FULL PAPER *Public Health*

Quantitative Analysis of *Staphylococcus aureus* in Skimmed Milk Powder by Real-Time PCR

Tetsuya IKEDA[1)], Naoto TAMATE[2)], Keiji YAMAGUCHI[1)] and Sou-ichi MAKINO[3)]

[1)]Hokkaido Institute of Public Health, Sapporo, Hokkaido 060–0819, [2)]Kucchan Public Health Center, Hokkaido 044–0001 and [3)]Department of Applied Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan

(Received 12 April 2005/Accepted 28 June 2005)

ABSTRACT. A large-scale outbreak of food poisoning caused by consumption of skimmed milk powder contaminated with staphylococcal enterotoxin A (SEA) occurred in Japan. No viable *Staphylococcus aureus* was detected in the skimmed milk powder, however, *sea* and *nuc* genes of *S. aureus* were detected in it by PCR. The number of *S. aureus* in skimmed milk powder was estimated by quantitative real-time PCR.

KEY WORDS: enterotoxin, food poisoning, real-time PCR, skimmed milk, *Staphylococcus aureus*.

J. Vet. Med. Sci. 67(10): 1037–1041, 2005

A mass outbreak of food poisoning caused by consumption of reconstituted milk, contaminated with staphylococcal enterotoxin (SE), occurred in Osaka, Japan, in June 2000, and more than 10,000 cases were reported [13]. Although the source implicated was reconstituted milk manufactured by company A. The raw material for this reconstituted milk was skimmed milk powder produced at a plant of the same company in Hokkaido in April 2000. In this episode, the presence and proliferation of *Staphylococcus aureus* in milk used to produce the skimmed milk powder were due to a malfunction in the temperature control system [13]. Although *S. aureus* was killed by heating, active SE remained in the milk as SE is heat resistant [4]. *Staphylococcus aureus* was not detected in the implicated foods by ordinary microbiological examinations, but SE was detected in concentrations of 0.05–1.6 *n*g/m*l* of the reconstituted milk by reversed passive latex agglutination (RPLA) and enzyme-linked immunosorbent assay (ELISA) [13].

Although 18 major antigenic types of SEs or SE-like putative toxins have been reported, SEA is the major cause of food poisoning [1, 6, 10, 11, 14, 16–21]. In an outbreak caused by SEA present in chocolate milk in the United States, twelve cartons each containing half pint (approx 0.28 l) contained SEA at a concentration of 94–184 *n*g, and SEA of 200 *n*g or less was presumed to have caused food poisoning [3]. However, the minimal dose of SEA that causes gastroenteritis is not known.

When a small number of *S. aureus* in present in food, rapid detection by ordinary methods is difficult. Therefore, PCR with primers designed from the DNA sequence of the *nuc* gene has been developed, and it is capable of detecting *S. aureus* in food even in low numbers [2]. However, estimating the bacterial count in foods by the normal PCR technique is not possible. Recently, real-time PCR has been developed and made it possible to detect bacteria and to quantitatively count the bacteria [9, 12, 15]. Therefore, the number of *S. aureus* cells in skimmed milk powder could be counted by real-time PCR if staphylococcal DNA is recovered from it.

MATERIALS AND METHODS

Skimmed milk powder having caused food poisoning: Ten batches of skimmed milk powders manufactured at the factory of company A on April 1, 2000 were the raw materials used to produce the food implicated in the outbreak of food poisoning, which occurred despite the heat treatment at 130°C for 3 sec. They were designated SM1, SM100, SM200, SM300, SM400, SM500, SM600, SM700, SM800 and SM830 in the order of the manufacturing time. The number of bacteria/m*l* of the reconstituted skimmed milk (10% w/v) was measured by standard plate count using Standard Method Agar (Nissui Co., Tokyo, Japan) [8]. The reconstituted skimmed milk (0.1 m*l*) was spread on mannitol salt agar (Oxoid Ltd., Basingstoke, England) containing egg yolk emulation (Oxoid), to count the number of *S. aureus*, by incubation for 48 hr at 37°C [8].

