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Investigation of *Staphylococcus aureus* positive for Staphylococcal enterotoxin S and T genes

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ABSTRACT. *Staphylococcus aureus* produces staphylococcal enterotoxins (SEs) and causes food poisoning. It is known that almost all SE-encoding genes are present on various types of mobile genetic elements and can mobilize among *S. aureus* populations. Further, plasmids comprise one of SE gene carriers. Previously, we reported novel SEs, SES and SET, harbored by the plasmid pF5 from Fukuoka5. In the present study, we analyzed the distribution of these SEs in various *S. aureus* isolates in Japan. We used 526 *S. aureus* strains and found 311 strains positive for at least one SE/SE-like toxin gene, but only two strains (Fukuoka5 and Hiroshima3) were positive for *ses* and *set* among the specimens. We analyzed two plasmids (pF5 and pH3) from these strains and found that they were different. Whereas these plasmids partially shared similar sequences involved in the *ser/selj/set/ses* gene cluster, other sequences were different. A comparison of these plasmids with those deposited in the NCBI database revealed that only one plasmid had the *ser/selj/set/ses* cluster with a stop mutation in *set* similar to that in pH3. In addition, the chromosomes of Fukuoka5 and Hiroshima3, positive for *ses* and *set*, were classified into different genotypes. Despite the low rate of gene positivity for these SEs, it is suggested that there is diversity in plasmids and strains carrying these two SEs. Consequently, regarding the entire feature of SE prevalence, we improved the multiplex PCR detection method for the SE superfamily to obtain further insight.

KEY WORDS: plasmid, *Staphylococcus aureus*, staphylococcal enterotoxin, staphylococcal food poisoning

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Staphylococcal food poisoning (SFP) is a food-borne disease caused by *Staphylococcus aureus*. It is a toxin-mediated disease, not an infectious disease. Heat- and proteolysis-stable protein toxins, namely staphylococcal enterotoxins (SEs), are its causative agents [1, 3]. Since the 1990s, many new toxins have been reported, and until now, more than 20 SEs and SE-like toxins (SEls) have been reported. The identification of new SEs continues and SFP outbreaks are being reported worldwide; moreover, SFP continues to be an important issue in public health and food safety. Thus, an understanding of all features of enterotoxigenic *S. aureus* and SEs is important for the prevention and control of SFP.

It has been reported that almost all SE and SEl genes reside on various mobile genetic elements, such as prophages, *S. aureus* pathogenicity islands, genomic islands, and plasmids [12]. Among these SE/SEl genes, *sed*, *selj*, *ser*, *ses*, and *set* are known to reside on plasmids. The history of the identification of these toxins is as follows; Bayles *et al*. described that *sed* genes are harbored by pIB485 [2], and Zhang *et al*. described that *selj* was harbored adjacent to *sed* on pIB485 [30]. On pF5 from food

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poisoning isolates, Omoe *et al*. reported a novel *ser* and Ono *et al*. described *ses* and *set* [16, 18].

The horizontal transfer of *S. aureus* plasmids contributes to the evolution of *S. aureus* [5, 6, 8, 11, 12, 14, 19, 28, 29]. Concerning SEs and SFPs, the transfer of plasmids harboring SE genes results in a recipient producing SEs, ultimately causing food poisoning outbreaks. This can threaten food safety and public health. Therefore, it is important to survey enterotoxigenic plasmids and *S. aureus*. However, to date, there is little information about these, and especially SES (staphylococcal enterotoxin S) and SET (staphylococcal enterotoxin T). Although we previously reported the vomit-inducing activity, superantigenic activity, and mRNA expression of these SEs, a genetic characterization of these enterotoxigenic plasmids has not been conducted. In the present study, to unveil the features of these newly identified SEs, we carried out an epidemiological analysis, comparison of plasmids, and molecular typing of strains carrying these plasmids.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and SE/SEl genotyping

In total, 526 *S. aureus* strains were used in this study for SES and SET gene detection. Forty-two strains were isolated from staphylococcal food poisoning outbreaks and 329 nasal swab isolates, as described in our previous study [22]. Seven methicillin-resistant *Staphylococcus aureus* (MRSA) isolates, 134 bovine mastitis isolates, and 14 mouse skin isolates were used in this study for the first time. All strains were collected in Japan. *S. aureus* was cultured overnight at 37°C in Soybean-casein-digest broth (Nissui, Tokyo, Japan) under shaking conditions and subjected to SES and SET detection. Lyse-n-Go (Thermo-Fisher Scientific, Waltham, MA, USA) was used for DNA extraction. New primers for the detection of *ses*, SES1 (5′-TCGGAATATACTATGGGGCAAA-3′) and SES2 (5′-GGTCTAACTCTTGAATTGTAGGTTC-3′), and primers to detect *set,* SET1 (5′-GGTTGGTGATTATGTAGATGCTTG-3′) and SET2 (5′-GTAGGCTTGTCTAAAGGGCTATG-3′), were used. PCR conditions were described in our previous report [17].

