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Positive Regulation of Staphylococcal Enterotoxin H by Rot (Repressor of Toxin) Protein and Its Importance in Clonal Complex 81 Subtype 1 Lineage-Related Food Poisoning

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We previously demonstrated the clonal complex 81 (CC81) subtype 1 lineage is the major staphylococcal food poisoning (SFP)-associated lineage in Japan (Y. Sato'o et al., J Clin Microbiol 52:2637–2640, 2014, http://dx.doi.org/10.1128/JCM.00661-14). Strains of this lineage produce staphylococcal enterotoxin H (SEH) in addition to SEA. However, an evaluation of the risk for the recently reported SEH has not been sufficiently conducted. We first searched for staphylococcal enterotoxin (SE) genes and SE proteins in milk samples that caused a large SFP outbreak in Japan. Only SEA and SEH were detected, while there were several SE genes detected in the samples. We next designed an experimental model using a meat product to assess the productivity of SEs and found that only SEA and SEH were detectably produced *in situ*. Therefore, we investigated the regulation of SEH production using a CC81 subtype 1 isolate. Through mutant analysis of global regulators, we found the repressor of toxin (Rot) functioned oppositely as a stimulator of SEH production. SEA production was not affected by Rot. *seh* mRNA expression correlated with *rot* both in media and on the meat product, and the Rot protein was shown to directly bind to the *seh* promoter. The *seh* promoter sequence was predicted to form a loop structure and to hide the RNA polymerase binding sequences. We propose Rot binds to the promoter sequence of *seh* and unfolds the secondary structure that may lead the RNA polymerase to bind the promoter, and then *seh* mRNA transcription begins. This alternative Rot regulation for SEH may contribute to sufficient toxin production by the CC81 subtype 1 lineage in foods to induce SFP.

Staphylococcus aureus produces several virulence factors causing human and animal diseases. Among virulence factors produced by *S. aureus*, staphylococcal enterotoxins (SEs) show emetic and superantigen activities and are the causative agents for staphylococcal food poisoning (SFP) and toxic shock syndrome (1, 2). Presently, 23 SEs and SE-like toxins (SEls) have been reported. SEA-SEE are classical SEs, while SEG-SElX are newly described SEs/SEls (1, 2). Of these, SEA is shown to be the most important in SFP outbreaks (2). Conversely, reports suggest the newly described SEs also contribute to SFP outbreaks (3–5), but contributions for these SEs remain unknown. Thus, it is necessary to evaluate the importance of the newly described SEs in SFP outbreaks.

Our previous report described the clonal complex 81 (CC81) subtype 1 lineage as the major SFP-associated lineage in Japan. Almost all of the CC81 subtype 1 isolates carried *sea* and showed high SEA production (6). Moreover, another unique genetic characteristic is the presence of *seh*. All CC81 subtype 1 isolates carried *seh* (all 30 strains), while other isolates rarely carried it (1 strain in 341 strains) (6).

Though limited in numbers, previous epidemiological reports found SEH (*seh*) in SFP cases. In 2000, a large outbreak involving 13,420 patients consuming contaminated low-fat milk occurred in Japan (7, 8). SEH and SEA were detected in the causative low-fat milk. A recent study in Germany showed almost all of the isolates from food poisoning by ice cream were positive for *seh* as well as *sea* (9). Further, an epidemiology study in South Korea showed that *S. aureus* strains positive for *seh* formed one of the dominant groups causing SFP, similar to findings of our previous study (6, 10). In the Netherlands, there was a report of an outbreak with *S. aureus* positive for *seh* only (3).

In addition to epidemiology, the emetic activity of SEs/SEls is an important characteristic as an etiological agent of SFP. Several studies concerning vomiting activities caused by SEs are reported (5, 11–14) where the emetic activity of SEH in primates was similar to that of SEA (2). Like SEA, SEH may play an important role in SFP, but there is little information about the production of SEH. Therefore, we explored the unique production mechanism of SEH in clone no. 10, which was classified into CC81 subtype 1.

MATERIALS AND METHODS

Detection of *se* genes and SE proteins in SFP-causing low-fat milk from a factory. To demonstrate the involvement of SEH in food poisoning, we first retrospectively determined the repertoires of SE/SEl genes and proteins in food samples. We used daily product samples from the historically largest outbreak in Japan (7, 8). Six milk samples processed during different periods in the factory that caused a large outbreak in Japan were used as samples 1 to 6. Multiplex PCR was carried out to detect the genes for

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SE/SEl in the samples as described previously (15). SEs/SEls in the samples were purified and concentrated for enzyme-linked immunosorbent assay (ELISA). The excess protein, e.g., casein, was removed by adding HCl (to pH 3.8) at room temperature for 10 min. After centrifugation (4,000 × *g*, 20 min, 4°C), the supernatant was filtered using a Millex-GP 0.22-μm filter (Millipore, MA). After neutralizing the supernatant with NaOH (pH 6.8), 10% (wt/vol, final concentration) chloroform (Wako Pure Chemical Industries, Osaka, Japan) was added to remove lipids from the supernatant. After centrifugation (4,000 × *g*, 20 min, 4°C), a 20% volume of 30% (wt/vol) trichloroacetic acid (Wako Pure Chemical Industries) was added and incubated at 4°C for 30 min. The precipitated protein was collected using centrifugation (4,000 × *g*, 20 min, 4°C). After discarding the supernatant and drying, the protein was resuspended in 0.1 M Tris-HCl (pH 8.0; Wako Pure Chemical Industries) and neutralized with NaOH (Wako Pure Chemical Industries) (pH 7.0 to 8.0). The sample was concentrated 25 times. Sandwich ELISA was performed to detect SEA, SEC, SEG-SEI, SEK, and SEM-SEQ as described by Omoe et al. and Sato'o et al. (6, 13, 16), with modifications. For detection, SuperSignal ELISA Femto maximum sensitivity substrate (Thermo Fisher Scientific, Waltham, MA) was used as the substrate.

