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A food poisoning caused by ST7 *Staphylococcus aureus* harboring sea gene in Hainan province, China

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ST7 *Staphylococcus aureus* is highly prevalent in humans, pigs, as well as food in China; however, staphylococcal food poisoning (SFP) caused by this ST type has rarely been reported. On May 13, 2017, an SFP outbreak caused by ST7 *S. aureus* strains occurred in two campuses of a kindergarten in Hainan Province, China. We investigated the genomic characteristics and phylogenetic analysis of ST7 SFP strains combined with the 91 ST7 food-borne strains from 12 provinces in China by performing whole-genome sequencing (WGS). There was clear phylogenetic clustering of seven SFP isolates. Six antibiotic genes including *blaZ*, *ANT (4')-Ib*, *tetK*, *lnuA*, *norA*, and *lmrS* were present in all SFP strains and also showed a higher prevalence rate in 91 food-borne strains. A multiple resistance plasmid pDC53285 was present in SFP strain DC53285. Among 27 enterotoxin genes, only *sea* and *selx* were found in all SFP strains. A Φ Sa3int prophage containing type A immune evasion cluster (*sea*, *scn*, *sak*, and *chp*) was identified in SFP strain. In conclusion, we concluded that this SFP event was caused by the contamination of cakes with ST7 *S. aureus*. This study indicated the potential risk of new emerging ST7 clone for SFP.

KEYWORDS

staphylococcus food poisoning, sequence type 7, whole-genome sequencing, enterotoxin A, prophage, plasmids

Introduction

Food-borne disease (FBD) caused by microorganisms is a major concern for food safety and it is a global health problem that is harmful to humans. *Staphylococcus aureus* an ubiquitous Gram-positive bacterium and a common causative pathogen of food poisoning (Hennekinne et al., 2012). A total of 314 outbreaks of staphylococcal food poisoning (SFP) were reported in

China between 2011 and 2016, involving 5,196 cases and 1 death (Liu et al., 2018). In 2020, the National Foodborne Disease Outbreak Surveillance System in China reported 4,662 outbreaks with confirmed etiology, of which bacterial pathogens were the most common cause of illnesses (41.7%) and *S. aureus* ranked third among the causative bacteria (75 outbreaks and 954 illnesses) (Li et al., 2021). The incubation period of SFP is 0.5–8 h, and the common symptoms are nausea and vomiting, with or without abdominal pain, dizziness, diarrhea, shivering, general weakness, and moderate fever (Hennekinne et al., 2012).

Staphylococcus aureus can produce different Staphylococcal enterotoxins (SEs) and SE-like (SEL) toxins. It was reported less than 1 µg of SE can cause SFP (Pinchuk et al., 2010). The most common SEs are SEA, SEB, SEC, SED and SEE, accounting for approximately 95% of SFP outbreaks (Bastos et al., 2017). Most of the SEs and SEL toxins are located on the mobile genetic elements (MGEs) (such as plasmids, prophages, genomic islands, and pathogenicity islands) or next to the staphylococcal cassette chromosome (Argudin et al., 2010). Among them, SEA and SEP are carried by prophages (Argudin et al., 2010).

ST7 *S. aureus* is an important circulating clone in China. ST7 is one of the most commonly isolated STs from the nasal swabs of healthy individuals and occupational livestock workers in China (Yan et al., 2015; Ye et al., 2015). ST7 is also the dominant type in healthy and diseased pigs, and environment (pigpen gates, soil, and ground), followed by ST398 and ST9 in China (Yan et al., 2014; Zhou et al., 2020). ST7 strains can cause a variety of diseases both in animals and humans. ST7 clones are common cause of bovine mastitis (Li et al., 2017). Among the human-related diseases, ST7 clones commonly cause skin and soft tissue infections, bacteremia, pneumonia, and musculoskeletal infection, especially in children (Gu et al., 2016; Li et al., 2018; Wu et al., 2018). Most importantly, ST7 is the dominant type in a variety of foods, such as ready-to-eat foods and vegetables (Liao et al., 2018; Rong et al., 2018).

At present, ST6, ST943, ST5, ST59, ST81, and ST8 were the predominant types of *S. aureus* that caused food poisoning outbreaks in many countries (Cha et al., 2006; Suzuki et al., 2014; Li et al., 2018; Chen and Xie, 2019; Lv et al., 2021). Until now, outbreaks of ST7 food poisoning have been only reported in Shijiazhuang and Guangzhou in China (Lv et al., 2021; Zhou et al., 2022). On May 13, 2017, an SFP outbreak caused by ST7 *S. aureus* strains occurred in two campuses of a kindergarten in Hainan Province, China, involving 26 children and one adult. ST7, as a new emerging SFP clone, is not well characterized up to now. In this study, we aimed to investigate the genomic characteristics of these ST7 strains, by using whole-genome sequencing (WGS). In particular, we analyzed the SFP strains for virulence, drug resistance, prophages, plasmid, and defense systems. Moreover, we conducted phylogenetic analysis of these SFP strains along with the 91 ST7 strains isolated from food in 12 provinces of China.