Purification and shotgun sequence of S. aureus plasmids harboring ses and set

Purification of plasmids from *S. aureus* strains positive for *ses* and *set* was carried out as described previously [16, 18]. The TOPO shotgun cloning kit (Invitrogen, Carlsbad, CA, USA), pCR4®Blunt-TOPO® (Invitrogen) and One shot® TOPO10 electrocomp™ *E. coli* (Invitrogen) were used for preparation of the shotgun library. White colonies were cultured in LB broth (Sigma Aldrich, St. Louis, MO, USA) containing 100 µg/ml ampicillin (Wako Pure Chemical Industries, Osaka, Japan) and cultured at 37°C under shaking conditions overnight. The cultured cells were subjected to plasmid extraction using the QIAprep8 (QIAGEN, Hilden, Germany) system. Nucleotide sequences of the shotgun library were obtained using an automatic DNA sequencer ABI3100Avant (Applied Biosystems, Foster City, CA, USA) and assembled using AGCT software, ver. 4.0 (Genetyx, Tokyo, Japan). Gaps of contigs were closed by primer walking with primers designed at contig ends.

Sequence analysis

Open reading frames (ORFs) were identified using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and *in silico* Molecular Cloning software (In Silico Biology, Inc., Yokohama, Japan), and ORFs were annotated with the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and through a search of the DNA Data Bank of Japan (DDBJ; http://blast.ddbj.nig.ac.jp/top-j.html). A comparison of plasmid sequences was performed using the Genome Matcher software (Version 3.012. [15]). Plasmid SAP047A was used for genomic comparison (No. GQ900405). RASTA was used for toxin-antitoxin detection [23].

Genotyping methods

agr typing of *ses*- and *set*-positive strains was carried out using previously reported primers [10]. The genotyping data of SE/SEl, coagulase, MLST, and genomic elements were already collected in our previous study [22].

Modification of multiplex PCR for SEs/SEls

To improve our previous multiplex PCR [17], the following modifications were used. To detect *ses* and *set*, SES1 and SES2 were added to previously reported multiplex PCR set 1, and SET1 and SET2 were added to multiplex PCR set 3. Further, to avoid non-specific bands, new primers detecting *fem*B, femB3 (5′-CACATGGTTACGAGCATCAT-3′) and femB4 (5′-TGTTTCGGGTGTTTTACCTT-3′), were used. A QIAGEN Multiplex PCR kit (Qiagen) was used to set up multiplex PCR. PCR was carried out in a 50 µl reaction volume, and all primer sets contained 0.4 µM of each primer as the final concentration. The PCR conditions were as follows. After 95°C for 15 min, 35 thermal cycles (94°C 30 sec, 57°C 90 sec, 72°C 90 sec) were carried out. After the cycles, a 72°C, 10 min final extension was carried out. Then, agarose gel electrophoresis was performed with 0.5× TBE buffer and a 3% agarose gel. To validate the modified PCR, we used 10 *S. aureus* genomic DNA samples (196E, S6, FRI-361, FRI-326, FRI-569, N315, Mu50, MW2, Fukuoka5, and RN4220). All strains except for Fukuoka5 were used as controls with our previous PCR method [17]. Genomic DNA was extracted as described previously [21]. After setup with the QIAGEN Multiplex PCR kit, KOD Multi & Epi (Toyobo, Osaka, Japan) was also used for multiplex PCR to confirm that primer sets worked well with another enzyme. This PCR was carried out in a 10 µl reaction volume with 0.2 U enzyme, and all primer sets contained 0.3 µM of each primer as the final concentration. The PCR conditions were as follows. After 94°C for 2 min, 25 thermal cycles (94°C 10 sec, 57°C 30 sec, 68°C 30 sec) were carried out.

Accession numbers

Nucleotide sequences of the two plasmids were deposited in DDBJ/EMBL/GenBank. The accession numbers of pF5 from Fukuoka5 and pH3 from Hiroshima3 are AB765928 and AB765929, respectively.