***Staphylococcus aureus* strains and assay for toxin production.** Eleven *Staphylococcus aureus* strains were used for toxin production. The SE/SEl genotypes of each strain are described in Table 1. We used nine clinical isolates from SFP, one human nasal swab isolate (IVM50), and MW2 as a control. *Staphylococcus aureus* was cultured in medium and on the meat product. Medium culture was performed as described previously (6), with some modifications. *Staphylococcus aureus* was precultured in 3 ml brain heart infusion (BHI) broth (Becton Dickinson, Sparks, MD) supplemented with 1% (wt/vol) yeast extract (Becton Dickinson) at 37°C overnight. About 6×10^9 precultured cells were inoculated into 60 ml fresh BHI broth supplemented with 1% yeast extract and cultured at 37°C for 24 h with constant agitation. If necessary, antibiotics (chloramphenicol final concentration, 10 μg/ml; tetracycline final concentration, 5 μg/ml) and/or xylose (final concentration, 1%, wt/vol) were added before inoculation of bacterial cells. After culture and centrifugation (15,600 × *g*, 20 min, 4°C), filtration was performed using a 0.2-μm-pore-size Minisart membrane filter (Sartorious, Göttingen, Germany) to prepare supernatant samples for sandwich ELISA. To evaluate SE production in foods we used a meat product, salted ham, because it is commonly associated with SFP (1) and it is relatively easy to measure SEs produced on the surface. For culture on meat product, about 10^8 *S. aureus* cells precultured overnight were spread on the surface of a 5-g salted ham slice. The bacterium-contaminated salted ham slices were incubated at 37°C for 24 h. After incubation, the slices were washed with 2 ml of Dulbecco's phosphate-buffered saline (pH 7.4) supplemented with 0.1% (wt/vol) bovine serum albumin (Sigma, St. Louis, MO). The washed buffer was centrifuged (15,600 × *g*, 20 min, 4°C) and filtered with a 0.2-μm-pore-size Minisart membrane filter to eliminate meat debris and bacteria, and the filtrates were used as ELISA samples. Sandwich ELISA was performed to detect the seven SE products (SEA to SED and SEG to SEI) as described above.

Genetic manipulation. We prepared gene deletion mutants, deletion mutants containing empty vector, and mutants complemented with the deleted *sarA*, *sarS*, *sarT*, *sarU*, *saeR*, and *rot* genes from the wild type of the CC81 subtype 1 lineage, no. 10. Allelic replacement and gene complementation were performed as described previously (17, 18). Plasmids and primers used in this study are listed in Tables 2 and 3. The constructed mutants in this study are listed in Table 1.

RNA extraction, reverse transcription, and real-time PCR. Bacterial culture was performed as described above. From medium culture, cells were collected from broth by centrifugation (2,800 × *g*, 10 min, 4°C). For meat culture, bacterium-contaminated salted ham incubated for 24 h was washed with 2 ml Dulbecco's phosphate-buffered saline (pH 7.4), and then the cells were pelleted by centrifugation (2,800 × *g*, 10 min, 4°C). The bacterial cells were subjected to RNA extraction.

TABLE 1 Bacterial strains used in this study

Strain	Relevant characteristic(s)	Source or reference
Staphylococcus aureus		
No. 10	CC81 subtype 1, *sea*, *seb*, *seh*, *sek*, *seq*	42
01240	CC81 subtype 1, *sea*, *seh*, *sek*, *seq*	6
Nagasaki	CC81 subtype 1, *sea*, *seb*, *seh*, *sek*, *seq*	16
Hiroshima3	CC5, *seg*, *sei*, *selj*, *sem*, *sen*, *seo*, *ser*	6
IVM50	CC508, *sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*	15
01235	CC96, *sea*, *sec*, *sel*	6
Oita1	CC508, *seg*, *sei*, *sem*, *sen*, *seo*	6
11727	CC8, *sea*, *sed*, *selj*, *ser*	15
MW2	CC1, *sea*, *sec*, *seh*, *sek*, *sel*, *seq*	43
196E	CC not assigned, *sea*, *sed*, *selj*, *ser*	44
S6	CC not assigned, *sea*, *seb*, *sek*, *seq*	45
RN4220	Genetic manipulation strain	46
No. 10 Δ*sarA*	Deletion mutant	This study
No. 10 Δ*sarA* pKAT	Vector control mutant	This study
No. 10 Δ*sarA* pKAT::*sarA*	Complemented mutant	This study
No. 10 Δ*sarS*	Deletion mutant	This study
No. 10 Δ*sarS* pWH1520	Vector control mutant	This study
No. 10 Δ*sarS* pWH1520::*sarS*	Complemented mutant	This study
No. 10 Δ*sarT*	Deletion mutant	This study
No. 10 Δ*sarT* pKAT	Vector control mutant	This study
No. 10 Δ*sarT* pKAT::*sarT*	Complemented mutant	This study
No. 10 Δ*sarU*	Deletion mutant	This study
No. 10 Δ*sarU* pKAT	Vector control mutant	This study
No. 10 Δ*sarU* pKAT::*sarU*	Complemented mutant	This study
No. 10 Δ*rot*	Deletion mutant	This study
No. 10 Δ*rot* pKAT	Vector control mutant	This study
No. 10 Δ*rot* pKAT::*rot*	Complemented mutant	This study
No. 10 Δ*saeR*	Deletion mutant	This study
No. 10 Δ*saeR* pWH1520	Vector control mutant	This study
No. 10 Δ*saeR* pWH1520::*saeR*	Complemented mutant	This study
Escherichia coli		
DH5α	Cloning strain	TaKaRa
BL21(DE3)	Expression strain	Novagen

