Characteristics of Staphylococcal Enterotoxin A Production and Growth of Staphylococcus aureus in Shaking and Stationary Cultures

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Staphylococcal food poisoning, which is still a serious health problem worldwide, is caused by staphylococcal enterotoxin (SE). Among many types of SE, staphylococcal enterotoxin type A (SEA) is known to be the most responsible for staphylococcal food poisoning. Production by SEA-producing strains in shaking culture has been studied by many investigators, whereas the kinetical differences between shaking and stationary cultures have not been studied intensively. Therefore, this difference was studied at various temperatures from 14°C to 46°C in the present study. Consequently the maximum SEA concentration and populations of SEA producer in shaking culture was higher than those in stationary culture. Of interest, however, the productivity, which is the maximum SEA amount produced by one cell, in shaking cultures was lower than that in stationary culture. Kinetic analysis clarified that SEA gene expression in staphylococcal cells preceded toxin production at optimal temperature. Next, several SEA producers were studied for the maximum toxin production at various temperatures from 14°C to 42°C in shaking culture. Consequently all strains showed different patterns, suggesting that the characteristics of SEA production of these strains would be strain-specific. These results in this study would provide useful, basic information to prevent staphylococcal food poisoning outbreaks.

Key words: Kinetics / Staphylococcal enterotoxin A / Staphylococcus aureus / Growth / Shaking and stationary cultures.

INTRODUCTION

Staphylococcus aureus food poisoning outbreaks are still a serious health problem worldwide (Asao et al., 2003; Schelin et al., 2011). Staphylococcal food poisoning is caused by staphylococcal enterotoxin (SE). To date, more than 20 SEs or SE-like proteins have been reported (Schelin et al., 2011; Hu and Nakane, 2014). Among these SEs or SE-like proteins, staphylococcal enterotoxin type A (SEA) is known as the most widely responsible for staphylococcal food poisoning outbreaks (Huang et al. 1987). SEA is also encoded by a bacteriophage (Betley and Mekalanos, 1985). It is well known that temperature is one of the most influential environmental factors for SE production by S. aureus as well as pH, water activity, atmospheric conditions, and salt level (Bergdoll, 1979). In addition, toxin production varies among staphylococcal strains and the SE type (Czop and Bergdroll, 1974; Otero et al., 1990). The optimal temperature for SE production might be also vary according to the strain, culture medium, culture with or without shaking, and other factors.

The detection method for SE may affect the results of quantitative measurements of the SE concentration of staphylococcal culture incubated in various conditions.
Previously SE had been detected using traditional assays such as the Ouchterlony plate technique and the double-gel-diffusion technique (Bergdoll, 1979). Woodburn et al. (1973) described that SEA production by strain S-6 with shaking culture at 37°C was more rapid than that without shaking. However, the toxin assay they used at that time was not quantitative; they determined SEA production from the positive sample number using the double-gel-diffusion test (Woodburn et al., 1973). Later, new technologies that have advanced in sensitivity and selectivity, such as fluoroimmunoassays, have been developed (Park et al., 1994; Bennett, 1995). These new technologies can provide quantitative results of the SE amount in samples using a standard curve for SE (Fujikawa and Morozumi, 2006; Sabike et al., 2014). On the other hand, most of the traditional SE assays are semi-quantitative, which give the quantity of SE with their sensitivities and the highest positive dilution of a sample.

Recently, Cao et al. (2012) divided SEA-producing strains into three groups: high, medium, and low SEA producers, based on the amount of SEA produced in a shaking culture at 37°C. Then they found that the difference in SEA production was associated with the expression level of the SEA gene, sea, encoded on a phage (Cao et al., 2012). S. aureus is a facultative aerobe. Thus, S. aureus cells in shaking culture would grow mainly by respiration with oxygen, whereas cells in stationary culture would grow mainly by fermentation without oxygen. Tsutsuura et al. (2013) also studied the temperature dependence of SEA production by S. aureus in shaking culture. The kinetic differences in SEA production and cell growth of S. aureus between shaking and stationary cultures have not been studied intensively thus far.

