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Food poisoning outbreak in Tokyo, Japan caused by *Staphylococcus argenteus*

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ABSTRACT

Staphylococcus argenteus is a novel species subdivided from *Staphylococcus aureus*. Whether this species can cause food poisoning outbreaks is unknown. This study aimed to investigate the enterotoxigenic activities of two food poisoning isolates suspected to be *S. argenteus* (Tokyo13064 and Tokyo13069). The results for phylogenic trees, constructed via whole genome sequencing, demonstrated that both isolates were more similar to a type strain of *S. argenteus* (MSHR1132) than any *S. aureus* strain. Moreover, the representative characteristics of *S. argenteus* were present in both strains, namely both isolates belong to the CC75 lineage and both lack a *crtOPQMN* operon. Thus, both were determined to be "*S. argenteus*." The compositions of the two isolates' accessory elements differed from those of MSHR1132. For example, the *seb*-related *Staphylococcus aureus* pathogenicity island, SaPIishikawa11, was detected in Tokyo13064 and Tokyo13069 but not in MSHR1132. Both isolates were suggested to belong to distinct lineages that branched off from MSHR1132 lineages in terms of accessory elements. Tokyo13064 and Tokyo13069 expressed high levels of *s(arg)eb* and produced S(arg)EB protein, indicating that both have the ability to cause food poisoning. Our findings suggest that *S. argenteus* harboring particular accessory elements can cause staphylococcal diseases such as food poisoning, similarly to *S. aureus*.

1. Introduction

Staphylococcus argenteus is a new species that was subdivided from *Staphylococcus aureus* in 2014 (Tong et al., 2015). The species epithet of *S. argenteus* reflects the color of its colony (silver) against *S. aureus* (golden) because this species is typified by the absence of production of the carotenoid pigment staphyloxanthin, which is encoded by the *crtOPQMN* operon (Holt et al., 2011; Xiong et al., 2015). Although some of the earliest reports mentioned that *S. argenteus* was linked to remote Aboriginal communities (Tong et al., 2010; Tong et al., 2013), it is currently recognized as having a global distribution between humans and animals (Dupieux et al., 2015; Jenney et al., 2014; Long et al., 2014; Thaipadungpanit et al., 2015; Tong et al., 2015). *S. argenteus* appears to be less virulent than typical *S. aureus* because it has a greater association with minor skin infections and a lesser association with serious and systemic infections such as sepsis (Holt et al., 2011). Moreover, it has not been reported that *S. argenteus* causes food poisoning, which is one of the representative staphylococcal diseases.

Multilocus sequence typing (MLST) provides data that are well suited for epidemiological and phylogenic studies of bacteria including staphylococci (Enright et al., 2000). *S. argenteus* was previously known as "*S. aureus* clonal complex (CC) 75" (Tong et al., 2013; Xiong et al., 2015), and the CC75 lineage (including ST75, ST850, ST883, and ST1223) is extremely distant from all other clones (Chantratita et al., 2016; Holt et al., 2011). MLST thus makes a clear distinction between *S. aureus* and *S. argenteus*. On the contrary, the original primers for MLST analysis of *S. aureus* cannot amplify several of the seven loci in *S. argenteus* (Ng et al., 2009); therefore, it is difficult to detect this species as a causative microbe using clinical specimens.

Staphylococcal strains Tokyo13064 and Tokyo13069 were isolated from the feces of a patient and food and identified as the causative microbe in the same food poisoning outbreak in Tokyo, Japan in 2010. These strains formed white colonies opposed to yellow colonies (Fig. S1). The sequence types (STs) of these strains were not determined because *aroE* (shikimate dehydrogenase), which is one of the seven loci, was not amplified using the conventional MLST method (Fig. S2). Hence, it was suspected that these strains were *S. argenteus* and that they caused the food poisoning outbreak.

Abbreviations: ANOVA, Analysis of variance; BHI, Brain heart infusion; BLAST, Basic Local Alignment Search Tool; CC, Clonal complex; CDS, Coding sequence; ELISA, Enzyme-linked immunosorbent assay; MLST, Multilocus sequence typing; NGS, Next-generation sequencing; PCR, Polymerase chain reaction; SaPI, *Staphylococcus aureus* pathogenicity island; SE, Staphylococcal enterotoxin; SEB, Staphylococcal enterotoxin B; SFP, Staphylococcal food poisoning; SNP, Single nucleotide polymorphism; ST, Sequence type

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In the present study, we conducted whole-genome analysis of the aforementioned two strains using next-generation sequencing (NGS) and compared their genomic structures and epidemiological properties to those of reported staphylococcal strains. Furthermore, we investigated whether these strains possessed the ability to cause staphylococcal food poisoning (SFP).

2. Materials and methods

2.1. Overview of the staphylococcal food poisoning outbreak that occurred in Tokyo, in 2010

In August 2010, two patients complained of vomiting, fever, and diarrhea 3 h after ingesting a box lunch prepared by a caterer at an industrial kitchen in Tokyo. Several other patients who ate the same box lunch also complained of vomiting, fever, and diarrhea. We examined fecal specimens from patients and caterer workers, and also examined suspected foods and swabs of the kitchen utensils and tableware in the caterer. We isolated 10 staphylococcal strains from the following specimens: fecal specimens from three patients and two workers, one suspected delicatessen food (contained eggplant, minced meat, and cheese), and four swabs each from a cold table, workbench, and empty lunch boxes. Bacterial identification was performed using a coagulase test from the staphylocoagulase antiserum kit (Denka Seiken, Tokyo, Japan). The detection and typing of the staphylococcal enterotoxin (SE) was performed by reversed passive latex agglutination for types A–E (Denka Seiken) according to the manufacturer's instructions. All 10 strains carried type VI coagulase and staphylococcal enterotoxin B (SEB). However, the colonies grown from the samples were not typical for *S. aureus*, because they formed white colonies instead of yellow colonies of *S. aureus* (Fig. S1). The investigation conducted by the health center, an administrative organization, demonstrated that the delicatessen cooked the food the day before the patients ate it and packed it in a lunch box without sufficient cooling; therefore, the food was left at a high temperature (at a temperature higher than room temperature in summer in Tokyo) for at least 12 h.