RESULTS

Prevalence of ses and set in a variety of S. aureus populations

By using PCR with the novel primers specific for *ses* and *set*, we analyzed their prevalence in a variety of *S. aureus* isolates. More than half of these were enterotoxigenic. Of the 526 strains analyzed, 311 (59.1%) were positive for at least one SE/SEl gene tested, and all food poisoning isolates, all MRSA isolates, 226 nasal swab isolates, and 36 bovine mastitis isolates were positive for one or more SE/SEl gene. In contrast, 103 nasal swab isolates, 98 bovine mastitis isolates, and all 14 mouse skin isolates were negative for all SE/SEl genes.

Focusing on plasmid-associated enterotoxins, *sed*, *selj*, and *ser* were not rare in *S. aureus* (*sed*: 25 strains; *selj*: 15 strains; *ser*: 15 strains), whereas *ses* and *set* were rare (Table 1). Only two isolates tested positive for *ses* and *set*. These strains that were positive for both *ses* and *set* were isolated from different food poisoning outbreaks in Hiroshima prefecture (Hiroshima3) and Fukuoka prefecture (Fukuoka5) in Japan. The SE genotype of Fukuoka5 has already been reported in our previous paper [18], whereas that of Hiroshima3 has not yet been reported. Hiroshima3 was also positive for other plasmid-associated SE/SEl genes, *selj* and *ser*, as well as *ses* and *set.* Concerning the enterotoxin profile, 12 strains carried only *sed*, 13 strains carried *sed*, *selj*, and *ser*, and two strains mentioned previously herein carried *selj*, *ser*, *ses*, and *set* as plasmid-associated toxin genes. Some strains harbored SEs/SEls as chromosomally associated SE/SEls in some combination.

Analysis of two plasmids harboring ses and set

Our previous study delineated the partial sequence of pF5 and confirmed that *ses* and *set* were present on pF5 [20]. However, in Hiroshima3, it was not clear whether these genes were present in the genome or plasmid. Therefore, we extracted plasmids from Fukuoka5 and Hiroshima3 and determined their complete sequences. Both Fukuoka5 and Hiroshima3 carried a single plasmid, namely pF5 and pH3, respectively. Both plasmids shared the coding sequences of four plasmid-associated SEs/SEls (SER, SElJ, SES and SET) but differed in other sequences. Plasmid maps and detailed information are shown in Fig. 1, Tables 2, and 3. pF5 was 43,265 bp in length and had 38 ORFs, whereas pH3 was 31,888 bp and had 33 ORFs. Similar to other *Staphylococcus* plasmids, these plasmids showed a low GC % (28.9% and 30.5% in pF5 and pH3, respectively). These two plasmids also shared partially similar sequences and carried similar *ser*/*selj*/*set*/*ses* clusters (ORF1–4 of pF5 and ORF1–4 of pH3), as shown Fig. 1. However, ORF3 in pH3 (encoding SET) was 204 bp, which was shorter than ORF3 in pF5, whereas ORF1, 2, and 4 of pF5 and pH3 were identical (Tables 2 and 3). These results were determined to be due to the deletion of T (position: 2,523 bp in pH3) and a subsequent frameshift mutation inside *set*. A further detailed comparative analysis is shown in Fig. 2. In addition to the *ser*/*selj*/*set*/*ses* cluster, there were other similar ORFs (ORF5–14 and ORF32–33 in pH3) indicated as a blue rectangle near this cluster (the second and third maps in Fig. 2). Of these, many proteins were predicted to encode hypothetical proteins, whereas the others were predicted to encode replication proteins, DNA-binding proteins, and alcohol dehydrogenase. This region around the *ser*/*selj*/*set*/*ses* cluster was conserved in both plasmids, but the other region was not. The two plasmids had different heavy metal resistance operons. pF5 had a cadmium resistance operon (ORF17–ORF18) and arsenic resistance operon (ORF33–ORF35), whereas pH3 had a mercury resistance operon (ORF19–ORF25), as shown in Tables 2, 3, and Fig. 1. In addition, pH3 had a penicillin resistance operon (ORF27–29), whereas pF5 had no antibiotic resistance operons. Furthermore, neither plasmid carried any type II toxin-antitoxin or *tra* genes.

