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Molecular interaction between methicillin-resistant *Staphylococcus aureus* (MRSA) and chicken breast reveals enhancement of pathogenesis and toxicity for food-borne outbreak

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ABSTRACT

To study pathogenesis and toxicity of *Staphylococcus aureus* in foods, FORC_062 was isolated from a human blood sample and complete genome sequence has a type II SCC*mec* gene cluster and a type II toxin-antitoxin system, indicating an MRSA strain. Its mobile gene elements has many pathogenic genes involved in host infection, biofilm formation, and various enterotoxin and hemolysin genes. Clinical MRSA is often found in animal foods and ingestion of MRSA-contaminated foods causes human infection. Therefore, it is very important to understand the role of contaminated foods. To elucidate the interaction between clinical MRSA FORC_062 and raw chicken breast, transcriptome analysis was conducted, showing that gene expressions of amino acid biosynthesis and metabolism were specifically down-regulated, suggesting that the strain may import and utilize amino acids from the chicken breast, but not able to synthesize them. However, toxin gene expressions were up-regulated, suggesting that human infection of *S. aureus* via contaminated food may be more fatal. In addition, the contaminated foods enhance multiple-antibiotic resistance activities and virulence factors in this clinical MRSA. Consequently, MRSA-contaminated food may play a role as a nutritional reservoir as well as in enhancing factor for pathogenesis and toxicity of clinical MRSA for severe food-borne outbreaks.

1. Introduction

Staphylococcus aureus is a well-known pathogen for human infection via cross contamination in hospitals or communities, causing cutaneous lesions, pneumonia, osteomyelitis, toxic shock syndrome, nausea, violent vomiting, and diarrhea (Lowy, 1998). Due to these virulence factors, clinical outbreaks were reported with 119,000 infections and almost 20,000 deaths in 2017 (Kavanagh, 2019). In addition, Staphylococcal food poisoning caused about 241,000 illnesses per year in the United States by the consumption of contaminated animal foods (Zeaki et al., 2019). In particular, it was reported that methicillin-resistant *S. aureus* (MRSA) strains are responsible for approximately 44% of cases and over 20% of excess mortality. Furthermore, the MRSA strains account for a proportion of more than 20% among *Staphylococcus aureus* isolates causing infections (Di Ruscio et al., 2019). Due to its pathogenicity and antibiotic resistance activity, understanding of its virulence factors at the genome level and the transcriptome level is urgently required to control the outbreaks caused by the pathogen (Baba et al., 2008).

Virulence factors of *S. aureus* are associated with adherence and invasion to a host cell surface, immune evasion system, type VII secretion system (T7SS), hemolysis, enterotoxin production, antibiotic resistance, and a toxin-antitoxin (TA) system. Host cell adherence to membrane glycoproteins and its invasion via host membrane by host tissue damage play a role in penetration to a host cell surface for bacterial infection, which is important as the first step in host cell infection (Foster et al.,

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2014; Priest et al., 2012). *S. aureus* has a specific immune evasion system to thwart the neutrophil in various ways and to produce capsular polysaccharides for efficient disruption of a host primary defense system (Foster, 2005; Nanra et al., 2013; van Kessel et al., 2014). In addition, T7SS is a complex protein system for secretion of several virulence factors including hemolysin, enterotoxin, and even antibiotic resistance proteins, which may be associated with promoting bacterial survival and the long-term persistence of staphylococcal abscess communities (Cao et al., 2016).

Hemolysin of *S. aureus* consists of three subunits: alpha (α), beta (β) and delta (δ) hemolysin proteins (Kielian et al., 2001). The α-hemolysin has an activity for pore formation in the blood cell membrane and δ-hemolysin for membrane lysis, associated with the formation of a spheroplast or a protoplast of blood cells (Bhakdi and Tranum-Jensen, 1991; Bhakoo et al., 1982; Freer and Birkbeck, 1982; Husmann et al., 2009). β-Hemolysin disrupts the formation by lysis activity (Glenny and Stevens, 1935; Huseby et al., 2007; Projan et al., 1989).