Strains and inoculated skimmed milk powder: *Staphylococcus aureus* strains No. 35, No. 39, 98–2–7, 52–1–2 and SEA100 were used. Strains No. 35 and No. 39 were kindly provided by Iwate University, strain 98–2–7 from Fukuoka Institute of Health and Environmental Sciences, and strain SEA100 from Tokyo Metropolitan Research Laboratory of Public Health. Strain 52–1–2 had been isolated from a patient with atopic dermatitis. These strains were cultured in Trypto-Soya broth (TSB; Nissui) for 24 hr at 37°C under shaker conditions and killed by boiling for 10 min. The CFUs of *S. aureus* were measured on nutrient agar (Oxoid) by incubation for 24 hr at 37°C. To obtain *S. aureus*-contaminated skimmed milk powder for experimental purposes killed *S. aureus* cells were added to skimmed milk powder, which was prepared in a usual way under strict temperature control in the factory of company A.

DNA extraction: Skimmed milk reconstituted (10% w/v)

in sterile water was centrifuged at 12,000 rpm for 3 min, and subsequently, DNA was extracted by using a DNeasy tissue kit (Qiagen GmbH, Hilde, Germany). DNA was purified from *S. aureus* cultured in TSB by using the DNeasy tissue kit.

PCR: The primers nuc-1 (5'-GCGATTGATGGTGATACGGTT-3') and nuc-2 (5'-AGCCAAGCCTTGACGAACTAAAGC-3') were used in this study to amplify a 267-bp fragment of *nuc* gene [2] specific to *S. aureus*, SEA-1 (5'-TTGGAAACGGTTAAAACGAA-3') and SEA-2 (5'-GAACCTTCCCATCAAAAACA-3') to amplify a 120-bp fragment of *sea* gene [7], essential for SEA production. Each 20-μl PCR mixture contained 2 μl of template DNA, 0.2 μM of each primer, and 0.5 U EX *Taq* (TAKARA BIO Inc, Shiga, Japan). Thirty-five amplification cycles (94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec) were performed in a GeneAmp PCR system 9700 (Perkin Elmer, Inc., Foster City, Calif.). To examine the detection limit of the *sea* gene by PCR using both SEA-1 and SEA-2 primers, 10-fold serial dilutions of the DNA extracted from each strain were prepared and PCR was performed with each dilution.

Real-time PCR: Real-time PCR was performed by using a TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, Calif.). Each 50 μl of PCR mixture contained 1 μl of template DNA, 1 μM of each primer (SEA-1 and SEA-2) and 100 nM of fluorogenic probe designated SaA (5' FAM-AATGTAACTGTTCAGGAGTTGGATCTTCAAG-CAAGA- TAMRA 3'). The following PCR cycle was used: 1 × 50°C for 2 min; 1 × 95°C for 2 min; 40 × (95°C for 15 sec followed by 60°C for 1 min) by using an ABI PRISM 7700 Sequence Detection System (Perkin Elmer). The data of real-time PCR were analyzed by the sequence detector software version 1.6.3 (Perkin Elmer). A standard curve for real-time PCR was established for serial dilutions of DNA purified from each strain cultivated in TSB, in which their CFU was measured. The sequence detector software produced a standard curve, which was then used to calculate the precise quantities of the starting template molecules for unknown sample.

ELISA: Skimmed milk powder was analyzed by using a commercial immunoassay kit Transia plate (Diffchamb, Lyon, France). The assay was carried out in accordance with the manufacturer's instructions. Briefly, skimmed milk reconstituted in PBS was used as sample. Highly purified SEA (AT101, Toxin Technology, Sarasota, Florida) was used as a standard to generate a standard curve.

RPLA: Skimmed milk powder was analyzed by using commercial immunoassay kit SET-RPLA (Denka Seiken Co., Ltd., Tokyo). First, 57% (w/v) skimmed milk reconstituted in PBS was adjusted to pH 3.8 by addition of HCl and centrifuged at 3,000 rpm for 20 min. Second, supernatant was adjusted to pH 7.0 by addition of NaOH and centrifuged at 3,000 rpm for 20 min. The supernatant was used as sample and highly purified SEA (AT101, Toxin Technology) was as a standard. Samples and standards were processed according to the manufacturer's instructions.