Therefore, in the present study, we first investigated the characteristics of SEA production and growth of a S. aureus strain at various temperatures in shaking and stationary cultures. The strain used was strain 11658, which had been studied intensively for its SEA production in pasteurized milk or cheese curd during a cheese-making process (Aoyama et al., 2008). Namely, in this study, the difference between shaking and stationary cultures was analyzed in terms of SEA production and growth of the SEA producer in broth at various temperatures. The toxin amounts of culture samples were detected quantitatively using the fluoroimmunoassay described above. Furthermore, the kinetics of the expression level of sea in S. aureus cells was studied in relation to SEA production. Secondly, SEA production by several SEA producers was studied at various temperatures to characterize the toxin production of these strains.

MATERIALS AND METHODS

S. aureus strains

S. aureus strains 11658 (Aoyama et al., 2008) and 11727 were kindly provided by Prof. M. Sugai and Dr. Y. Sato of Hiroshima University, Japan. Strain 13008 was also kindly provided by Dr. A. Hira of Tokyo Metropolitan Institute of Public Health, Japan. Strains f4 and St.1 were isolated from humans in our laboratory. These strains were all SEA producers and did not produce any other classical staphylococcal enterotoxin types B to E, as determined using a reversed passive latex agglutination test (Denka Seiken, Tokyo, Japan).

Culture

Bacterial strains were grown on Baird-Parker agar plates (Oxoid, Hants, UK) at 37°C for 2 days. Some well-grown colonies were then inoculated into brain heart infusion (BHI) broth (Becton, Dickinson and Company, Sparks, USA) and then cultured with shaking at 110 strokes/min and 37°C for 1 day on a shaker (MK161, Yamato, Tokyo, Japan). Cells were washed with saline (0.85% NaCl solution) by centrifugation at 15,000 × g for 15 min. Cells were thoroughly suspended in saline and then diluted to 10^6 CFU/ml with saline. The cell suspension was inoculated into BHI broth at a ratio of 1:100 in volume to give a final concentration of 10^3 CFU/ml and then dispensed into test tubes in 8 ml aliquots. The test tubes were stored with shaking at 110 strokes/min or without shaking at given temperatures in an incubator for certain periods. For each condition, three independent experiments were performed with one sample per experiment.

SEA measurement

The SEA concentrations in the supernatants of cultured samples were measured using an enzyme-linked fluorescence assay, VIDAS Staph enterotoxin II (bioMerieux, Marcy-l’Etoile, France) using a mini-VIDAS automated system. The supernatants of the samples were obtained by centrifugation at 15,000 × g for 15 min. The toxin estimation was done with a standard curve obtained with purified SEA (Fujikawa and Morozumi 2006; Sabike et al. 2014). Purified SEA was kindly provided by Denka Seiken Co. (Tokyo, Japan).

Cell counting

Viable cell counts of samples were measured using a plating method. Namely, samples were serially diluted 10-fold with saline, and then 0.1 ml portions of dilution were plated on nutrient or standard agar plates (two plates per dilution) (Nissui Pharmaceuticals, Tokyo, Japan). After incubation at 37°C for 2 days, the colonies on the agar plates were counted, and the mean popula-
The total RNA concentration was confirmed using a real-time PCR (Miyazaki et al., 2008; Wong and Medrano, 2005).

(i) RNA was extracted from cells using a modification of the method by Lövenklev et al. (2004). Briefly, cells were collected from 2 ml of cell suspension cultured as described above by centrifugation and subsequently phenol and glass beads (acid-washed, $diameter \leq 106 \mu m$) were added into the cells to homogenize them. Other steps in RNA extraction were conducted in accordance with the manufacturer’s protocol (TaKaRa, NIPPON GENE, Toyama, Japan). After RNA extraction, DNase treatment with DNase I (TaKaRa Bio, Kusatsu, Japan) was conducted to purify the samples for measurement. The total RNA concentration was confirmed using a Qubit assay (Life Technology, Eugene, USA). The samples were then stored at -80°C until use for cDNA construction.