Recently, 17 types of staphylococcal enterotoxins (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ and SEU) were identified (Aydin et al., 2011). It has been suggested that they may have the biological effects of superantigens, causing toxic shock syndrome by initiating the activation and proliferation of T cells, pyrogenicity, enhancement of lethal endotoxin shock, and induction of inflammatory cytokines (Bohach et al., 1990; Marrack and Kappler, 1990; Miethke et al., 1992). In particular, SEA has been known to be associated with food poisoning causing gastroenteric syndrome in humans (Balaban and Rasooly, 2000; Letertre et al., 2003).

The staphylococcal cassette chromosome *mec* (SCC*mec*) is a mobile genetic element specific for *Staphylococcus*, associated with methicillin resistance activity. SCC*mec* contains two essential components, methicillin-resistant gene (*mecA*) complex and cassette chromosome recombinase (*ccr*) gene complex (Sani et al., 2014). The *mec* gene complex consists of *mecA* gene encoding a penicillin binding protein 2 A (PBP2A) with low affinity to beta-lactam antibiotics and regulatory genes. This low binding affinity to antibiotics endows the host with its antibiotic resistance activity by protection from the inhibition of cell wall synthesis, substantiating that the *mecA* gene is responsible for staphylococci resistant to penicillin-like antibiotics (Fogarty et al., 2015). The *ccr* gene complex (*ccr*C or the pair of *ccr*A and *ccr*B) encoding recombinases provides the mobility of a SCC*mec* genetic element on the host chromosome via excision, integration, and ligation (Huda et al., 2017). According to the combination of *mec* gene complex and *ccr* gene complex, SCC*mec* genetic element could be classified into types I to VIII (Kennedy and DeLeo, 2009; Zhang et al., 2009).

To further understand the virulence, pathogenesis, and antibiotic resistance of *S. aureus*, their genomes have been sequenced and their functionalities were analyzed according to the development of next-generation sequencing (NGS) technology (Durand et al., 2018; Nair et al., 2011; Zubair et al., 2015). To date (Sep 2019), complete genome sequences of 443 *S. aureus* strains are available in the GenBank database. Recently, the genome of *S. aureus* MCRF184 was completely sequenced and analyzed with bioinformatics, revealing that this genome has an enterotoxin gene cluster, a superantigen/hemolysin gene cluster, an immune evasion gene cluster, and a putative antimicrobial resistance gene cluster, regarding its pathogenesis and antibiotic resistance, but not a SCC*mec* genetic element (Aswani et al., 2019). This putative antimicrobial gene cluster consists of a type III restriction-modification (RM) system, efflux pump, acetyltrasferase, regulators, and mobile elements, probably instead of a SCC*mec* cluster for antibiotic resistance. Therefore, this *S. aureus* genome study is important to extend our knowledge on *S. aureus* virulence and pathogenesis activities for control of this pathogen.

According to the sources of MRSA, it categorized into three groups: healthcare-associated MRSA (HA-MRSA), community-associated MRSA (CA-MRSA), and livestock-associated MRSA (LA-MRSA) (Abolghait et al., 2020). HA-MRSA has been well-known human pathogen for infections in the hospitals. Because of its various antibiotic resistance, clinical treatment of HA-MRSA has been serious issues in the patients. Although it is a primary MRSA for human infections, it has decreased for the last decade, probably due to development of healthcare technology. However, CA-MRSA and LA-MRSA have increased every year. Therefore, clinical MRSA human infection via ingestion of the contaminated foods is probably getting worse and it is one of the major safety topics for human infections (Kluytmans, 2010). In particular, MRSA is generally transmitted to humans via various contaminated food, but it is not clearly understood the role of the MRSA for survival, propagation and even toxicity of clinical MRSA in the food environments, even though those properties of HA-MRSA has been widely studied for human infections (Sergelidis and Angelidis, 2017). In addition to clinical MRSA infection to human in hospitals, it has been often detected in contaminated chicken, indicating that clinical MRSA propagation and infection to human via food, which could be a threat to food safety and public health (Fox et al., 2017; Hennekinne et al., 2012). Therefore, it is necessary to understand the interaction between clinical MRSA and the food environment to control food poisoning. Due to the development of NGS technology and the accumulation of *S. aureus* genome information, transcriptome analysis has recently been available to extend our understanding of their interaction for food poisoning at the genomic level. The most recent paper reported that *S. aureus* increased utilization activities of amino acids and sugars from chicken breast, comparing with Luria-Bertani medium, suggesting that *S. aureus* can propagate in specific food as a nutrient reservoir and then could cause food poisoning via enterotoxin production after ingestion of the contaminated chicken breast (Dupre et al., 2019). Therefore, understanding of the behavior of *S. aureus* when exposed to raw chicken breast may be important to elucidate the survival and pathogenesis of *S. aureus* in frequently contaminated raw chicken for food safety.