RNA extraction was performed with the FastRNA pro blue kit (MP Biomedicals, Irvine, CA). The remnant DNA was treated using RQ1 RNase-free DNase (Promega, Madison, WI). Subsequently, reverse transcription from purified RNA was performed with a Transcriptor first-strand cDNA synthesis kit (Roche, Basel, Switzerland). All cDNA preparation procedures were performed according to the manufacturer's directions. All cDNA solutions were 10-fold diluted with TE buffer (con-

TABLE 2 Plasmids used in this study

Plasmid name	Purpose	Source or reference
pKFT	Genetic manipulation	17
pKFT (*sarA* FR)	Genetic manipulation	This study
pFK25 (pKFT (*sarS* FR))	Genetic manipulation	17
pKFT (*sarT* FR)	Genetic manipulation	Sugai laboratory
pKFT (*sarU* FR)	Genetic manipulation	Sugai laboratory
pKFT (*rot* FR)	Genetic manipulation	In this study
pKFT (*saeR* FR)	Genetic manipulation	Sugai laboratory
pKAT	Genetic manipulation	Sugai laboratory
pKAT::*sarA*	Genetic manipulation	Sugai laboratory
pKAT::*sarT*	Genetic manipulation	Sugai laboratory
pKAT::*sarU*	Genetic manipulation	Sugai laboratory
pKAT::*rot*	Genetic manipulation	This study
pWH1520	Genetic manipulation	MoBiTec
pWH1520::*sarS*	Genetic manipulation	18
pWH1520::*saeR*	Genetic manipulation	Sugai laboratory
pGEM-T Easy	EMSA	Promega
pGEM-T Easy/SEH promoter	EMSA	This study
pET28a	EMSA	Novagen
pGrot[a]	EMSA	20

[a] Reconstructed in this study.

taining 10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and 4 μl of the diluted cDNA solution was subjected to real-time PCR assay (20-μl [total volume] reaction mixture containing 0.5 μM each primer set in each tube). Real-time PCR was performed with SsoAdvanced SYBR green supermix (Bio-Rad, Hercules, CA) and a Bio-Rad CFX96 system (Bio-Rad). The real-time PCR primers are listed in Table 3. Real-time PCR conditions included denaturing at 95°C for 1 min, 40 cycles of 95°C for 15 s and 60°C (*rot* and *seh*) or 62°C (*gyr*B) for 15 s, and then 72°C for 30 s. Fluorescence was measured at the end of every extension step. After cycling, melt curves analysis was performed between 70°C and 90°C. All quantitative PCR (qPCR) data were analyzed using CFX Manager software version 3.0 (Bio-Rad) according to the manufacturer's instructions. The relative expression level of *rot* and *seh* was calculated by relating the target gene expression to the constant expression of the reference gene, *gyrB*. To determine the PCR amplification efficiency of each qPCR, the cDNA solution was serially diluted with 10 mM Tris-HCl (pH 7.5) containing 0.5% Tween 20 (Wako Pure Chemical Industries). No inhibition of PCR amplification was observed, and PCR amplification efficiency of all samples was between 90 and 110%.

Electrophoretic mobility shift assay (EMSA). We adapted a previous method with modification to perform the mobility shift assay (19). The SEH promoter sequence was amplified with PrimeSTAR GXL DNA polymerase (TaKaRa, Shiga, Japan) with the primers listed in Table 3. The PCR product was purified using gel extraction with a FastGene gel/PCR extraction kit (Nippon Genetics, Tokyo, Japan), and then a 3′ A was added to the purified DNA with a Mighty TA cloning reagent set for PrimeSTAR (TaKaRa). TA cloning was performed with the DNA fragment with the added A by using pGEM-T Easy (Promega) and the Ligation high Ver. 2 kit (Toyobo, Osaka, Japan). Blue-white selection used DH5α and LB agar supplemented with ampicillin (Wako Pure Chemical Industries), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Wako Pure Chemical Industries), and isopropyl-β-D-thiogalactopyranoside (IPTG; Wako Pure Chemical Industries). The inserted sequence was confirmed by sequencing with a BigDye Terminator v 3.1 kit (Life Technologies Corporation, Carlsbad, CA), T7 promoter primer, SP-6 promoter primer (Table 3), and Hi-Di formamide (Life Technologies Corporation) using a 3130 genetic analyzer (Life Technologies Corporation). Promoter cloning (construction of pGEM-T Easy/SEH promoter; Table 2) was performed according to the manufacturer's directions.

The DNA probe was amplified with the pGEM-T Easy/SEH promoter, PrimeSTAR GXL DNA polymerase, T7 promoter primer (5′-cy3 labeled or nonlabeled), and SEH upstream AS primer (Table 3) and purified using gel extraction with a FastGene gel/PCR extraction kit. DNA concentration was measured using a NanoDrop 1000 (NanoDrop Technologies, Inc., Wilmington, DE). Rot protein was prepared according to a previously described method (20) using the vectors and primers listed in Tables 2 and 3, respectively. SEH promoter probe and Rot protein were incubated under previously described conditions (20). The reaction mixture was electrophoresed with a 6% polyacrylamide gel and 0.5× Tris-borate-EDTA (TBE) buffer under cold conditions. After electrophoresis, the gel was imaged using a Molecular Imager FX system (Bio-Rad).