(ii) First-strand cDNA was synthesized in two separate reverse transcription assays using the reverse primers for sea and gyrB reference gene in a final volume of 20 µl in accordance with the manufacturer’s protocol (TaKaRa Bio). The reverse transcription reaction for each sample was performed using a thermal cycler (TaKaRa Bio).

(iii) Primers and probe for the sea gene were newly designed using Primer Quest software on the basis of consensus sequences constructed from multiple alignment results of 41 strains of S. aureus sea gene sequences deposited in the NCBI database (Integrated DNA Technologies, Iowa, USA) as reported by Masuda et al. (2015). The primers and probe for sea developed in this study were:

Forward primer: 5'-CGGTiTTCTGACTCTTCTCACGAAACGCGG-3'  
Probe: 5'-CGTGGTTCAATCTACGACTGACT-3'  
Reverse primer: 5'-CGTGTTCTGACCACCCTAATAA-3'.

To normalize the expression level of the sea gene, the mRNA of gyrB gene was also quantified using the primers and probe reported by Voyich et al. (2016):

Forward primer: 5'-CAAATGATCAGCTACATTGATTA-3'  
Probe: 5'-AATCGGTGCGGACTTTGATCTCAGCAGAAAG-3'  
Reverse primer: 5'-CGGCATCGTCTATAATGACCG-3'

All probes were labeled with the fluorescent reporter dye FAM (6-Carboxyfluorescein) at the 5’ end and with the fluorescent quencher dye TAMRA (6-Carboxytetramethylrhodamine) at the 3’ end. TaqMan real-time PCR was performed by mixing of 12.5 µl of TaKaRa premix Ex Taq (TaKaRa Bio), 2 µl of diluted cDNA, a primer-probe mixture (4 µl and 2 µl of mixture were added for sea assay and gyrB assay, respectively), and nuclease-free water to a final volume of 25 µl. Nuclease-free water and genomic DNA extracted from S. aureus were used as negative and positive controls, respectively. Gene expression was measured using a real time thermal cycler (TP800, TaKaRa Bio), and the thermal conditions were as follows: the first denaturation step was 1 cycle at 95°C for 30 sec, and then monitored 40 cycles by holding at 95°C for 5 sec and 63°C for 30 sec. The relative quantification of the sea and gyrB genes was calculated with each standard curve in accordance with the manufacturer’s protocol (TaKaRa Bio).

Data analysis.

The average and standard error at data points were calculated for three independent experiments. The averages of growth and SEA production at data points were analyzed using an extended logistic model (Fujikawa et al., 2004; Fujikawa and Morozumi, 2006). The relationship between SEA production (or microbial population) and temperature was analyzed using Spearman’s rank correlation method (Tsutsuura et al., 2013) with statistical analysis software, R.

RESULTS AND DISCUSSION

SEA production and growth of strain 11658 at various temperatures

Strain 11658 was studied as an example of an SEA producer to determine its characteristics in toxin production at various temperatures ranging from 14°C to 46°C in shaking and stationary cultures. The time points when this strain produced SEA at maximum concentrations in shaking and stationary cultures at these temperatures are shown in Table 1. Toxin production reached its maximum fastest at 30°C in both the shaking and stationary cultures. No staphylococcal growth or subsequent SEA production were observed at 46°C in both culture conditions. The pattern of the maximum SEA concentration of the strain in the shaking culture at various temperatures showed an upwardly convex shape with a peak at 30°C (Fig.1A), showing that the optimal temperature for SEA production by this strain was 30°C. In the stationary culture, the maximum SEA concentrations at these temperatures were much lower than those in shaking cultures and did not have a peak (Fig.1A). Of interest, the maximum toxin concentration in stationary cultures increased gradually with temperature (Fig.1A).