In this study, a clinical isolate, *S. aureus* FORC_062, was isolated from an infected patient's blood sample. To understand its pathogenesis and antibiotic resistance at the genomic level, its genome was completely sequenced and compared with other *S. aureus* genomes, and its genome functionality was analyzed using bioinformatics tools. In addition, transcriptome analysis of FORC_062 with raw chicken breast was performed to elucidate its interaction and adaptation with a frequently contaminated food sample for survival and pathogenesis in the food as a reservoir or carrier for propagation. This result would be useful for providing a novel insight into pathogenic characteristics of *S. aureus* and the development of a new regulation approach for food safety.

2. Materials and methods

2.1. Strain isolation, growth conditions, and identification

S. aureus FORC_062 was obtained from Samsung Medical Center (Seoul, South Korea) and the strain was designated as FORC_062 by its deposition to the culture collection of the Food-borne Pathogen Omics Research Center (FORC). The methicillin-resistant *S. aureus* (MRSA) N315 and methicillin-sensitive *S. aureus* (MSSA) ATCC 29213 were obtained from a culture collection of the Department of Food Science and Biotechnology, Seoul National University (Seoul, South Korea). They were cultivated at 37 °C with shaking (220 rpm) in tryptic soy medium (TSB; Difco, USA) and modified M9 minimal medium containing histidine 0.004% (w/v) (Burke et al., 1972) and casamino acid 0.1% (w/v) (Lincoln et al., 1995), and the agar medium was prepared with 1.5% Bacto Agar (Difco, USA). Bacterial identification was performed using capillary sequencing of 16 S rRNA gene with a 27 F/1492 R universal primer set. A phylogenetic tree analysis of 16 S rRNA gene sequence was conducted using a MEGA6 program with the neighbor-joining method under 1000 bootstrap replicates. (Tamura et al., 2013).

2.2. Purification of DNA and RNA

Genomic DNA was extracted and purified using a DNeasy Blood & Tissue Kit (Qiagen, USA) according to the manufacturer's protocol. Total RNAs were isolated and purified using a miRNeasy Mini Kit and RNeasy MinElute Cleanup Kit (Qiagen) with the given standard procedures, and DNA was removed using TURBO DNase (2 U/μl concentration; AMbion, USA). The quality of the extracted RNA was confirmed using an Agilent 2100 Bioanalyzer with Agilent RNA 6000 Nano reagents (Agilent Technologies, Germany).

2.3. Polymerase chain reaction (PCR) and quantitative real-time PCR (qRT-PCR)

Previously reported PCR primers targeting *mecA, nuc* genes (Fang and Hedin, 2003; Mehrotra et al., 2000) were chemically synthesized and purified (Bionics, South Korea) and listed in Table S1. After DNA gel extraction, the sizes of PCR products were monitored using 1.5% agarose gel electrophoresis with Geldoc™ EZ Image (Bio-Rad, USA).

The extracted RNA was converted to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad, USA) and then Real-time PCR was performed with the cDNA a Chromo 4 Real-time PCR Detection system (Bio-Rad, USA) with SYBR Green I (Kim et al., 2013). The primers for qRT-PCR in this study are listed in Table S1. Normalization was conducted with the *recA* gene expression level as an internal reference (Ma et al., 2015). qRT-PCR data are presented as mean ± standard deviation of three independent experiments. The differences between groups were determined using two-tailed t-tests in SigmaPlot 12 (SYSTAT Software Inc., USA).