RESULTS

Staphylococcus aureus was not isolated on mannitol salt agar with egg yolk added, because the samples had been pasteurized. SM600 and SM700 showed possible non-*S. aureus* contamination by standard plate count (Table 1). To examine whether the dead bacterial cells in all samples were *S. aureus* cells, the total DNA in each sample was isolated and PCR was performed by using primer sets to amplify *sea* [7] and *nuc* [2] genes. *nuc* and *sea* genes were amplified from nine samples but not from SM1 (Fig. 1), thus indicating that these skimmed milk powder samples contained dead *S. aureus* and that the total DNA for PCR could be extracted from the dead cells even after ultra high temperature pasteurization. Therefore, the number of SEA-producing *S. aureus* cells in these skimmed milk samples could be estimated if the copy numbers of *sea* gene in the samples was estimated by PCR. The amounts of SEA in the 10

Table 1. Number of *S. aureus*, *sea* genes, SPC[a] and the amount of SEA in each sample of skimmed milk powder that caused food poisoning

Sample no.	Date of manufacture	SPC (CFU/g)	Number of *S. aureus* (CFU/g)	Number of *sea* genes as determined by real-time PCR (copy/g)	PCR		Amount of SEA	
					sea	*nuc*	ELISA (*n*g/g)	RPLA (*n*g/g)
SM1	2000.4.1	<300	<50	$<1.6 \times 10^4$	–	–	<2.0	<2.2
SM100	2000.4.1	<300	<50	2.7×10^7	+	+	2.9	2.2
SM200	2000.4.1	<300	<50	5.8×10^6	+	+	2.0	2.2
SM300	2000.4.1	<300	<50	1.2×10^6	+	+	<2.0	<2.2
SM400	2000.4.1	<300	<50	1.2×10^5	+	+	<2.0	<2.2
SM500	2000.4.1	<300	<50	2.3×10^4	+	+	<2.0	<2.2
SM600	2000.4.1	6.2×10^3	<50	4.7×10^7	+	+	20.8	17.5
SM700	2000.4.1	4.0×10^2	<50	5.2×10^7	+	+	8.5	8.8
SM800	2000.4.1	<300	<50	1.2×10^7	+	+	6.4	4.4
SM830	2000.4.1	<300	<50	4.3×10^7	+	+	4.3	4.4

a) Standard plate counts.