The cell populations of the culture samples were measured at the times when the maximum toxin concen-
The differences in the maximum concentrations between the two cultures at these temperatures are shown in Table 2. The ratio of shaking culture over stationary culture in SEA production varied from 3.48 to 22.6 by the temperature. The maximum populations in shaking culture were higher than those in stationary culture at all temperatures examined, as shown in Fig. 1B. The differences in maximum population between the two culture methods were almost constant with an average of $9.91 \pm 1.85 \text{ log}$ or $0.99 \pm 0.029$ in log in the temperature range shown in Table 2.

SEA productivity, which was defined as the maximum SEA amount per cell in the present study, was examined between the two culture methods at various temperatures. Namely, toxin productivity (ng/CFU) was calculated by dividing the maximum SEA concentration (ng/ml) by the maximum population (CFU/ml), the results of which are presented in Fig. 1. Toxin productivity was generally higher at higher temperatures over 30°C for both culture methods (Table 3). This increase was more remarkable in the stationary culture than in the shaking culture. Thus, the toxin productivity in the stationary culture was higher than that in the shaking culture at higher temperatures from 26°C to 42°C, as shown by ratios of $<1$ in Table 3. This could come from the fact that toxin productivity was strongly affected by the population. Namely, a difference of a factor of ten (one log) in the population was observed between the two culture methods, whereas the difference in SEA production was not so great, especially at higher temperatures (Fig. 1).

SEA production, expression of sea gene, and growth of strain 11658 in shaking and stationary cultures at the optimal temperature.

The kinetics of SEA production and growth of strain 11658 at the optimal temperature (30°C) were further studied for both culture methods. Microbial growth in the shaking culture was similar to that in the stationary culture (Fig. 2A). Namely, the periods of the lag phase for the two cultures were very short and close to each other, being 1.6 h and 1.4 h in shaking and stationary cultures, respectively. The rate constants of growth, which are the slopes of the log phase, for the two culture methods were also similar, at 1.1 (1/h) and 1.2 (1/h) in shaking and stationary cultures, respectively. The only difference between the cultures was observed in the late log and the stationary phases of growth. The maximum population in the stationary phase in the shaking culture was about 1 log CFU/ml higher than that in the stationary culture (Fig. 2A and Table 2). The toxin production curves in the shaking and stationary cultures were both sigmoidal (Fig. 2A).

### Table 1

| Temperature (°C) | 14 | 18 | 22 | 26 | 30 | 34 | 38 | 42 |
|-----------------|----|----|----|----|----|----|----|----|
| Shaking culture | 18 | 6  | 6  | 4  | 2  | 2.6| 2.6| 2.6|
| Stationary culture | 22 | 15 | 8  | 6  | 2.6| 3  | 3  | 3  |

Unit: days.
Compared with cell growth, SEA production was delayed in both cultures; the time differences in the lag period between cell growth and toxin production in the shaking and stationary cultures were both about 12 h. Some quantitative differences were observed between the cultures. SEA production in the shaking culture was more remarkable in the late log phase of cell growth. The rate constant of SEA production, which is the slope of the toxin production curve, in the shaking culture (190 ng/ml/h) was higher than in the stationary culture (31 ng/ml/h). A large difference was also seen in the maximum SEA concentration between the cultures; the maximum concentration in the shaking culture (2,000 ng/ml) was 10 times higher than that in the stationary culture (200 ng/ml) (Fig.2A). The toxin concentration reached its maximum in the stationary phase of growth (about 48 hours of incubation) in the shaking culture. This was also observed for other SEA-producing strains (Cao et al. 2012; Tsutsuura et al. 2013; Wallin-Carquist et al. 2010). The time in the stationary culture was not clear because of its low production, but was estimated to be also about 48 hours of incubation (Fig.2A). This length of time was the same as in the shaking culture. To our knowledge, there have been no reports on the period required to reach the maximum SEA concentration in stationary culture of a staphylococcal strain.