2.4. Next-generation sequencing (NGS)

Genome sequencing was conducted at ChunLab, Inc. (Seoul, South Korea) using a PacBio RS II system (Pacific Biosciences, USA), and assembled by PacBio SMRT Analysis 2.3.0 software (Pacific Biosciences) according to the manufacturers' protocols. After total RNA isolation, mRNA was purified using a Ribo-Zero™ rRNA Removal Kit (Epicentre, USA). Then, a cDNA library was generated from enriched mRNA using a TruSeq Stranded mRNA Sample Preparation Kit (Illumina, USA) following the manufacturer's instructions. The quality of the cDNA library was validated using an Agilent 2100 Bioanalyzer (Agilent Technologies).

2.5. Bioinformatics analyses

Open reading frames (ORFs) and annotations were predicted by the Rapid Annotation using Subsystem Technology (RAST) server (Aziz et al., 2008) and the GeneMarkS program (Besemer et al., 2001). The ribosome binding sites were predicted using a RBSfinder (J. Craig Venter Institute, USA). Functional analysis and categorization of the predicted ORFs was carried out using InterProScan 5 (Jones et al., 2014) and Clusters of orthologous groups (COGs) based WebMGA programs (Wu et al., 2011). The genome map was visualized using the GenVision program (DNASTAR, USA). Analyses of virulence factors and antibiotic resistance genes were conducted using the Virulence Factor Database (VFDB; Liu et al., 2019) and the Antibiotic Resistance Genes Database (ARDB; Liu and Pop, 2009). Bacteriophage composition analysis was performed using a PHASTER database (Arndt et al., 2016) and insertion sequences (IS) and transposons were identified using the ISfinder database (Siguier et al., 2006). The average nucleotide identity (ANI) analysis of FORC_062 was conducted to reveal the DNA-based sequence relationship with completely sequenced *S. aureus* strains using the JSpecies program and R program (Richter and Rossello-Mora, 2009). A comparative genome analysis between FORC_062 and N315 was conducted with the Artemis Comparison Tool (ACT) (Carver et al., 2005). RNA-Seq analysis was performed using CLC Genomics Workbench 7.5.1 (CLC Bio, USA). The COG numbers were used to produce the metabolic pathways of the *S. aureus* using the iPath program ver. 3 (Darzi et al., 2018).

2.6. Antibiotic susceptibility test

The susceptibility of FORC_062 to various antibiotics was identified by the agar disk diffusion method on Muller-Hinton agar (Difco) through the Kirby-Bauer disk susceptibility test (Hudzicki, 2009). Antibiotic discs (90 mm diameter; Oxoid, UK) were placed on Mueller-Hinton agar plates, incubated at 37 °C for 24 h, and the diameter of each zone was measured in millimeters. The following antibiotics discs were used in this study: amoxicllin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefazolin (30 μg), and tetracycline (30 μg).

2.7. Transcriptome condition on chicken breast for total RNA preparation

FORC_062 was grown to mid-log phase (OD600, 0.7) with the modified M9 minimal medium containing histidine 0.004% (w/v) and casamino acid 0.1% (w/v) at 37 °C. Then, 5 ml of FORC_062 ($1.41 \times 10^8 \pm 12.12$ CFU/ml) was inoculated to the 35 ml modified M9 media as a negative control or the modified M9 media containing 25 g of raw chicken breast as a test, respectively. The cultures were incubated at 37 °C for 2 h or 4 h. After incubation, the cells were harvested by centrifugation at 5000×*g* and 4 °C for 10 min. The pellets were resuspended in 5 ml 0.1% diethyl phosphorocyanidated-treated 1 x phosphate-buffered saline (0.1% DEPC-treated 1 x PBS), and the solutions were then mixed with 10 ml of RNAprotect Bacteria Reagent (Qiagen, USA) and incubated at room temperature for 5 min. Total RNA was prepared as mentioned above. In addition, viable cell count of FORC_062 in each sample using trypticase soy agar (TSA) plates was conducted at 0, 2, 4, and 6 h incubation time points, with the standard protocol (Li et al., 2014). All RNA sample preparations and viable cell counts were performed in triplicate.