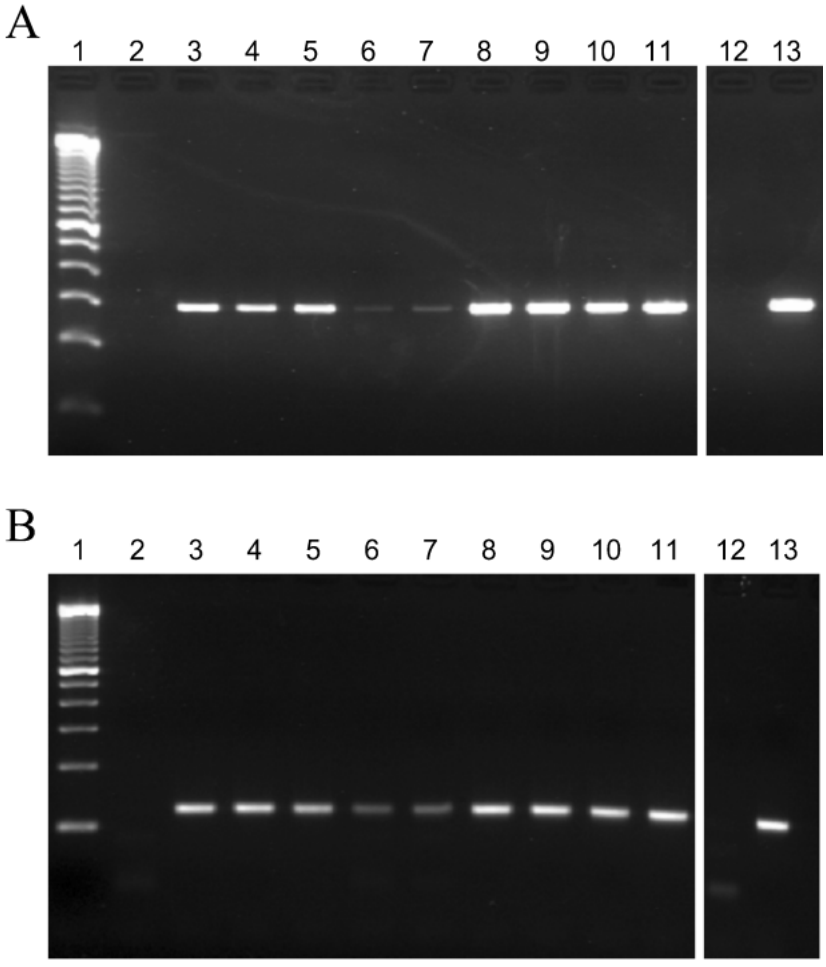

Fig. 1. Detection of *sea* and *nuc* genes in skimmed milk powder by PCR. (A) Detection of *nuc* gene. (B) Detection of *sea* gene. Lane 1, 100 bp size maker; lane 2, SM1; lane 3, SM100; lane 4, SM200; lane 5, SM300; lane 6, SM400; lane 7, SM500; lane 8, SM600; lane 9, SM700; lane 10, SM800; lane 11, SM830; lane 12, negative control; lane 13, positive control.

skimmed milk powder samples were also measured by ELISA and RPLA; no SEA was detected in four samples (Table 1).

Five *S. aureus* strains were cultivated in TSB and their CFUs were measured. Each bacterial culture was boiled to kill the cells and artificially contaminated with an SEA-negative 10% skimmed milk suspension of the dead cells. *sea* gene was detected by PCR using both SEA-1 and SEA-2 primers as the number of *S. aureus* added to 10% skim milk suspension was more than 1.4×10^5–1.5×10^6/m*l*. The number of fluorescence signals dependent on the copy number of *sea* gene was then detected by the real-time PCR by using the primer and probe sets for *sea* gene. Each standard curve was established from the relationship between the fluorescence signal and bacterial counts. By using these standard curves, the bacterial counts were estimated in the pasteurized skimmed milk powder samples. The number of heat-killed bacterial cells was estimated at approximately 30% as compared to live cells in skimmed milk powder (Tables 2 and 3).

To estimate the bacterial count in nine *Staphylococcus*-positive samples listed in Table 1, real-time PCR was performed. DNA extracted from strain No. 39 was used as a standard in this experiment. From the standard curve, the copy number of *sea* gene in each sample was measured by the fluorescent signals; the number of *sea* genes was 2.3×10^4–5.2×10^7 copies/*g* in these skimmed milk powder samples (Table 1).

DISCUSSION

It was believed that the outbreak of food poisoning in Osaka in 2000 was due to SEA in the skimmed milk powder [13]. Recently, it was confirmed that the food poisoning was caused by SEA and SEH [5]. However, it was difficult to detect SEA and SEH by ELISA in all skimmed milk pow-

Table 2. Measurement of *sea* gene by real-time PCR in skimmed milk powder with added dead bacteria

Strain	Number of *sea* genes as determined by real-time PCR (/*g*)[a,b)]	Number of dead *S. aureus* added (cell/*g*)	Real-time PCR/added dead *S. aureus*
No. 39	4.4×10^8	1.5×10^9	28.4%
98–2–7	4.7×10^8	1.3×10^9	35.0%
No.35	5.7×10^8	8.8×10^8	64.9%
SEA100	2.1×10^7	1.7×10^8	12.7%
52–1–2	9.0×10^8	3.2×10^9	28.4%
			Average 33.9%

a) DNA extracted from the viable strain No. 39 in TSB was used as a standard.
b) DNA was extracted from the strain resuspended in 10% skimmed milk for real-time PCR.