The kinetics of the expression of the SEA gene, sea, in microbial cells during the incubation period in shaking and stationary cultures was studied to the time when the SEA concentration reached its maximum (Fig.2B, C). Here the relative expression quantity of sea gene transcription was determined in reference to the housekeeping gene encoding gyrB. The toxin gene expression levels in the two cultures had both peaked at 20 hours of incubation, which was in the early stage of toxin production (Fig.2B, C). Sea expression amounts at data points in microbial cells were accumulated along with the incubation time to see the dynamics of sea gene expression followed by SEA synthesis. The toxin production curves and the total, accumulated gene expression curves with time were all sigmoidal (Fig.2B, C). Total gene expression in the cells preceded toxin production in both culture methods. Namely, the time lags between total sea expression and SEA production were observed in both cultures (Fig.2B, C).

The kinetics between the gene expression and the production of a given protein has rarely been reported, to the best of our knowledge. The time lags measured in the present study could be for the process between messenger RNA transcription and protein synthesis in the central dogma of molecular biology proposed by Crick (1958). Cao et al. (2012) reported that the time difference between the build-up of sea mRNA and that of extracellular SEA was approximately 6 h in their strain, Mu 50. It appears that they measured the time difference between the peaks of the two factors during incubation, which definition for which differed from our kinetic analysis.

The Mu 50 strain analyzed by Cao et al. (2012) produced an extremely high amount of SEA (about 10,400 ng/ml) after a 24-hour incubation with shaking in BHI at 37°C. The base sequences for SEA and the relative quantification test used by Cao et al. (2012) were different from those in the present study, because we could not utilize the method of Cao et al. (2012) in our preliminary studies. However, the patterns of gene expression and toxin production of strain 11658 and Mu 50 during the incubation period were similar. This suggested that the kinetics of sea mRNA expression and subsequent SEA production observed in our study
The characteristics of SEA production and the growth of several SEA-producing strains (13008, St.1, f4, and 11727) were studied at various temperatures in shaking culture. The maximum concentrations of SEA and the maximum cell populations of bacterial strains were studied at various temperatures ranging from 22°C to 42°C. Bacterial cells of the tested strains were incubated until the toxin concentrations reached the maximum at each temperature. The incubation times at 22°C, 26°C, 30°C, 34°C, 38°C, and 42°C were 6, 4, 2, 2, 2, and 2 days, respectively.

SEA production during the incubation was also measured in shaking and stationary cultures. SEA gene expression was measured in a culture and then summed over time. RE shows the relative expression of sea. The data points and the SEA production curve shown in Fig.2A are depicted again in Fig.2B, C for reference. Bars show the standard errors at data points. Curves are described with an extended logistic model.

The characteristics of SEA production and the growth of several SEA-producing strains (13008, St.1, f4, and 11727) were studied at various temperatures in shaking culture. The maximum concentrations of SEA and the maximum cell populations of bacterial strains were studied at various temperatures ranging from 22°C to 42°C. Bacterial cells of the tested strains were incubated until the toxin concentrations reached the maximum at each temperature. The incubation times at 22°C, 26°C, 30°C, 34°C, 38°C, and 42°C were 6, 4, 2, 2, 2, and 2 days, respectively.

SEA production in this temperature range varied with the strains (Fig.3A). Strain 13008, which produced very high amounts of SEA at various temperatures, showed the maximum concentration at 38°C. Strain St.1 also produced high amounts of SEA at 22 to 34°C and had a peak at 26°C. Strain f4, a high producer, did not show a clear peak in this temperature range, whereas the concentration at 22°C was slightly higher than those at other temperatures. Strain 11727, a low SEA producer, did not show a peak in this temperature range, whereas the SEA concentration at 22°C was slightly higher than those at other temperatures.
SEA production of strain 11658, which was studied precisely above, was an intermediate producer among the strains examined in the present study.

