybridization. Southern blot analysis with the *ser* probe showed that the *ser* gene exists on the pF5, pF6, and pF7 plasmids. As well, an *sej* probe revealed the same signals on the same membrane. We concluded that 2.8 kbp of the *EcoRI* fragment of pKO311 must be a part of the pF5 plasmid (Fig. 6). *EcoRI*-digested pIB485-like *sed*-harboring plasmids showed almost the same pattern as the original pIB485 plasmid. However, several types of length polymorphism were observed. All *sed*, *ser*, and *sej* probes hybridized to 4.5 kbp of the *EcoRI* fragment of pIB485-like plasmids, leading us to the conclusion that pIB485-like *sed*- and *sej*-harboring plasmids also contain the *ser* gene (Fig. 6). pIB485 was originally described as a *sed*-carrying plasmid by Bayles and Iandolo (3). Then, Zhang et al. reported the *sej* gene as a new SE gene which existed on a *sed*-carrying plasmid, pIB485 (31). We have also shown that *sed* and the *sej*-carrying pIB48-like plasmids also carry *ser*. At present, the evolutionary relationship between pF5 and pIB485 is unknown. In recent years, several studies have shown that most *S. aureus* strains harbor one or more SE genes (16, 21). The presence of multiple SE genes in *S. aureus* makes it less clear whether any single toxin is responsible for staphylococcal food poisoning. Previous studies have shown that SEA and SED are common causes of staphylococcal food poisoning (5). Because SED-producing *S. aureus* strains should harbor pIB485-like plasmids, the SED-producing *S. aureus* involved in food poisoning would produce the additional enterotoxin-related toxins, SER and SEJ. If SER and SEJ are proved to be emetic toxins, the food poisoning caused by SED-producing *S. aureus* may be assumed to be a complex phenotype of intoxication by multiple SEs.

The benefit for *S. aureus* to maintain multiple SAg-related genes, such as the SE gene, *tst-I*, and *set*, on genomes and plasmids is not fully understood. Ferens and Bohach (9) hypothesized that SAgS play an important role in modulating the host immune response and that they may contribute to the maintenance of a suitable environment for colonization of *S. aureus* on host mucosal membranes. As well, Arcus et al. (1) showed that SET3, i.e., staphylococcal exotoxin-like protein 3, did not exhibit any of the properties of an SAg. They also suggested that the SET family may have an entirely different function from SAgS, although SETs have a conserved SAg-like three-dimensional structure. It seems that these SAg and SAg-related proteins have multiple functions that contribute to their survival and localization in hosts and to the pathogenicity

of *S. aureus*. SEs and TSST-1 are important toxins because they cause food poisoning and toxic shock syndrome. Moreover, it is also important to understand the functions of these SAgS and SAg-related toxins per se in order to clarify the nature of persistent infection of *S. aureus* and their pathogenicity.

**Nucleotide sequence accession number.** The nucleotide sequence of pKO311 was submitted to the GenBank, EMBL, and DDBJ databases and was assigned accession number AB075606.

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