Materials and methods

Epidemiological investigation

On May 13, 2017, an outbreak of food poisoning in campuses A and B of a kindergarten in the Hainan Province, China was investigated. The outbreak involved 26 children and one adult. The symptoms had

appeared after consumption of breakfast. The most common symptoms were vomiting (27/27, 100%), followed by periumbilical abdominal pain (14/27, 51.85%), and fever (3/27, 11.11%). The incubation period for symptoms was 1–3 h. The cakes purchased from a cake shop were suspected of being related to the SFP. The leftover food and anal swabs of patients were collected for bacterial isolation. All samples were processed based on *S. aureus* isolation methods for foods according to the national standards in China (GB/T 4789.10–2017). Five *S. aureus* strains were isolated from leftover cake and lean porridge in campus A, the cream from cake shop, and leftover cake in campus B, while two strains from patients were isolated from campus A.

Bacterial collection

Ninety-one food-borne ST7 *S. aureus* strains were collected from 12 provinces of China between 2006 and 2019 (Supplementary Figure S1). All the strains in the study were confirmed by PCR detection for *nuc* and *mecA* (Merlino et al., 2002).

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of ceftiofloxacin, linezolid, clindamycin, erythromycin, doxycycline, gentamicin, penicillin, rifampicin, sulfamethoxazole, tigecycline, ciprofloxacin, tetracycline, vancomycin, fusidic acid, quinupristin/dalfopristin, daptomycin, teicoplanin, florfenicol, tiamulin, and nitrofurantoin for 7 SFP strains were determined by microdilution method. *S. aureus* ATCC29213 was used as the quality control strain. The MICs values were interpreted according to Clinical and Laboratory Standard Institute (CLSI) Performance Standards for Antimicrobial Susceptibility Testing (31st edition).

DNA extraction, genome sequencing, assembly, and annotation

Genomic DNA was extracted from pure cultures using a commercial kit (QIAGEN, Germany) and quantified by Qubit 4.0 (USA Invitrogen ABI). Genomic DNA was sequenced using Illumina NovaSeq PE150 by the Beijing Novogene Bioinformatics Technology Co. Ltd. All sequences were preprocessed by READFQ V10 (Chen et al., 2018) to delete data with mass value ≤ 20 . Clean data were assembled using SPAdes V3.13 (Bankevich et al., 2012), from which only contigs greater than 500 bp in length were selected for further analysis. The Nanopore Oxford MinION platform was used for three strains, i.e., DC53285, DC53206, and DC52998 (You et al., 2018). Unicycler v0.5.0 (Wick et al., 2017) was used for genome assembly based on ONT long reads and Illumina short reads. All genomes were annotated by Prokka V1.14.5 (Seemann, 2014). The closed genome was confirmed by PCR (Supplementary Table S4).

Phylogenetic analysis

The genomes of seven SFP strains, 91 food-borne strains, and seven ST7 published sequences from NCBI (Supplementary Table S1)

were included in the phylogenetic analysis. DC51277 (CC7-ST943) was used as an outgroup. The core genome was extracted by Roary 3.13.0 (Page et al., 2015). Maximum-likelihood phylogenetic trees were constructed using RAxML V8.2.12 (Stamatakis, 2014) based on 2,387 core genes (shared by >99% of *S. aureus* isolates) and the evolutionary tree was visualized by ITOL¹ (Letunic and Bork, 2019).

Multilocus sequencing typing (MLST), detection of virulence, resistance, restriction-modification genes (R-M), prophage, and plasmid

MLST was performed by PubMLST² website. VFDB and CARD libraries were downloaded to construct the local database, and the search within blastp was conducted with parameters of >80% identity and >80% coverage. According to the copy number of virulence and resistance genes, heatmap was drawn using the heatmap package of R V4.1.2. (Chan, 2018). To better assess the carriage rate of virulence genes, our data were compared with the data of a previously published manuscript (Slingerland et al., 2020), in which genome of 10,288 *S. aureus* strains were included. Statistical analysis was performed by Chi-squared test for independence using SPSS Statistics 25.0 (IBM). *p* values <0.05 were considered indicative of statistical significance.

R-M genes were searched on Restriction-Modification Finder 1.1.³ *hsdM* and *hsdS* were verified by PCR (Supplementary Table S4). CRISPR-cas genes were searched on CRISPRCas Finder (Couvin et al., 2018).

The prophage structure of four strains was compared, including one SFP strain (DC53285) and three non-SFP strains which carried *sea* (DC53206, DC52998, and DC52929). Prophages and plasmids were identified by PHASTER⁴ and PlasmidFinder.⁵ The prophage and plasmid nucleotide sequences were compared with sequences in the GenBank database using BLASTN⁶ and were visualized by Easyfig (Sullivan et al., 2011). The closed plasmid was confirmed by PCR (Supplementary Table S4).