Compared with SEA production, no clear differences in the maximum cell population were observed among the SEA producers at temperatures between 22°C and 42°C (Fig.3B); the averages of the maximum populations of the five strains at 22°C, 26°C, 30°C, 34°C, and 38°C were 10.0±0.25, 10.0±0.18, 10.0±0.18, 9.9±0.11, 9.6±0.31, and 9.2±0.29 log CFU/ml, respectively.

The characteristics of SEA production with regards to optimal temperature, maximum SEA concentration, and population, and the toxin productivity of the strains were then analyzed with the above data shown in Fig.3. A variety of the toxin production characteristics were observed for the strains (Table 4). Among the strains clear differences were observed in the optimal temperature and maximum SEA concentration of the strains, but there were small differences in the maximum cell population.

The optimal temperature range for SE production by S. aureus has been reported to be between 30°C and 40°C (Vandenbosch et al., 1973) or between 34°C and 40°C (Schelin et al., 2011). The optimal temperature of strain 11658 studied above (30°C) was within the range given by Vandenbosch et al. (1973), while those of the other three strains in Table 3 were also lower than these temperature ranges. The maximum SEA concentrations differed extensively according to the strain, from 180 to 5240 ng/ml (Table 4). Cao et al. (2012) also found a huge difference in SEA production among S. aureus strains incubated at 37°C.

Large differences in SEA productivity were observed among the five strains; the highest SEA productivity (0.64 fg/CFU), which was observed for strain 13008, was 21 times higher than the lowest (0.03 fg/CFU) by strain 11727 (Table 4). Tsutsuura et al. (2013) also reported that SEA productivity in shaking culture varied with the temperature, strain, and inoculum size. Although the strains and culture conditions in the present study were different from those of Tsutsuura et al. (2013), the values shown in Table 3 were within their ranges.

Some of the above strains were also used in preliminary studies of SEA production in stationary culture (data not shown). Consequently, the SEA productivities of strains 13008 and f4 in stationary culture at 30°C, 34°C, and 38°C were higher than in shaking culture, similar to that of strain 11658 (Table 3). On the other hand, the SEA productivity of strain 11727 in stationary culture was lower than in shaking culture at these temperatures. These results also suggested that toxin productivity differed according to the culture conditions and was strain specific.

Cao et al. (2012) divided their SEA producers into three groups: a high producer group (>1,000 ng/ml), an intermediate group (10-1,000 ng/ml), and a low group (<10 ng/ml) according to toxin production at 37°C. When these categories were applied to our strains, the results of the culture at 38°C, which was the closest to 37°C, indicated that 3 of the 5 strains (strains 13008, f4, and 11658) were high producers and two (strains 11727 and St.1) were intermediate producers (Fig.3). However, strain St.1 produced higher amounts of SEA at lower temperatures (Fig.3); it produced as much as 3780 ng/ml at 26°C, showing that this strain is potentially a high producer. Therefore, the present study suggested that each staphylococcal strain has its own specific optimal temperature for SEA production. Namely, toxin production by a given strain at a single temperature, such as at 37°C, might not be suitable to fully characterize that strain.

The present study showed the diversity of SEA production among staphylococcal strains; SEA production varied with the environmental conditions including the temperature and culture method. The reasons for this diversity were unknown. Cao et al. (2012) demonstrated that sea expression in a SEA-producing strain is correlated with the clonal lineage of sea-carrying phages. Therefore, it can be expected that the dynamics of the growth of the phages in staphylococcal cells in various conditions could be a clue to understand the variety of SEA production.

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CONFLICT OF INTEREST

No conflict of interest declared.

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