RESULTS

Detection of SE genes and SE proteins in SFP-causing samples of a large SFP outbreak. We first attempted to detect SE/SEl genes and proteins in milk samples reported to contain SEA and SEH (7, 8). Including these two genes, nine toxin genes (*sec*, *seg*, *sek*, *sei*, *sem*, *sen*, *seo*, *sep*, and *seq*) were detected in four of the six milk samples (see Fig. S1 in the supplemental material). To confirm toxin proteins, we used sandwich ELISA, which detects >0.2 ng/ml SE protein, and detected only SEA and SEH (0.46 ng/ml and 0.63 ng/ml, respectively) but not the other serotype SEs. This suggested the samples associated with SFP contain inactivated *S. aureus* cells carrying a variety of SE genes besides *sea* and *seh* or their DNA, but only limited types of enterotoxins were detectably produced. We questioned if there is a preference in the production of enterotoxin serotypes by SFP-causing *S. aureus*, especially in foods, and this led us to investigate SE production in food samples.

Comparison of SE production in media and on salted ham. We compared the production of SEs by *S. aureus* grown on salted ham and in media. The data are shown in Fig. 1. Only SEA and SEH, but no other serotype SEs, were detectably produced on meat product after incubation (Fig. 1). SEH was detected in all samples, and its amounts ranged from 0.025 to 0.28 μg/5 g meat product. Also, SEA was detected in six of eight strains, and its amounts ranged from 0.061 to 0.22 μg/5 g meat product. These results indicated not all SEs were produced at detectable levels on meat products under this condition. Of note, the amount of SEH was similar to that of SEA, the most important SE in SFP (Fig. 1). Conversely, various SEs were detected in the media (Fig. 1). Among SEs, the level of SEB was the highest, followed by SEC, SEA, SEH, and SED. These data further support the idea that SEH, as well as SEA, is preferentially produced in (on) foods and is therefore an etiologically important SE in SFP.

Identification of the regulatory pathway of SEH. We determined the factors affecting SEH production in media using a series of regulatory gene mutants. The data from the ELISA for SEH are shown in Fig. 2. The deletion of *sarA*, *sarT*, *sarU*, *rot*, and *saeR* decreased SEH production, while complementation of mutants recovered production. In contrast, *sarS* deletion increased production while its complementation decreased production. The schematic image of the regulatory pathway of SEH expression, based on our data and previous studies, is illustrated in Fig. 3. Rot was named after the function "repressor of toxins" in *S. aureus* (21). However, our results clearly demonstrated that Rot is an enhancer of SEH production. In addition, SEA production was not affected by Rot (see Fig. S2 in the supplemental material).

mRNAs of *rot* and *seh* were expressed in both media and meat product. Subsequently, we performed a temporal sampling of *S. aureus* from media and meat product and conducted qPCR to

TABLE 3 Primers used in this study

Primer name	Sequence (5′-3′)	Purpose
gyrB forward	AGGTCTTGGAGAAATGAATG	qPCR
gyrB reverse	CAAATGTTTGGTCCGCTT	qPCR
seh forward	TCAAGGTGATAGTGGCAAT	qPCR
seh reverse	CCAATCACCCTTTCCTGT	qPCR
rot forward	TGCAGTATTTCAACCACACAC	qPCR
rot reverse	GTATCGTTAATGCGCCAGT	qPCR
M13 forward	GTAAAACGACGGCCAGT	Genetic manipulation
M13 reverse	CAGGAAACAGCTATGAC	Genetic manipulation
SarA1	AGGATCCAAAAATTCTAATGGA	Genetic manipulation
SarA2	GGATCCTCAACTCATTCTTAAG	Genetic manipulation
SarA3	GGTTTTGCACTAATGGCACT	Genetic manipulation
SarA4	TTGCTCGATACATTTGCCCGA	Genetic manipulation
sarS1	GCCAAAGCTTATACATGGCTAGTCGG	Genetic manipulation
sarS4	TCAAGGATCCATAGAAGGCGCTTTG	Genetic manipulation
sarT F-F	GGCTGCAGGATAGTGACTTAATGTTCTT	Genetic manipulation
sarT F-R	CCGGATCCGTCTTCATCCCTTACTTTTAA	Genetic manipulation
sarT R-F	GGGGATCCAGTATGTTTCGAGATTTTAAT	Genetic manipulation
sarT R-R	CCGAGCTCTACACCCTGTGGTGCAGTGTC	Genetic manipulation
sarT F	CGGGATCCCGTTATGTTTCATTAATATTTATTTC	Genetic manipulation
sarT R	CCCAAGCTTGGGCCTTACATTCTCCTACTA	Genetic manipulation
sarU F-F	CGGGATCCCGTTATGTTTCATTAATATTTATTTC	Genetic manipulation
sarU F-R	GGCTGCAGCTTACATTCTCCTACTACTATTTTC	Genetic manipulation
sarU R-F	GGCTGCAGATTTAACAGATTTACCTCTTG	Genetic manipulation
sarU R-R	CCCAAGCTTGTGACGATATTGTTGAATCTG	Genetic manipulation
sarU F	GGGTTCGAAGTCTTCATCCCTTACTTTTAA	Genetic manipulation
sarU R	CCCAAGCTTTTAAAAGAAAAATTTTCTTGG	Genetic manipulation
rot1	AATGGATCCACAAGGTATTA	Genetic manipulation
rot2	GGGGATCCTGTTAATTTCTCCT	Genetic manipulation
rot3	TCAAATGTTGACTACTCAAT	Genetic manipulation
rot4	ATAAACTTGCTTTCTATTCA	Genetic manipulation
rot comp S	GCCGAAGCTTTAAGGTTGAGAATGTATATC	Genetic manipulation
rot comp AS	GCCGAAGCTTTTACACAGCAATAATTGCGT	Genetic manipulation
SaeF	TGGAAAGCTTATGATTTCACAGCACCC	Genetic manipulation
SaeSR	TTGCAAGCTTGATACAAGTAATTGGTC	Genetic manipulation
T7 promoter-1	TAATACGACTCACTATAGGG	EMSA
T7 promoter-1 cy3[a]	TAATACGACTCACTATAGGG	EMSA
SP6 promoter-1	CAAGCTATTTAGGTGACACTATAG	EMSA
SEH upstream S	TCTAACTACTATAGCAACTG	EMSA
SEH upstream AS	TTTAAAACTCCTCAATATAT	EMSA
g-rot-f-NcoI	GCGCCATGGTGAAAAAAGTAAATAACGACACT	EMSA
g-rot-r-XhoI	GCGCTCGAGCACAGCAATAATTGCGTTTA	EMSA