Results

Phylogenetic analysis of SFP strains and non-SFP strains

The 106 genomic sequences (including one outgroup sequence) in this study shared 2,387 core genes. The phylogenetic tree can be mainly divided into two clusters (Figure 1). The tree demonstrated that the SFP outbreak strains isolated from the food matrix (DC53286, DC53288, DC53289, DC53349, and DC53362) clustered together with those of the human cases (DC53284 and DC53285) in a single

clade carried by one branch in cluster 1. Further analysis of SFP strains in the phylogenetic tree showed that food isolates from campus A were closely clustered with campus B, and the cream from cake shop closely clustered with patient isolates from campus A.

The geographically clustered stains were from Hainan, Anhui, and Jilin Provinces in cluster 1 and 2, respectively. These strains were mainly isolated from meat with adjacent isolation years. The one NCBI sequence from human in Zhejiang Province in China that was closely clustered with Chinese isolates from food matrix in cluster 1. In cluster 2, six NCBI human sequences of foreign countries clustered as a subclade with an imported food isolate from Uruguay and a few food isolates from China.

Phenotype and genotype of antibiotic resistance

All SFP isolates were methicillin-sensitive *S. aureus* (MSSA) and only resistant to penicillin and tetracycline. The heatmap showed the same resistance gene profiles of the seven SFP strains, all of which carried one copy of *blaZ*, *tetK*, *ANT (4')-Ib*, and *hnuA*, *lmrS*, and *norA* gene (Figure 2).

In this study, 91 food-borne strains carried a total of 15 resistance genes. Six antibiotic genes showed a higher prevalence rate, i.e., *norA*, *lmrS*, *blaZ*, *ANT (4')-Ib*, *tetK*, and *hnuA* (Supplementary Table S2). Only two strains carried two copies of resistance genes, which were *AAC (6')-Ie-APH (2'')-Ia*, *catA*, and *blaZ*, respectively (Figure 2). No special antibiotic genes were observed for the 7 SFP strains compared with 91 food-borne strains.

Combined with phylogenetic tree, *ermC*, *hnuA*, *ANT (4')-Ib*, and *tetK* were mainly distributed in cluster 1 strains (*p*<0.05). And most of strains were from poultry and livestock meat in Anhui, Hainan, and Yunnan Province. While *AAC (6')-Ie-APH (2'')-Ia* and *ermB* genes were mainly found in cluster 2 strains (*p*<0.05), and most of which were from poultry and livestock meat in Jilin and Gansu Province (Supplementary Table S2; Figure 1).

Distribution of virulence genes

All SFP isolates had the same virulence gene profile (Figure 3; Supplementary Table S3). Among the 27 enterotoxin genes, only *sea* and *selx* were found in all SFP strains. The *chp*, *sak*, *scn*, *lukD*, *lukE*, *lukS-PV*, and *lukF-PV* were also present in all SFP strains.

The 91 non-SFP isolates showed a significantly higher prevalence rate of ten virulence genes, i.e., *fnbA*, *fnbB*, *sdrD*, *lukD*, *lukE*, *sep*, *lukF-PV*, *lukS-PV*, *sak*, and *sdrE* compared with the reference data (*p*<0.05). Six virulence genes (*tsst-1*, *sea*, *sec*, *sel*, *chp*, and *sspB*) had a lower prevalence rate (*p*<0.05, Supplementary Table S3). Almost all the virulence genes were one copy, except for *eap/map*, *hysA*, and *nuc*.

Genomic structure of prophage carrying *sea*

One SFP strain (DC53285) and three non-SFP strains (DC53206, DC52998, and DC52929) were selected for the prophage structure analysis. The identified prophages of DC53285, DC53206, DC52998,