Subsequently, we compared our two plasmids and another plasmid that was positive for *ses* and *set*. To date (Oct, 2020), the plasmid SAP047A isolated from human clinical blood in the USA had been the only plasmid deposited in the NCBI database. The *ser*/*selj*/*set*/*ses* cluster in this plasmid was similar to that of our two plasmids. Of note, the same stop mutation in *set* was found in plasmids SAP047A and pH3. In addition, the penicillin resistance operon (similar to that in pH3) and cadmium resistance operon (similar to that in pF5) were found in plasmid SAP047A. In addition, the sequences around *selj* and *ser* of the two plasmids were almost identical (approximately 98%) to that of pIB485, which carried *ser, selj*, and *sed*, although the sequence of *ser* was partial

Table 1. Staphylococcal enterotoxin/staphylococcal enterotoxin like toxin (SE/SEl) distribution in each population

		SFP*,** (n=42)	MRSA (n=7)	Nasal swab* (n=329)	Bovine mastitis (n=134)	Mouse skin (n=14)	Total
Any SE/SEl							
	se/sel positive	42	7	226	36	0	311
	se/sel negative	0	0	103	98	14	215
SES/SET							
	ses positive	2	0	0	0	0	2
	set Positive	2	0	0	0	0	2

*SE/SEl detection without SES and SET in these isolates was done in our previous paper [22]. **Staphylocccal food poisoning.