[a] cy3 labeled at the 5′ end.

investigate the expression of *rot* and *seh* genes. As shown in Fig. 4, mRNA expression of *rot* and *seh* correlated (correlation coefficient [R^2] of 0.99 in media and 0.44 on meat product) in *S. aureus* both in media and on meat product.

Rot protein-*seh* promoter sequence interaction. We next determined if Rot directly binds to the *seh* promoter sequence. The nucleotide sequences of *rot* and *seh* promoters in no. 10 were the same as those of MW2 (data not shown). Recombinant Rot was purified with a His tag (Fig. 5a) to show binding of His-tagged Rot to the promoter sequence of *seh*. Using EMSA, the binding of Rot caused an apparent mobility shift of the band (Fig. 5b). Also, the addition of redundant nonlabeled DNA totally cancelled the shift. Taken together with mutation/complementation experiments and qRNA analysis, the data strongly suggested Rot protein directly binds to the *seh* promoter and positively enhances *seh* mRNA transcription.

DISCUSSION

Gene regulation and virulence factor production are closely related to the virulence of *S. aureus* (22, 23). Among regulatory proteins, Rot was first identified in 2000, and several reports showed Rot represses many secretory proteins but enhances cell wall proteins (21, 24–27). The origin of the name of Rot came from its function as a repressor of the expression of toxin genes. Until now, the Rot-dependent inhibitory expression of toxins, including SEB, SED, α-hemolysin, β-hemolysin (truncated), and Panton-Valentine leukocidin, is demonstrated.

Other global regulators, such as the two-component system and the SarA family, interact with Rot (28–32). Among these, the interaction between the Agr system and Rot was characterized. The Agr system is one of the two-component regulatory mechanisms in *S. aureus* that sense cell density. RNAIII, functional RNA of the Agr system, prevents the translation of *rot* and indirectly