1 <https://itol.embl.de>

2 <https://pubmlst.org/>

3 <https://cge.cbs.dtu.dk/services/Restriction-ModificationFinder/>

4 <http://phaster.ca/>

5 <https://cge.food.dtu.dk/services/PlasmidFinder/>

6 <http://blast.ncbi.nlm.nih.gov/blast>

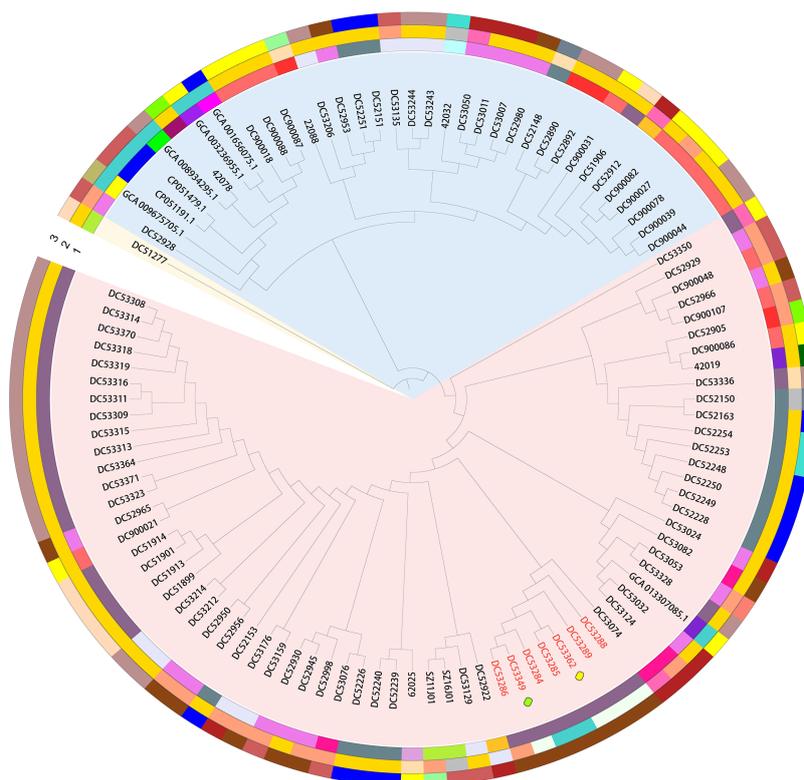


FIGURE 1
 Maximum-likelihood phylogenetic tree based on 2,387 core genes of the 106 *S. aureus* isolates. The outgroup ST943 strain is indicated in yellow bottom; cluster 1 and 2 is shown in red and blue bottom, respectively. Isolates