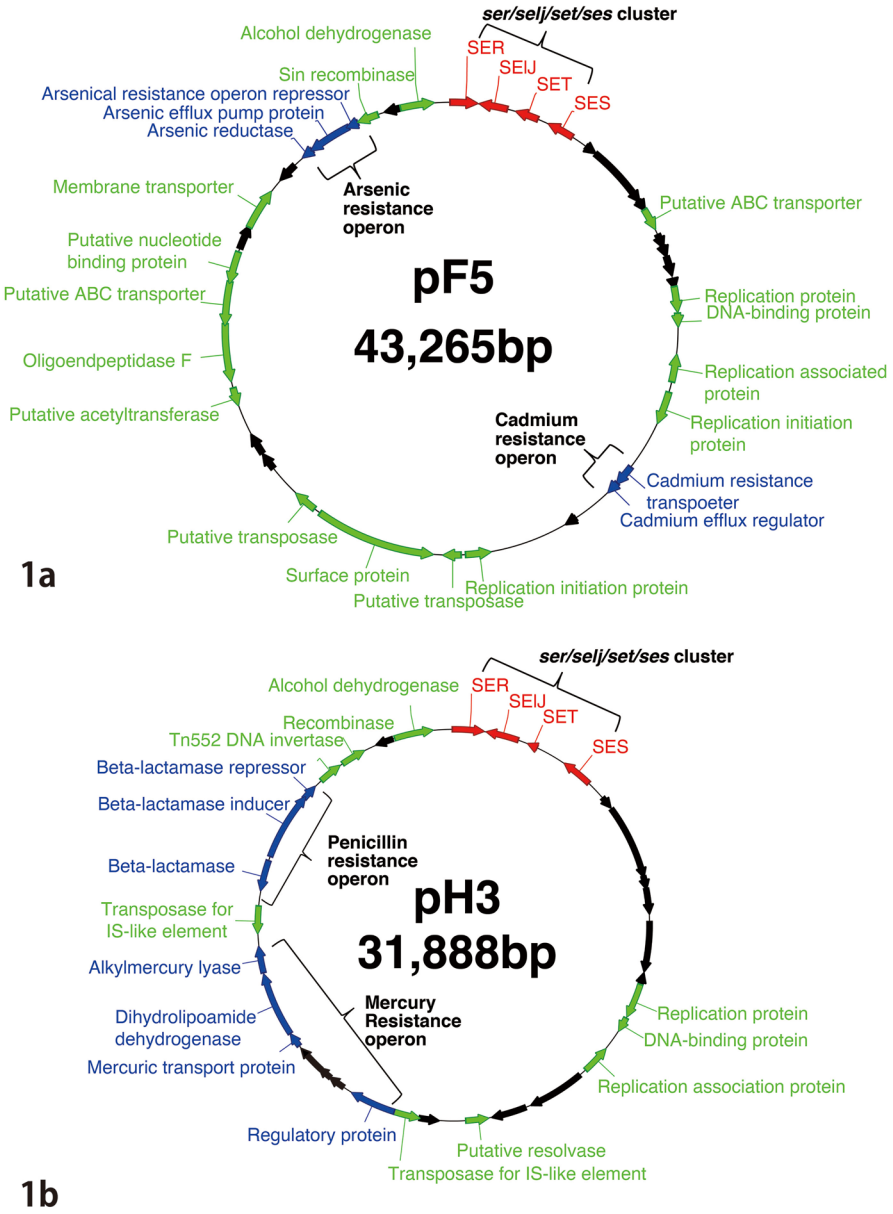

Fig. 1. Open reading frame (ORF) maps of the two plasmids. The maps of the two plasmids harboring *ses* and *set* are shown. *ser/selj/set/ses* cluster, red; antibiotic resistance operons and heavy metal resistance operons, blue; other known functional genes, green; hypothetical genes, black. a: Plasmid map of pF5 from Fukuoka5. b: Plasmid map of pH3 from Hiroshima3. ApE (ApE Plasmid Editor, version 2.0.53c by M. Wayne Davis) was used to draw plasmids.

(corresponding to 663–1,667 bp in accession AF053140.1). These results indicated that enterotoxin-associated plasmids shared similar sequences, including enterotoxin cassettes.

Genetic background of Fukuoka5 and Hiroshima3

Next, a comparison by molecular typing methods for chromosomes, not plasmids, was carried out. The results are shown in Table 4. These two strains had different genetic backgrounds. Fukuoka5 was classified into *agr*I, CoaIII, and Sequence type (ST) 8 based on *agr* typing, Coa typing, and MLST, respectively. Conversely, Hiroshima3 was classified into *agr*II, CoaII, and an ST5 single locus variant. None of the strains had *S. aureus* pathogenicity islands. This result indicated that there were two types of genetically distinct isolates positive for *ses* and *set*.

Modified multiplex PCR

Lastly, we tried to improve the multiplex PCR for SE/SEl genotyping. Novel primers for detecting *ses*, *set*, and *fem*B were included. The primers for *ses* and those for *set* were added to primer sets 1 and 3, respectively. Novel primers for *fem*B, instead of previous primers,

Table 2. Open reading frame (ORF) map of pF5

ORF No.	Start	Stop	Length	Direction	Products
ORF1	1	780	780	+	SER
ORF2	861	1,667	807	-	SElJ
ORF3	1,993	2,643	651	-	SET
ORF4	3,053	3,826	774	-	SES
ORF5	4,426	4,719	294	+	Hypothetical protein
ORF6	4,794	6,728	1,935	+	Hypothetical protein
ORF7	6,732	7,043	312	+	Hypothetical protein
ORF8	7,052	7,681	630	+	Putative ABC transporter
ORF9	7,919	8,308	390	+	Hypothetical protein
ORF10	8,296	8,574	279	+	Hypothetical protein
ORF11	8,642	9,187	546	+	Hypothetical protein
ORF12	9,338	9,556	219	+	Hypothetical protein
ORF13	9,578	10,306	729	+	Replication protein
ORF14	10,393	10,752	360	+	DNA-binding protein
ORF15	11,612	12,403	792	-	Replication associated protein
ORF16	12,826	13,770	945	+	Replication initiation protein
ORF17	15,392	16,009	618	+	Cadmium resistance transpoeter
ORF18	16,028	16,375	348	+	Cadmium efflux regulator
ORF19	17,681	17,983	303	+	Hypothetical protein
ORF20	20,445	21,161	717	-	Replication initiation protein
ORF21	21,393	21,878	486	+	Putative transposase
ORF22	22,211	25,858	3,648	-	Surface protein
ORF23	26,162	26,836	675	+	Putative transposase
ORF24	27,924	28,412	489	-	Hypothetical protein
ORF25	28,492	29,100	609	+	Hypothetical protein
ORF26	30,078	30,578	501	-	Putative acetyltransferase
ORF27	30,841	32,553	1,713	-	Oligoendpeptidase F
ORF28	32,550	33,845	1,296	-	Putative ABC transporter
ORF29	33,846	34,781	936	-	Putative nucleotide binding protein
ORF30	34,992	35,705	714	+	Hypothetical protein
ORF31	35,692	36,909	1,218	+	Membrane transporter
ORF32	37,372	37,938	567	-	Hypothetical protein
ORF33	38,345	38,656	312	-	Arsenic reductase
ORF34	38,674	39,963	1,290	-	Arsenic efflux pump protein
ORF35	39,963	40,277	315	-	Arsenical resistance operon repressor
ORF36	40,338	40,937	600	-	Sin recombinase
ORF37	41,205	41,624	420	-	Hypothetical protein
ORF38	41,748	42,719	972	+	Alcohol dehydrogenase