Table 3. Effect of the concentration of the dead strain No. 39 added in real-time PCR

Number of *sea* genes by real-time PCR (/*g*)[a,b)]	Number of added *S. aureus* (cells/*g*)	Number of *sea* genes/Number of added *S. aureus*
2.1×10^9	1.1×10^{10}	18.8%
4.4×10^8	1.5×10^9	28.4%
1.3×10^7	1.1×10^8	11.1%
2.3×10^5	1.1×10^6	20.4%

a) DNA extracted from the viable strain No. 39 in TSB was used as a standard.
b) DNA was extracted from the strain resuspended in 10% skimmed milk for real-time PCR.

der samples because they were present only in small amounts and viable *S. aureus* was undetectable in the implicated skimmed milk powder samples because of the heat treatment. Thus, PCR was used to amplify *sea* gene instead of detecting SEA in skimmed milk powder; *sea* gene was detected in nine skimmed milk powder samples, including SM300, SM400 and SM500, in which SEA was not detected by using ELISA. Thus, detecting *sea* gene was useful as an index of SEA contamination in dairy products such as reconstituted milk and skimmed milk powder.

DNA was extracted from skimmed milk powder containing heat-killed *S. aureus* cells. The number of *S. aureus* estimated by real-time PCR was not equal to that added to skimmed milk. The recovery rate of each of the five strains was 12.6%–64.9% (33.9% on average). These results indicate that inspecting isolates of *S. aureus* from skimmed milk powder is necessary to determine the recovery rate and to correctly count the CFUs by real-time PCR. However, even if skimmed milk powder is contaminated by *S. aureus*, as was the case in Osaka food poisoning, it should have already been killed and may not have been isolated. When *S. aureus* is not isolated nor the recovery rate is unknown, by producing a recovery rate of approximately 30%, it is possible to calculate the approximate number of *S. aureus*; the average recovery rate of the five strains was approximately 30% in this study. No significant change was observed in the recovery rate of *S. aureus* even if the number of *S. aureus* in skimmed milk powder changed. In brief, real-time PCR is used to a large extent for comparing the number of *S. aureus* among different skimmed milk powder samples when they are contaminated with the same strain, as was the case in the Osaka food poisoning incident.

Although real-time PCR was used for 10 skimmed milk powder samples that were believed to be the source of food poisoning, no *sea* gene was detected in SM1, which showed that the contamination of SEA in milk occurred after manufacturing processes of SM1. *Staphylococcus aureus* multiplied and produced toxins during the manufacturing of SM1 and SM100, but the *S. aureus* cell number gradually decreased during the manufacturing process of SM100 and SM500. Further, *S. aureus* cell numbers increased and produced toxins during the manufacturing process of SM500 and SM600. These results indicated that *S. aureus* might have increased and contaminated skimmed milk powder twice during the manufacturing process. The manufacturing process might have malfunctioned during manufacturing SM1 and SM100, and also SM500 and SM600.

Skimmed milk powder containing SEA was carried into the Hazard Analysis and Critical Control Points (HACCP) system in the factory, which produced reconstituted milk from this skimmed milk powder without detecting the contamination by SEA. Methods that rapidly evaluate the safety of skimmed milk powder are necessary to prevent food poisoning. The method described in this study is rapid, supersensitive and can quantitatively confirm the contamination by *S. aureus*. This method can be used to count viable and dead *S. aureus* cells. We believe that this method is very effective in estimating the source of food poisoning

and carrying out risk assessment of foods.

ACKNOWLEDGMENTS. We are grateful to Kunihiro Shinagawa and Miyoko Endoh for providing the *S. aureus* strains used in this study.