from food poisoning are indicated by red text. The yellow and green boxes represent strains from the cake shop and campus B, respectively. Isolates from food poisoning are from campus A. Region, time, and source type are indicated by means of numbers and the colored rings. NCA_013307085.1, GCA_001656075.1, GCA_003236955.1, GCA_008934295.1, GCA_009675705.1, CP051191, and CP051479 are sequences from NCBI.

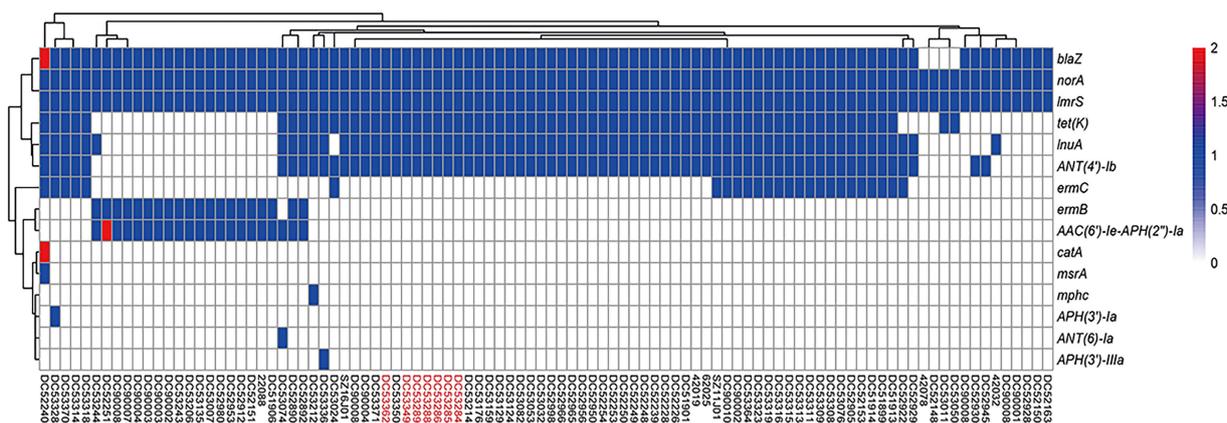
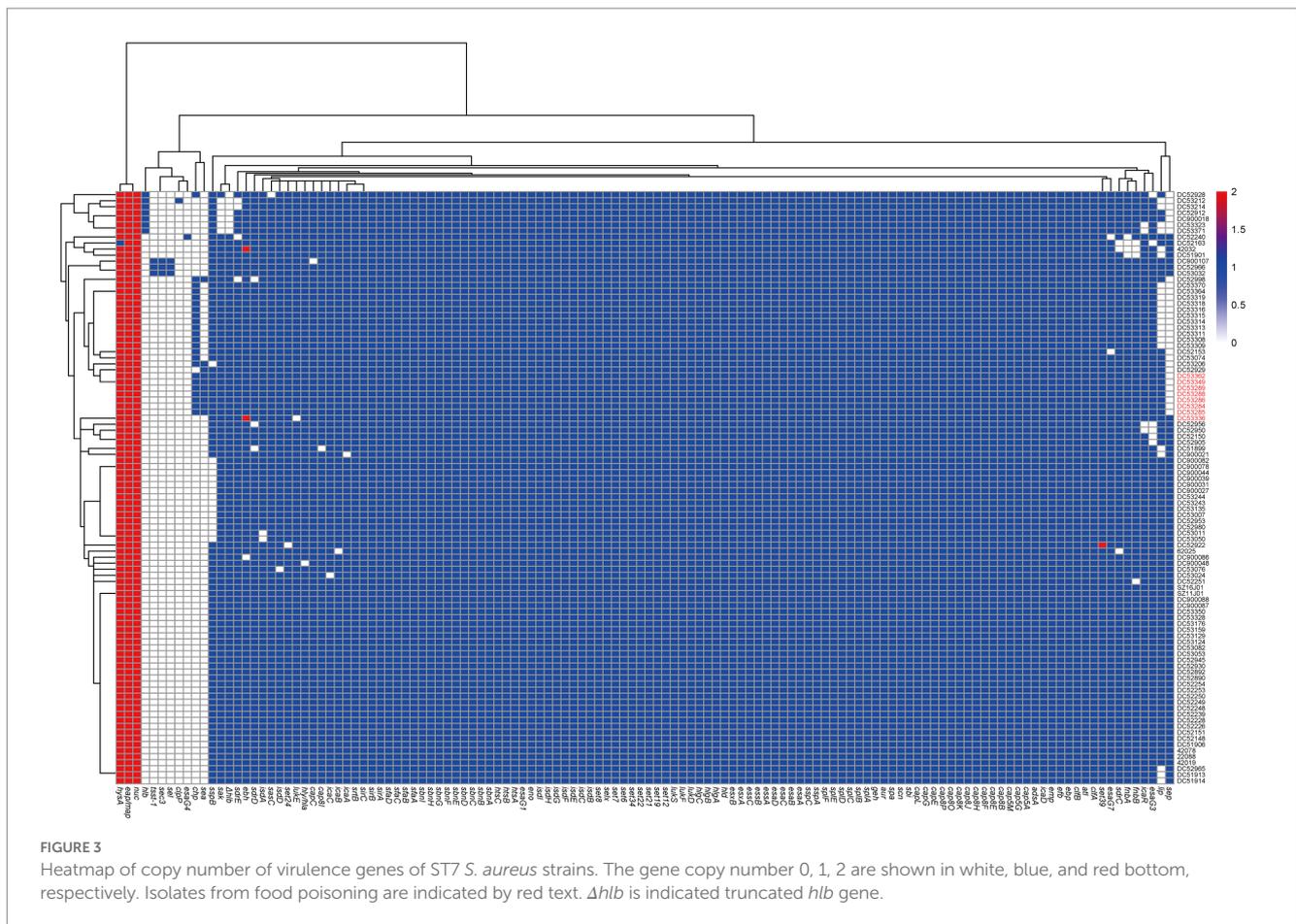