Table 3. Open reading frame (ORF) map of pH3

ORF No.	Start	Stop	Length	Direction	Product
ORF1	1	780	780	+	SER
ORF2	861	1,667	807	-	SElJ
ORF3	1,993	2,196	204	-	SET
ORF4	3,052	3,825	774	-	SES
ORF5	4,423	4,716	294	+	Hypothetical protein
ORF6	4,791	6,725	1,935	+	Hypothetical protein
ORF7	6,729	7,040	312	+	Hypothetical protein
ORF8	7,052	7,678	627	+	Hypothetical protein
ORF9	7,916	9,184	1,269	+	Hypothetical protein
ORF10	9,226	9,570	345	-	Hypothetical protein
ORF11	9,569	10,429	861	+	Replication protein
ORF12	10,516	10,857	342	+	DNA-binding protein
ORF13	11,447	12,157	711	-	Replication association protein
ORF14	12,431	13,882	1,452	+	Hypothetical protein
ORF15	14,073	14,942	870	+	Hypothetical protein
ORF16	15,047	15,595	549	-	Putative resolvase
ORF17	16,329	16,805	477	-	Hypothetical protein
ORF18	16,829	17,503	675	-	Transposase for IS-like element
ORF19	17,547	18,710	1,164	+	Regulatory protein
ORF20	19,009	19,416	408	+	Hypothetical protein
ORF21	19,433	19,786	354	+	Hypothetical protein
ORF22	19,750	20,463	714	+	Hypothetical protein
ORF23	20,589	20,924	336	+	Mercuric transport protein
ORF24	20,982	22,625	1,644	+	Dihydrolipoamide dehydrogenase
ORF25	22,707	23,357	651	+	Alkylmercury lyase
ORF26	23,706	24,380	675	-	Transposase for IS-like element
ORF27	24,820	25,665	846	-	Beta-lactamase
ORF28	25,772	27,529	1,758	+	Beta-lactamase inducer
ORF29	27,519	27,899	381	+	Beta-lactamase repressor
ORF30	28,163	28,741	579	+	Tn552 DNA invertase
ORF31	28,868	29,479	612	+	Recombinase
ORF32	29,828	30,247	420	-	Hypothetical protein
ORF33	30,371	31,342	972	+	Alcohol dehydrogenase

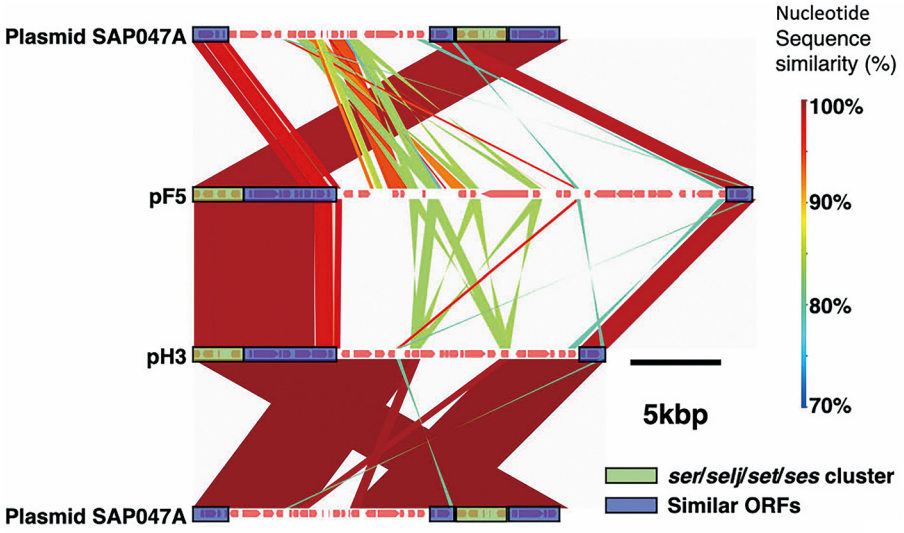

Fig. 2. Comparative analysis of the similarity among the three plasmids, pF5, pH3, and plasmid SAP047A. The genome matcher program was used. Arrows indicate open reading frames (ORFs), and their orientation is the ORF direction. Sequence homology (%) is shown by the color scale. If there was 100% homology, the sequences are connected by red lines. Moreover, if there was less than 70% homology, there are no lines. The color range is red (100%) to blue (70%). Green and blue highlights indicate the *ser/selj/set/ses* cluster and similarly shared ORFs, respectively. The scale bar indicates 5 kb. The accession numbers for pF5, pH3, and plasmid SAP047A are AB765928, AB765929, and GQ900405, respectively.