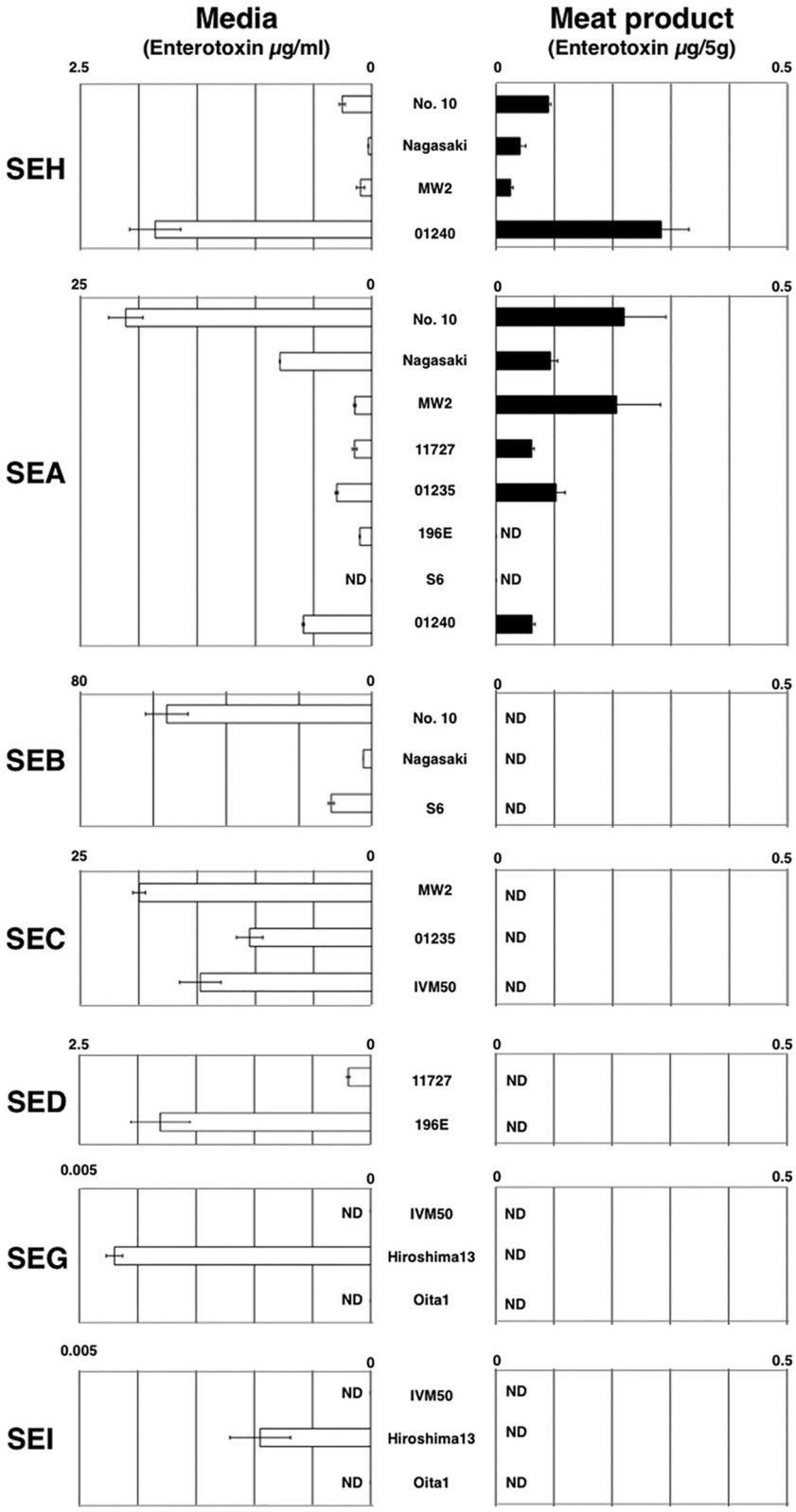

FIG 1 Comparison of SE production in media and on meat product. Briefly, various *S. aureus* strains were cultured in media and on salted ham slices, and the filtrates of culture broth and wash from the slices were used for ELISA. The averages and standard errors from each sample are shown. All cultures and all ELISAs were repeated two times ($n = 4$ per sample). White bar, SEs in media (μg/ml); black bar, SEs on meat product (μg/5 g). ND, not detectable (<0.2 ng/ml or 5 g salted ham). (A portion of the data for SEB, SEH, SEG, and SEI were a reexamination of the studies by Omoe et al. and Sato'o et al. using improved ELISA [16, 42]).

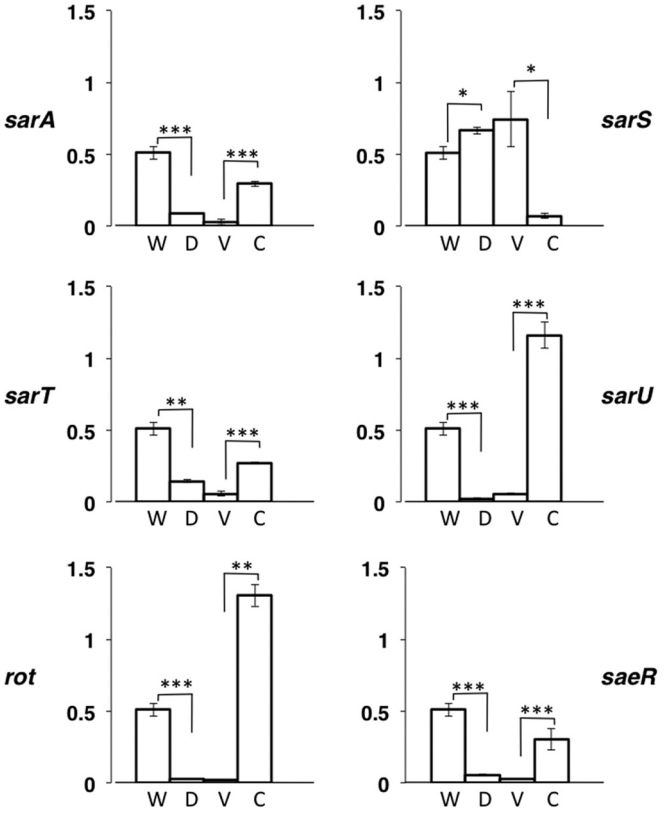

FIG 2 Effects of global regulators on SEH production. SEH production of global regulator mutants in medium was compared with that of the wild type. *S. aureus* SFP clone no. 10 and its global regulator mutants were cultured in medium, and the filtrates of the cultures were used for ELISA. The averages and standard errors from each sample are shown. All cultures and all ELISAs were repeated two times ($n = 4$ per sample). W, no. 10 wild type (the value in this figure is the same as that in Fig. 1); D, global regulator deletion mutants; V, vector control mutants (deletion mutants containing pKAT or pWH1520); C, complemented mutants (deletion mutants containing complementing vector). Significance was determined by Student's *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

changes gene expression (30, 32). This interactive mechanism also affects some SE expression. The repression of SEB and SED by Rot is inhibited by RNAIII (26, 27). In addition, the upstream sequence of *sec* has imperfect 10-bp inverted repeats (in MW2 [GenBank accession number BA000033], positions 851652 to 851660 and 851689 to 851698), closely similar to the inverted repeats important for Rot binding to the *seb* promoter (27). Similar to SEB and SED, the expression of SEC seems to be repressed by Rot, and this repression may be cancelled by RNAIII (33). Effects of the Agr system on the expression of *egc*-related SEs, such as SEG and SEI, remains to be investigated. However, the expression of these genes changes when the cell density changes (34). Therefore, *egc*-related SEs also may be affected by RNAIII (and Rot). Of note, these levels of SE production were much lower than those of others in media (Fig. 1). As a possible reason for this, it may be that these SEs could not be detected on meat product, on which the production of other SEs also decreased (Fig. 1). In contrast, we found *sarA*, *sarT*, *sarU*, *rot*, and *saeR* are upregulators and *sarS* is a downregulator for SEH production (Fig. 2 and 3). In addition, we observed SEA was not repressed by Rot. The expression of SEA and SEH was reported to be unaffected by *agr* (35, 36).