FIGURE 2
 Heatmap of copy number of resistance genes of ST7 *S. aureus* strains. The gene copy number 0, 1, 2 are shown in white, blue, and red bottom, respectively. Isolates from food poisoning are indicated by red text.

and DC52929 were 42,263 bp, 47,075 bp, 46,733 bp, and 47,230 bp in length and contained 67, 67, 68, and 68 coding sequences (CDSs), respectively. They were all integrated within the *hlyB* and belonged to Φ Sa3int integrase gene group. Overall, the structure of DC53285 shared 98.97% of nucleotide sequence identity and 85% coverage with Φ NM3 (NC_008617.1), and shared 96.87% identity and 78% coverage with

SA1014ruMSSAST7 (NC_048710.1) (Figure 4). DC52929 prophage showed low nucleotide similarity with the above three prophages. Type A immune evasion cluster (IEC) genes (*sea*, *sak*, *chp*, and *scn*) were found in DC53285, DC53206, and DC52998 prophage, while type D IEC genes (*sea*, *sak*, and *scn*) were found in DC52929. The *lukF*-PV and *lukS*-PV were located downstream of the four prophages (Figure 4).



ST7 SFP strain carried a multi-antibiotic resistant plasmid

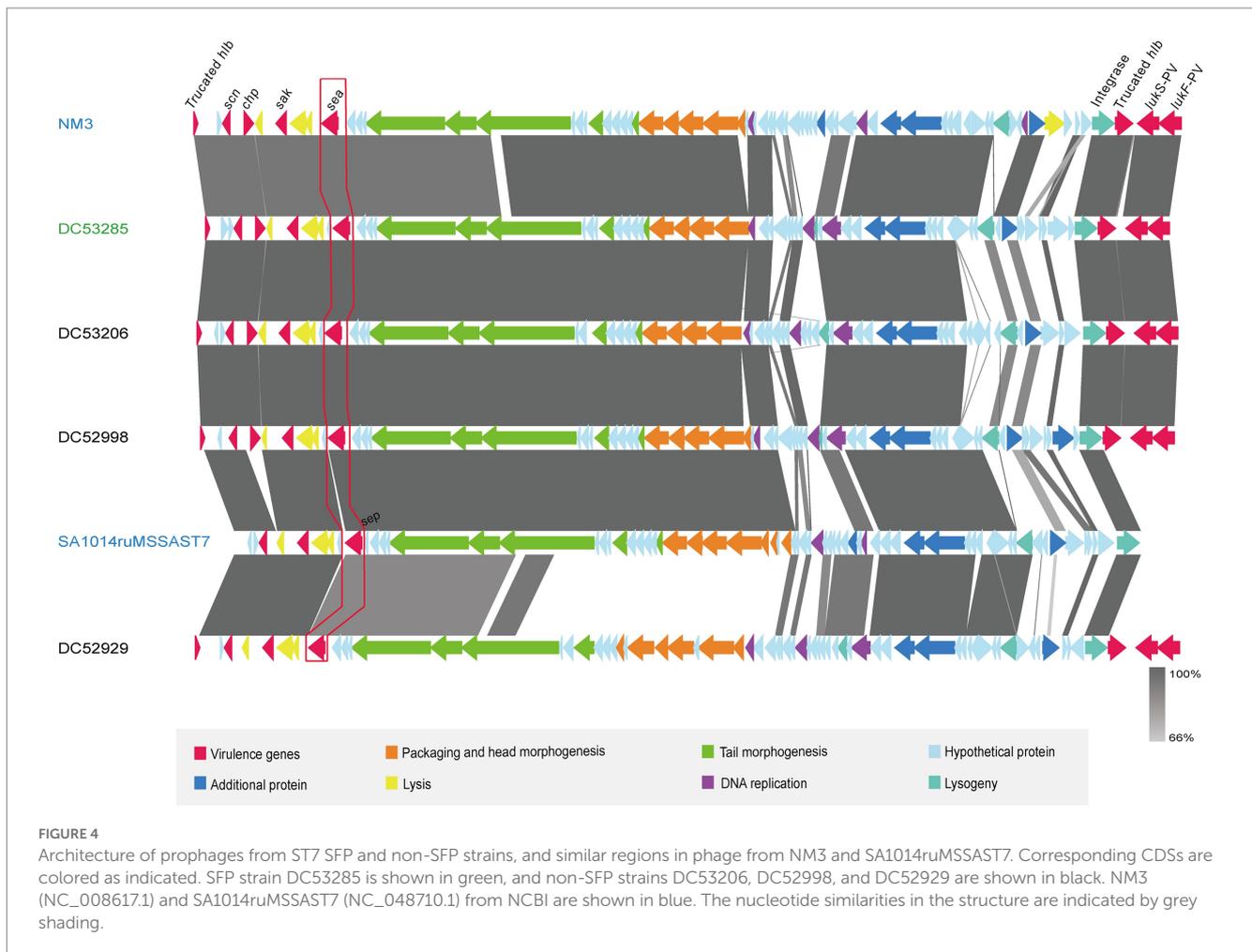
A multiple resistance plasmid pDC53285 was present in the SFP strain DC53285. The complete sequence of pDC53285 was 35,024 bp in size and had a G+C content of 29% (accession no. NMDC60045017). Sequence analysis identified 43 CDSs. pDC53285 carried four antibiotic resistance genes, including *ANT(4')-Ib*, *tetK*, *lunA*, and *blaZ*. Plasmid pDC53285 shared 99% coverage (99.97% nucleotide identity) with the plasmid MJ015 (NZ_CP038185.1) from Zhejiang Province in NCBI database. The genetic structure of pDC53285 also showed high nucleotide similarity (71% identity) and the main ORF features with two plasmids pSR02 (NZ_CP048645) and pMW2 (NZ_CM007996). The 1–18,870 bp and 33,717–35,024 bp regions of pDC53285 were highly equivalent to the 1–16,494 bp, and 24,746–26,055 bp regions of plasmid pSR02. The pDC53285 plasmid retained around two-thirds of the genes from pSR02, mainly including *ANT(4')-Ib*, *tetK*, *lunA*, *oriT* (the origin of transfer site), and a relaxase protein. The 18,853–25,717 bp region of pDC53285 was highly equivalent to the 2,173–20,100 bp region of pMW2, mainly including *blaZ* and a relaxase protein. The two relaxase protein encoding genes belonged to MOB_V family (Figure 5A). Further sequence analysis showed that the two plasmids (pMW2 and pSR02) harbored a 17 bp upstream homologous region and an 835 bp downstream homologous region (Figure 5B).

ST7 SFP isolates carry intact type I and IV restriction-modification (RM) systems

All SFP strains contained complete type I and type IV RM systems. Analysis of CRISPR-cas system revealed that none of the SFP strains carried CRISPR-cas system encoding genes.