Table 4. Chromosomal type of *Staphylococcus* carrying *ses/set* plasmid

Strains	SE/SEl genes on chromosome**	agr	Coa***	MLST****	SaPI*****	Plasmids
Fukuoka5	(*selj, ser, ses, set*)	I	III	ST8	none	pH3
Hiroshima3	*seg, sei, sem, sen, seo,* (*selj, ser, ses, set*)	II	II	ST5 SLV*	none	pF5

*Single locus variant; ***selj, ser, ses* and *set* were on plasmids; ***coagulase type; ****ST8 and ST5 belongs to Clonal complex 8 (CC8) and CC5, respectively; *****Staphylococcus aureus* pathogenicity island.

were used to avoid nonspecific bands. To confirm that PCR was performed properly, 10 *S. aureus* strains, which were known to be of various SE/SEl genotypes, were subjected to multiplex PCR. The results are shown in Fig. 3. All PCR products corresponding to every gene, including *ses* and *set*, were obtained, and the sizes of all products were consistent with the predicted sizes. This result confirmed that the modified multiplex PCR performed well. In addition, it was also confirmed that these primer sets worked well with another PCR enzyme (Supplementary Fig. 1).

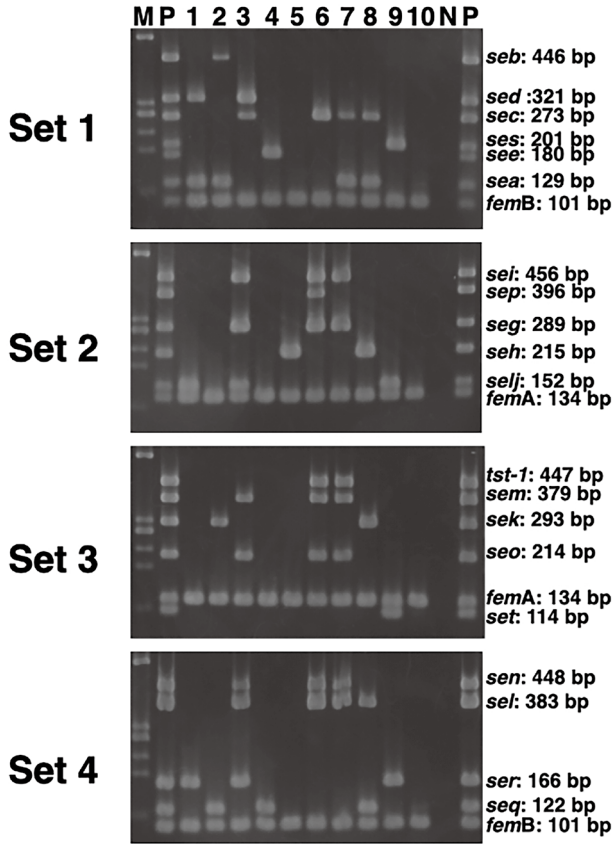

Fig. 3. Improvement of multiplex PCR in this study. Electrophoresis result of multiplex PCR modified in this study. Primers to detect *ses* and *set* were added to primer sets 1 and 2, respectively. The primers used to detect *fem*B in sets 1 and 4 were changed to new primers. Primers for set 3 were the same as those in a previous study [17]. PCR M, φx174/*Hae*III digest; P, positive control (mixture of nine genomic DNA samples, including 196E, S6, FRI-361, FRI-326, FRI-569, N315, Mu50, MW2, and Fukuoka5); N, no DNA (pure water); 1, 196E (*sea*, *sed*, *selj*, *ser*); 2, S6 (*sea*, *seb*, *sek*, *seq*); 3, FRI-361 (*sec*, *sed*, *seg*, *sei*, *selj*, *sel*, *sem*, *sen*, *seo*, *sep*, *ser*); 4, FRI-326 (*see*, *seq*); 5, FRI569 (*seh*), 6, N315 (*sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *sep*, *tst-1*); 7, Mu50 (*sea*, *sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *sep*, *tst-1*) 8, MW2 (*sea*, *sec*, *seh*, *sek*, *sel*, *seq*); 9, Fukuoka5 (*selj*, *ser*, *ses*, *set*); 10, RN4220 (no SE/SEl genes). The SE/SEl genotype of each strain was from our previous study [17].