REFERENCES

1. Bergdoll, M. S. 1983. Enterotoxins, pp. 559–598. *In*: Staphylococci and Staphylococcal Infections, (Easton, C. S. F. and Adlam, C. eds.), Academic Press, London, United Kingdom.
2. Brakstad, O. G., Aasbakk, K. and Maeland, J. A. 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. *J. Clin. Microbiol.* **30**: 1654–1660.
3. Evenson, M. L., Hinds, M. W., Bernstein, R. S. and Bergdoll, M. S. 1988. Estimation of human dose of staphylococcal enterotoxin A from a large outbreak of staphylococcal food poisoning involving chocolate milk. *Int. J. Food. Microbiol.* **31**: 311–316.
4. Hilker, J. S., Heilman, W. R., Tan, P. L., Denny, C. B. and Bohrer, C. W. 1968. Heat inactivation of enterotoxin A from *Staphylococcus aureus* in veronal buffer. *Appl. Microbiol.* **16**: 308–310.
5. Ikeda, T., Tamate, N., Yamaguchi, K. and Makino, S. 2005. A mass outbreak of food poisoning disease caused by small amounts of SEA and SEH. *Appl. Environ. Microbiol.* **71**: 2793–2795.
6. Jarraud, S., Peyrat, M. A., Lim, A., Tristan, A., Bes, M., Mougel, C., Etienne, J., Vandenesch, F., Bonneville, M. and Lina, G. 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J. Immunol.* **166**: 669–677.
7. Johnson, W. M., Tyler, S. D., Ewan, E. P., Ashton, F. E., Pollard, D. R. and Rozee, K. R. 1991. Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. *J. Clin. Microbiol*. **29**: 426–430.
8. Japan Food Hygiene Association. 1990. Standard Methods of Analysis in Food Safety Regulation Biology.
9. Keya, S. 2000. Rapid identification of *Yersinia enterocolitica* in blood by the 5' nuclease PCR assay. *J. Clin. Microbiol.* **38**: 1953–1958.
10. Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J. Q., Ito, T., Kanamori, U., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N. K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K,. Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H. and Hiramatsu, K. 2001. Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet* **357**: 1225–1240.
11. Letertre, C., Perelle, S., Dilasser, F. and Fach, P. 2003. Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus*. *J. Appl. Microbiol.* **95**: 38–43.
12. Lyons, S. R., Griffen, A. L. and. Leys, E. J. 2000. Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. *J. Clin. Microbiol.* **38**: 2362–2365.
13. Ministry of Health and Welfare and Osaka City. 2001. A massive outbreak of *Staphylococcus aureus* enterotoxin A associated with milk product. *Food Sanit. Res*. **51**: 17–36.
14. Munson, S. H., Tremaine, M. T., Beteley, M. J. and Welch, R. A. 1998. Identification and characterization of staphylococcal enterotoxin type G and I from *Staphylococcus aureus*. *Immunology*. **66**: 3337–3348.
15. Oberst, R. D., Hays, M. P., Bohra, L. K., Phebus, R. K., Yamashiro, C. T., Paszko-Kolva, C., Flood, S. J. A., Sargeant, J. M. and Gillespie, J. R. 1998. PCR-Based DNA amplification and presumptive detection of *Escherichia coli* O157:H7 with an internal fluorogenic probe and the 5' nuclease (TaqMan) assay. *Appl. Environ. Microbiol.* **64**: 3389–3396.
16. Omoe, K., Hu ,D.L., Takahashi-Omoe, H., Nakane, A. and Shinagawa, K. 2003. Identification and characterization of a new staphylococcal enterotoxin-related putative toxin encoded by two kinds of plasmids. *Infect. Immun.* **71**: 6088–6094.
17. Orwin, P. M., Leung, D. Y. M., Donahue, H. L., Novick, R. P. and Schlievert, P. M. 2001. Biochemical and biological properties of staphylococcal enterotoxin K. *Infect. Immun.* **69**: 360–366.
18. Ren, K., Bannan, J. D., Pancholi, V., Cheung, A. L., Robbins, J. C., Fischetti, V. A. and Zabriskie, J. B. 1994. Characterization and biological properties of a new staphylococcal enterotoxin. *J. Exp. Med.* **180**: 1675–1683.
19. Su, Y. C. and Wong, A. C. L. 1995. Identification and purification of a new staphylococcal enterotoxin, H. *Appl. Environ. Microbiol.* **61**: 1438–1443.
20. Yarwood, J. M., McCormick, J. K., Paustian, M. L., Orwin, P. M., Kapur, V. and Schlievert, P. M. 2002. Characterization and expression analysis of *Staphylococcus aureus* pathogenicity island 3. *J. Biol. Chem.* **277:** 13138–13147.
21. Zhang, S., Iandolo, J. J, and Stewart, G. C. 1998. The enterotoxin D plasmid of *Staphylococcus aureus* encodes a second enterotoxin determinant (*sej*). *FEMS Microbiol. Lett.* **168**: 227–233.