Discussion

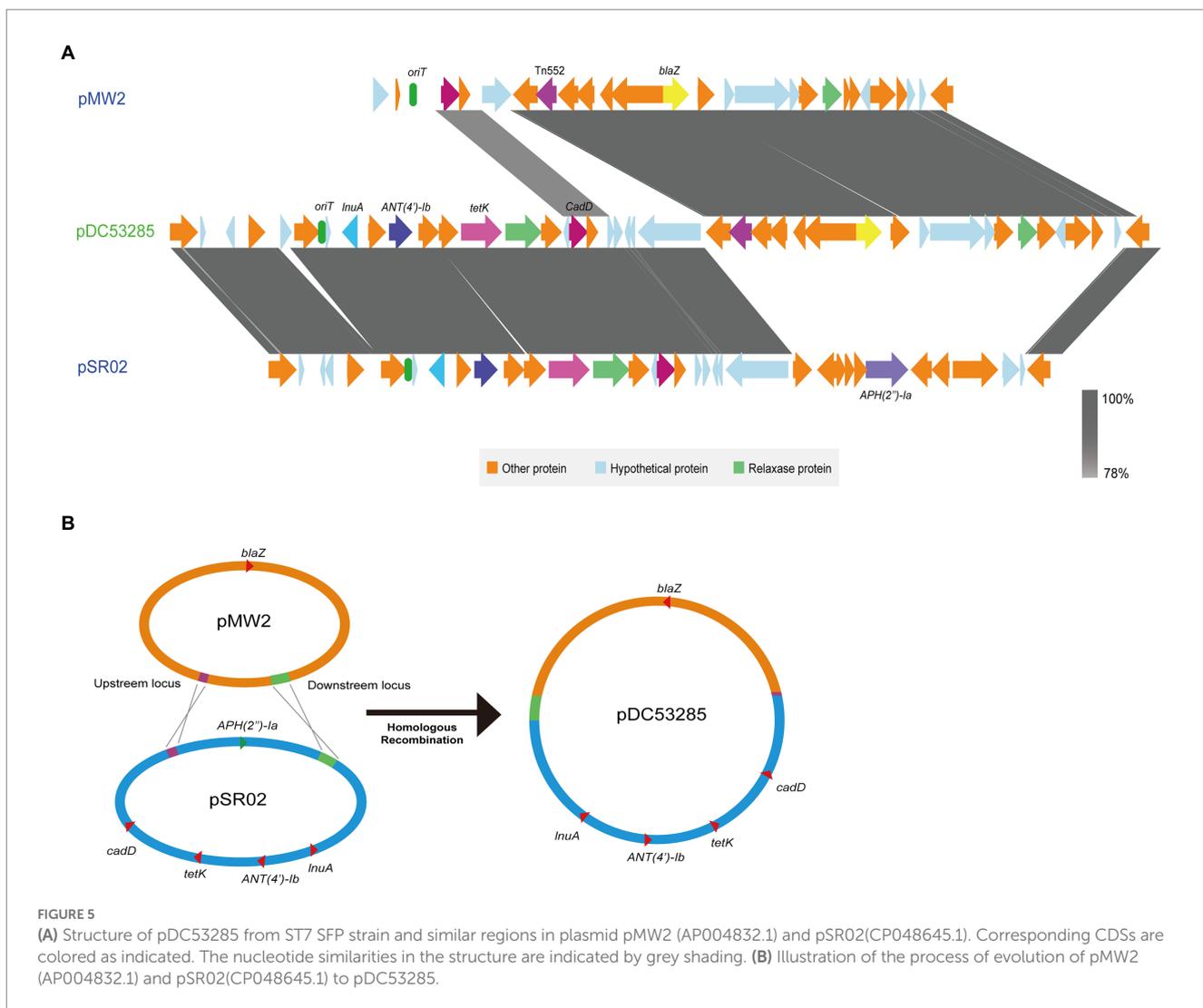
In this study, we used WGS to investigate *S. aureus* involved in the outbreak in Hainan Province, China. We confirmed seven ST7 SFP isolates with a clear phylogenetic clustering in cluster 1. Further analysis of SFP strains in the phylogenetic tree showed that food isolates from campus A were closely clustered with campus B, and the cream from cake shop closely clustered with patient isolates from campus A. Therefore, we concluded that this SFP event was caused by the contamination of cakes from cake shop with *S. aureus* carrying *sea*. The strain isolated from the porridge was also related with this SFP, which may be due to cross contamination of porridge with cakes. SFP investigations have typically relied on epidemiological data and molecular typing; however, WGS is increasingly used in recent years (Mossong et al., 2015; Durand et al., 2018; Nouws et al., 2021). In this study, we also confirmed that WGS can be a high discriminative tool to support epidemiological outbreak-investigations.



SEA was the main cause of food poisoning caused by ST7 *S. aureus* in this study. SEA, alone or in association with other SE(s), is the most frequently involved (>75%) in SFP outbreaks worldwide (Hennekinne et al., 2012; Guillier et al., 2016). The *sea* is the most frequently identified enterotoxin gene in China (Yan et al., 2012; Chen and Xie, 2019) and is always located on the prophage (Argudin et al., 2010), which formed the IEC together with the different combinations of *scn*, *chp*, *sak*, and *sep* (van Wamel et al., 2006). All SFP isolates in this study carried Φ Sa3int prophages belonging to the family *Siphoviridae* and genus *Biseptimavirus*. In addition, type A IEC, including *sea*, *scn*, *sak*, and *chp* gene were found on the Φ Sa3int prophages. Studies have shown that the main difference between human and animal strains of *S. aureus* is that human strains always carry IEC (McCarthy and Lindsay, 2013). Thus, the real source of the contamination of cakes in this study was probably human based on the IEC type of the SFP strains. However, we do not have strains isolated from the staff in the cake shop to support this inference. The prophage of SFP strain DC53285 showed high nucleotide similarity with NM3 (NC_008617.1) which was carried by ST254 (CC8) *S. aureus*. Phage Φ phiNM3 has been reported in different MLST type strains, including ST1, ST5, ST7, ST8, ST30, ST72, ST109, and ST508 (Nepal et al., 2021). Therefore, the Φ Sa3int prophage of SFP strains may have been exchanged by horizontal gene transfer (HGT) from the same or different ST strains. A previous study found a significantly higher prevalence of Φ NM3 in

chronic rhinosinusitis patients with high disease severity compared to those with low disease severity (Nepal et al., 2021). This implies that the prophage of SFP strains can increase the pathogenicity of strains.