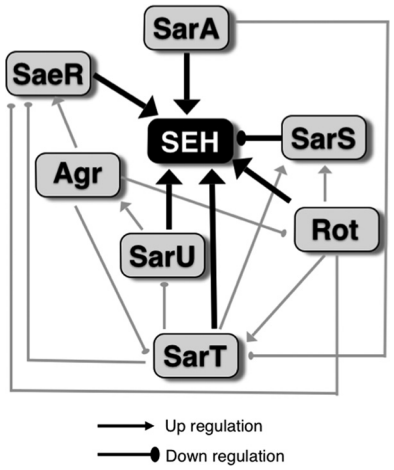

FIG 3 Regulatory pathway of SEH. Arrowheads, upregulation; circle heads, downregulation; black lines, found in this study; gray lines, found in previous studies (22, 25, 28–32, 47).

This suggests Rot-Agr interaction does not directly interfere with these cases of expression, unlike the other SEs mentioned above. We constructed the mutants from a single strain, no. 10, classified into CC81 subtype 1. We likewise observed similar SEH production in media and meat product with both of the other CC81

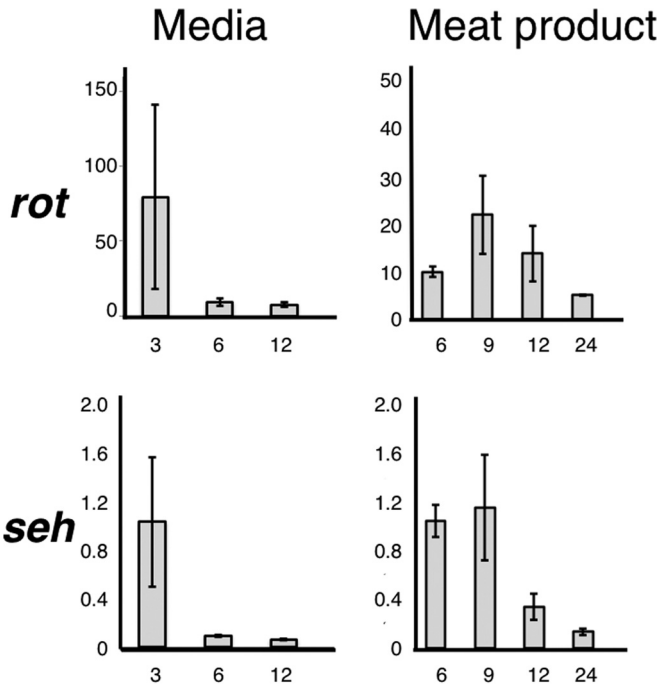

FIG 4 Expression time course of *seh* and *rot* in *S. aureus* SFP clone no. 10. The bacterium was cultured at the indicated times and recovered from the broth culture (or the wash from ham slices) by centrifugation. RNA purification, cDNA synthesis, and subsequent qPCR were performed as described in Materials and Methods. Vertical lines, relative expression (*rot* or *seh* and *gyrB*); horizontal lines, incubation time (hours). Two independent cultures and two independent assays were performed, and a total of four pieces of data per sample were averaged. Standard errors are shown. Correlation coefficients of relative rates (*seh* and *gyrB* as well as *rot* and *gyrB*) were $R^2 = 0.99$ (in media) and $R^2 = 0.44$ (on meat product).

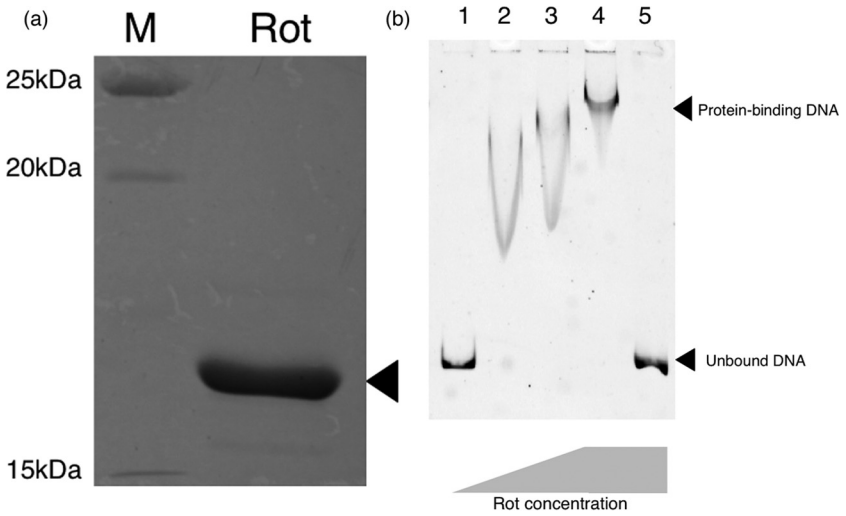

FIG 5 Gel shift assay of *seh* promoter in the presence and absence of Rot. (a) Purified Rot protein. (b) Gel shift assay. EMSA was performed with *seh* promoter sequence DNA probe (50 ng/reaction) incubated with various concentrations of Rot (ng/reaction). Lane 1, 0 ng; lane 2, 1 ng; lane 3, 2 ng; lanes 4 and 5, 10 ng. Nonlabeled DNA probe (with 50-fold more nonlabeled than labeled DNA) was added to lane 5 in the reaction sample. Electrophoresis was carried out with 6% polyacrylamide gel and 0.5× TBE buffer at a 100-V constant voltage for 2.5 h under cold conditions. Subsequently, the gel was imaged by the Molecular Imager FX system (Bio-Rad).

subtype 1 strains (Nagasaki and 01240) and the closely related CC1 lineage (MW2) (Fig. 1). Further, the nucleotide sequences of the Rot and *seh* promoter in no. 10 was the same as those of MW2. Although further investigations are needed, we speculate that an alternative control mechanism for SEH production by Rot is common among these closely related lineages.