DISCUSSION

Plasmids comprise an important factor associated with evolving processes in *Staphylococcus*. The majority of *S. aureus* strains has one or more plasmids and their plasmids carry various virulence factors [9, 12]. These virulence factors include antibiotic resistance genes, heavy metal resistance genes, and toxin genes. The SE/SEl family is a plasmid-related toxin. The horizontal gene transfer of these plasmids is responsible for acquiring enterotoxigenicity and might render recipient cells food poisoning pathogens. In the present study, we focused on and analyzed newly described plasmid-encoding SEs, *ses* and *set*, and delineated their detailed genetic background.

Although Ono *et al*. reported a partial sequence of plasmid pF5 from Fukuoka5 [20], the whole nucleotide sequence of an *ses/set*-positive plasmid isolated in Japan was not available. In this study, we conducted complete genome sequencing of two plasmids in Japan, including pF5, and identified that these two plasmids were typical enterotoxigenic plasmids in *Staphylococcus*. *S. aureus* plasmids can be divided into three types [12]. According to this classification, both plasmids sequenced in this study were type II plasmids because of the intermediate length, resistance genes, and lack of *tra* responsible for conjugation. Although some reports have shown that some plasmids carry the toxin-antitoxin system responsible for plasmid maintenance, our plasmids did not. As described previously herein, it seems that *selj* is associated with *ser* as a cassette in the same plasmids, whereas *sed* is independent of them. Although only two strains were identified and analyzed in this study, it seems that *ses* and *set* form a similar homologous cassette in those two strains; that is, two nearly identical gene sequences are aligned in the same direction. The recombination of these three enterotoxin cassettes might cause divergence of the enterotoxigenic plasmid in *S. aureus*.

S. aureus strains positive for *ses* and *set* were rare (only two strains from food poisoning), compared with those harboring other SEs/SEls, especially the plasmid-encoding SE/SEl genes *sed*, *selj*, and *ser*. Consistent with our reports, other groups reported *S. aureus* positive for these three genes [4, 7, 26]. In contrast, only two of 526 strains were positive for *ses* and *set* and carried plasmids (approximately 0.5%). A recent study by Vu *et al*. analyzed the distribution of several superantigens in *S. aureus* from diabetic foot ulcers and demonstrated that the positivity rates of *ses* and *set* were relatively lower than those of other SE genes, similar to our present result [27]. We speculate that few *S. aureus* strains are positive for *ses* and *set*. There might be some explanations for this;

specifically, our sequenced plasmids did not have any *tra* and toxin-antitoxin systems. *tra* mediates plasmid spreading among the *S. aureus* population [13]. The toxin–antitoxin system confers stability to the plasmid in cells [24]. A lack of these genes in plasmids appears to be a disadvantage for plasmid distribution in the *S. aureus* population.

Moreover, Fukuoka5 and Hiroshima3 are classified into different backgrounds, clonal complexes (CC) 8 and CC5, respectively. However, these CCs are well known SE-plasmid associated CCs. Our recent studies indicated that *sed*, *selj*, and *ser* were strongly associated with the specific lineages CC5 and CC8 in SFP outbreaks [25]. Two strains positive for *ses* and *set* were also classified into these groups, indicating the lineage specificity of these SEs. With respect to plasmids associated with another toxin, exfoliative toxin type B, recent reports indicated that strains carrying the ETB (exfoliative toxin B) plasmid from impetigo patients could be classified into a specific clonal complex, CC121 [20]. McCarthy *et al*. suggested a lineage-associated plasmid [13]. Since each CC carries a unique restriction-modification system, each CC tends to accept unique MGEs, including plasmids, and shows their own MGE profiles.

This is the first study to shed light on two new plasmid-encoding SEs, *ses* and *set*. To date, these toxins have been assessed in only a few studies as a part of molecular epidemiology [27]. We found that there is variability in the plasmids and strains carrying these plasmids, although *ses* and *set* are thought to be rare in the *S. aureus* population. This indicates that the plasmids and strains do not represent the spread of a single clone. However, it remains unclear how much variation exists. Considering staphylococcal enterotoxin E, its positive rate is known to vary regionally, being less common in Japan and more common in Europe and USA [1, 22, 25]. That is why the positive rate of these novel SEs in other countries might show a different trend. Because we used isolates only in Japan, differences in prevalence rates by region are still unknown. In other words, the features of these plasmids and these newly identified SEs remain to be elucidated. Therefore, further studies are needed to understand this. Our new modified multiplex PCR is easy to use and will facilitate future studies, because reactions including our primers, used to detect these SEs, can be performed well with at least two different PCR kits.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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