A multiple resistant plasmid pDC53285 with *rep5a* was found in ST7 SFP strain. pDC53285 carried *oriT* and relaxase protein, which were similar to that in plasmid pMW2 and pSR02. The conjugative mobilization plasmids usually have a Type-IV secretion apparatus, an origin of transfer (*oriT*) site, and a relaxase protein. Interestingly, it has recently been demonstrated that mobilization transfer can be carried out through the relaxase-*in trans* mechanism for the plasmid only with *oriT* and relaxase protein, and it is a frequent event that most *S. aureus* plasmids have evolved to take advantage of (O'Brien et al., 2015; Ramsay et al., 2016). Therefore, the plasmid pDC53285 in this study may be a mobilizable plasmid through the relaxase-*in trans* mechanism of mobilization. pDC53285 showed the highest nucleotide similarity with the plasmid carried by MJ015 (NZ_CP038185.1), which is an MSSA ST7 strain isolated from a patient in Zhejiang, China. Therefore, pDC53285 may potentially be a mobilizable plasmid through a horizontal transfer manner. Further studies should investigate the distribution of pDC53285 in Chinese strains. Based on the genetic structure of pDC53285, it is speculated that pDC53285 was generated as a result of fusion between plasmids pSR02 and pMW2. On further comparative analysis, two plasmids (pMW2 and pSR02) were found to harbor a 17bp upstream homologous region



and an 835 bp downstream homologous region, facilitating homologous recombination of the two plasmids, and resulting in formation of a 35,024 bp integrated multi-antibiotic resistance plasmid. The same recombination mechanism was reported for a conjugative virulence plasmid, p15WZ-82_Vir, from a clinical *Klebsiella variicola* strain (Yang et al., 2020).

ANT(4)-Ib, also called *aadD2*, encoding an aminoglycoside 4'-O-nucleotidyltransferase was first described in *Bacillus clausii* and rarely reported in *S. aureus* (Bozdogan et al., 2003; Ullah et al., 2021). In this study, the high prevalence of *ANT(4)-Ib*, together with *blaZ*, *tetK*, and *InuA* were found in ST7 food-borne and SFP strains. It may be that these food-borne strains carry the similar multiple resistant plasmid as SFP strains as well. These antibiotic resistance genes were mainly distributed in strains of cluster 1 and closely associated with region and poultry/livestock-derived foods. Whether these resistance genes originated in local poultry/livestock associated strains is worth to be further investigated.

The *sep* gene is always located on the prophage and can also cause SFP (Omoe et al., 2005; Chiang et al., 2008; Argudin et al., 2010). The carriage rate of *sep* carried by ST7 *S. aureus* in this study (69.4%, 68/98) was much higher than that in previous studies (Omoe et al.,

2005; Bania et al., 2006; Chiang et al., 2008; Wang et al., 2021). Therefore, the ST7 strains isolated from food in this study are at high risk of causing SFP. This is the first study to identify a high carriage rate of *lukFS-PV* (100%, 98/98) in the ST7 *S. aureus*. The *lukFS-PV* plays an important role in pathogenicity in the context of bacteremia, keratitis, skin and soft tissue infections, and other related diseases (Sueke et al., 2013; Knudsen et al., 2016; Xiao et al., 2019). Such a high carriage rate of *lukFS-PV* among ST7 isolates is liable to increase the risk and severity of diseases.

In summary, we concluded that this SFP event was caused by the contamination of cakes from cake shop with ST7 *S. aureus*. The food poisoning was found to be caused by enterotoxin A and a type A IEC (*sea*, *scn*, *chp*, and *sak*) was located on the Φ Sa3int prophage. Six antibiotic genes including *blaZ*, *ANT(4)-Ib*, *tetK*, *InuA*, *norA*, and *lmrS* were present in all SFP strains and also showed a higher prevalence rate in 91 food-borne strains. In addition, a multi-resistant plasmid pDC53285 was found in ST7 SFP strains, which may act as a vector for the dissemination of antimicrobial resistance among strains. Due attention should be accorded to the high prevalence rate of *sep*, *lukF-PV*, and *lukS-PV* in ST7 strains isolated from food, which is liable to increase the risk of SFP and other diseases.

Limitations

Because this is a retrospective study, samples from the staff in the cake shop, food handlers, and environment in the canteen in the kindergarten were not available. It is difficult to conclude whether the contamination comes from human.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA917243, and modified BioSample Accession is available in the [Supplementary material](#).

Ethics statement

This study was approved by Ethical Committee of National Institute for Communicable Disease Control and Prevention Chinese Center for Disease Control and Prevention (approval No. ICDC-202111). The patients/participants provided written informed consent to participate in this study.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

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Supplementary material

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