2.2. Bacterial strains and culture

The bacterial strains used in this study are listed in Table S1. These strains were isolated from the feces of patients or foods suspected to have caused food poisoning outbreaks in Tokyo, Japan. At first, we performed the typing of the enterotoxin by reversed passive latex agglutination for SEA–SEE and confirmed that both Tokyo13064 and Tokyo13069 produced only SEB protein (Fig. S3). Staphylococcal strains were cultured overnight in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, MD, USA) or BHI broth supplemented with 1% yeast extract (Becton Dickinson) at 37 °C with shaking (100 rpm) to extract genomic DNA (gDNA) or total RNA (tRNA), respectively. The staphylococcal strain cultures were grown in BHI broth supplemented with 1% yeast extract at 37 °C for 48 h with shaking (100 rpm) to assess SE production. *Escherichia coli* DH5α was purchased from Promega (Madison, WI, USA). The *E. coli* strain was cultured overnight in Luria–Bertani broth (Sigma, St. Louis, MO, USA) containing 50 μg/ml ampicillin (Wako Pure Chemicals, Osaka, Japan) at 37 °C with shaking for plasmid isolation.

2.3. Preparation of gDNA

gDNA was extracted from the cultured cells after treating the cells with lysostaphin (Wako Pure Chemicals) using a QIAamp DNA mini kit (QIAGEN GmbH, Hilden, Germany). The concentration of the extracted gDNA was determined using a QuantiFluor ONE dsDNA System (Promega), its purity was determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and its integrity was investigated by performing 0.8% agarose gel electrophoresis.

2.4. Construction of plasmids

Plasmids and the primers for construction are presented in Tables S2 and S3. The plasmids pGTgyrB, pGTsodA, pGTftsZ, pGTrpoB, pGTseb, pGTsei, pGTsem, pGTsen, pGTseo, and pGTselu were constructed by cloning the individual polymerase chain reaction (PCR) products, which were amplified from the previously extracted gDNA, into pGEM-T easy TA cloning vectors (Promega). *E. coli* DH5α was transformed with the cloning plasmids. Plasmids were extracted from the cultured cells using a QIAprep Spin Miniprep kit (QIAGEN) according to the manufacturer's instructions.

2.5. Genomic library preparation and whole genome sequencing

The whole genomes of Tokyo13064 and Tokyo13069 were sequenced. For each gDNA, index-tagged libraries were prepared using a Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and sequenced using Illumina MiSeq with 300-bp paired-end reads according to the manufacturer's instructions.

To ensure that only the highest quality data were used for assembly, reads were trimmed and filtered using CLC Genomics Workbench 9.0 (QIAGEN) set to a minimum length of 100 bp and a quality score threshold of 30. These trimmed reads were then mapped to the *S. argenteus* MSHR1132 genome (Accession Number: FR821777) with mode “Map Reads to Reference.” Moreover, trimmed reads were de novo assembled via the “De Novo Assembly” mode with default settings. Each assembled contig was aligned to the MSHR1132 genome and combined as a single pseudocontig using CONTIGuator2 (http://contiguator.sourceforge.net/), producing pcg13064 and pcg13069.

2.6. Phylogeny based on k-mer diversity

A k-mer is a substring of length k from a read obtained through NGS, and counting the occurrences of all such substrings is a central step in many DNA sequence analyses. The reads of suspected *S. argenteus* isolates (Tokyo13064 and Tokyo13069) were phylogenetically compared with reference genomes including *S. argenteus* MSHR1132 using the diversity of k-mer as the index. Using the “Create K-mer Tree” program of Microbial Genomics Module in CLC Genomics Workbench 9.0, pairwise distances among Tokyo13064, Tokyo13069, and references were calculated using Feature Frequency Profile via Jensen–Shannon divergences, and the phylogenetic tree was constructed using the neighbor-joining method. The k-mer length was set to be 16 bases for this analysis.

The sequences of the following *S. argenteus* strain and 10 *S. aureus* strains were used for comparative analysis: MSHR1132, MRSA252 (BX571856), MW2 (BA000033), N315 (BA000018), Mu3 (AP009324), COL (CP000046), NCTC8325 (CP000253), Newman (AP009351), TW20 (FN433596), T0131 (CP002643), and M013 (CP003166).

2.7. Phylogeny based on single-nucleotide polymorphisms

Assembled contigs described in the “Genomic library preparation and whole genome sequencing” section were further compared with the reference genomes MSHR1132, MRSA252, MW2, N315, Mu3, COL, NCTC8325, Newman, TW20, T0131, and M013 based on single-nucleotide polymorphism (SNP) composition. First, assembled contigs and reference genome sequences were, respectively, split into 10,000-bp “reads” using Pyfasta (ver. 0.5.2) (Massachusetts Institute of Technology) and mapped to the MSHR1132 sequence as a reference using BWA-MEM (ver. 0.7.5a) (Li and Durbin, 2009). SNPs and indels were called using SAMtools (Li et al., 2009). After format conversion using VarScan (ver. 2.3.9) (Koboldt et al., 2009), indels were omitted using Vcftools (Danecek et al., 2011). The bases in which SNPs were