Recently, Benson et al. reported Rot binds the promoters of superantigen-like protein (SSL) and activates SSL expression (37). They also suggested Rot contributes to the stabilization of promoters and then may aid the binding of SaeR to the promoters. In this study, a similar phenomenon was observed. There was a positive correlation of mRNA expression between SEH and Rot under both conditions (Fig. 4), and the data suggest Rot regulates SEH production at the transcriptional level and was essential for expression of *seh*. Additionally, Rot and SaeR both activated SEH production, similar to a previous study (37). We analyzed the secondary structure of the *seh* promoter sequence. As shown in Fig. 6a, part of the sequence includes putative Shine-Dalgarno (SD), −10, and −35 sequences that were predicted to form a possible loop-like structure, indicating that loop formation interferes with the access of the RNA polymerase. There are two sequences important for binding of Rot, similar to a previous study (27), at the loop region in this structure, depicted as Rot recognition 1 and 2. Based on the analysis of the *seh* promoter structure, we hypothesized the following mechanism, as shown in Fig. 6b: (i) RNA polymerase cannot bind to the promoter, (ii) Rot unwinds and prevents the formation of the loop structure, and (iii) this leads RNA polymerase to bind to the promoter sequence, and then the transcription begins. Rot may rewind the SEH promoter to stabilize the structure of the promoter, aiding binding of RNA polymerase to −10 and −35 sequences (Fig. 6). The data suggest alternative Rot regulation is crucial for SEH production on (in) foods and in causing SFP.

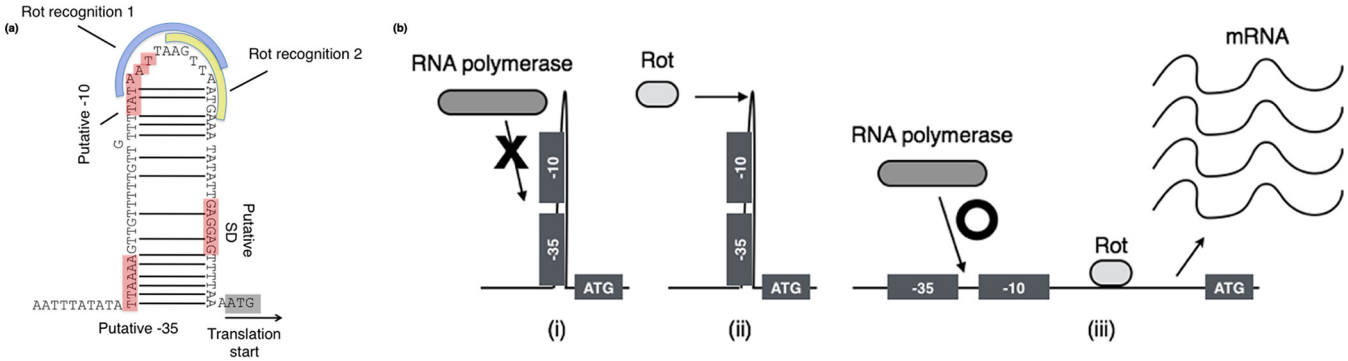

FIG 6 Structure of upstream sequence of *seh* and schematic image of *seh* transcription. (a) Prediction of secondary structure of *seh* promoter region. GENETYX-MAC v.15 (Software Development Co., Ltd., Tokyo, Japan) was used for analysis. The loop-like structure of the promoter sequence is shown. The sequence of no. 10 was identical to that of MW2. −35, −10, and SD sequences and transcription start codons (ATG) are boxed. Two nucleotide sequences important for Rot binding are represented by curved bars. The sequences are similar to those in a previous study (27), but orientation and order differed. (b) Hypothetical interaction scheme of Rot and the promoter sequence of *seh*. Topological images are shown. (i) The hairpin-like structure inhibits binding of RNA polymerase. (ii) Rot binds to free non-base-pairing regions. (iii) Structure rewinds. RNA polymerase is able to bind the promoter sequence.

Vomiting activity associated with SEs has been investigated using animal models. SEH was reported to have relatively lower vomiting-inducing activity than others against *Suncus murinus* but similar to that of SEA against the primates (2). Evenson et al. estimated that the minimum amount of SEA for an SFP outbreak is 144 ng/person (38). Assuming that the vomiting activity caused by SEH is comparable to that associated with SEA in humans, consumption of about 5 to 15 g meat product incubated for 24 h with all four *seh*-positive strains used in this study is enough to cause SFP symptoms. All strains positive for *seh* in this study except MW2 were classified into CC81, which is the major SFP lineage in Japan, as described previously (6, 39). We claim that this clone has significant potential to cause SFP.

As described above, the large SFP outbreak in Japan was caused by SEA and SEH. Other than Japan, articles reported there were outbreaks caused by *S. aureus* positive for the *seh* gene in France, Netherlands, China, Germany, and South Korea (3, 9, 10, 40, 41). Among these, the epidemiological study in South Korea showed *S. aureus* strains positive for *seh* were one of the dominant groups. SEH-producing strains belonging to CC81 subtype 1 or related clones may cause frequent SFP outbreaks in far-east Asia. Further epidemiological studies on this important SFP lineage may be necessary to show the spread of the clone positive for *seh*.

In this study, we demonstrated that a novel positive upregulation mechanism of SEH production by Rot may be one of the virulence mechanisms contributing to the highly frequent onset of SFP outbreaks caused by CC81 subtype 1. Our conclusion has some limits due to a single experimental condition mimicking food contamination using salted ham; however, our data show we can claim SEH and SEA are important SEs. The relationships between newly described SEs and SFP are not fully understood, and further studies are necessary to evaluate potential risks of the newly described SEs in SFP.

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