2.8. Nucleotide sequence accession number

The whole genome sequence of FORC_062 was deposited in the GenBank of NCBI (http://www.ncbi.nlm.nih.gov/genbank) under the accession number CP022582 for the chromosome. Raw RNA sequence information for transcriptome analysis in FORC_062 was deposited in the NCBI Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA508464.

3. Results

3.1. General characteristics and genome properties of S. aureus FORC_062

FORC_062 was isolated from human patient blood and identified as *S. aureus* by 16 S rRNA sequence analysis (data not shown). In addition, PCR was performed with *mecA* and *nuc* gene-targeting primer sets (Fang and Hedin, 2003; Mehrotra et al., 2000), showing that this strain has methicillin-resistant *Staphylococcus aureus* (MRSA) specific genes (Fig. S1). A subsequent antibiotic screening test with methicillin resistance (oxacillin resistance) confirmed its methicillin resistance activity, following the procedure of the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2015) (Fig. S2).

The genome of FORC_062 has a one circular double-stranded DNA chromosome with 2,905,353 bp with a GC content of 32.92%. The chromosome contains 2721 predicted open reading frames (ORFs), 60 tRNA genes, and 5 rRNA operons with an extra 5 S rRNA gene. Among them, 2293 ORFs (84.27%) were predicted to be functional, and 428 ORFs (15.73%) were predicted to be unknown (Table S3). The genome of FORC_062 contains several MGEs and they have type II SCC*mec* (staphylococcal cassette chromosome *mec*; FORC62_0030 -

FORC62_0054), transposons/IS elements, prophage, or pathogenicity island (PI) in the genome. In addition, it has one complete transposable element (Tn*554*) and many other mobile elements (IS3, ISL3, IS1182, and IS6). Three complete prophages were detected in the genome (Fig. 1) and one of them has a type II toxin-antitoxin (TA) system. Three pathogenicity islands (PIs) of SaPIn1, SaPIn2, and SaPIn3 were also detected (Fig. 1) and they encode various kinds of virulence factors. These MGEs are summarized in Table S2.

3.2. Pathogenesis and virulence factors

To determine the virulence features of FORC_062, virulence factors were found in the genome annotation result using the virulence factor database (VFDB) (Chen et al., 2005) and they were categorized into five groups: antibiotic resistance, biofilm development, host immune evasion, secretion systems, and toxins (Table 1). The results of genome annotation data and the Antibiotic Resistance Genes Database (ARDB) search showed that this genome has 15 antibiotic resistance genes containing methicillin, tetracycline, and fluoroquinolone, confirming that this strain is MRSA.

For biofilm generation, the genome has host adhesion related genes, serine protease-related genes, and cysteine protease-related genes. For host adhesion, it has autolysin encoding gene *atl* (FORC62_1014), fibrinogen-binding protein encoding genes *efb* (FORC62_1184), *fnbB* (FORC62_2508), and *fnbA* (FORC62_2509), and clumping factor encoding genes *clfA* (FORC62_0778), and *clfB* (FORC62_2638), suggesting that these genes may be responsible for survival and pathogenicity. In addition, serine protease related genes *splA* (FORC62_1900), *splB* (FORC62_1899), *splC* (FORC62_1898), *splD* (FORC62_1897), *splF* (FORC62_1896), and *sspA* (FORC62_1010) were detected, which may be related to virulence roles in its colonization or infection to the host (Paharik et al., 2016). Furthermore, cysteine protease-related *sspB* (FORC62_1009) and *sspC* (FORC62_1008) were detected, probably for *S. aureus* biofilm development. In addition, bacterial capsular polysaccharide synthesis genes were found, which might be important for host immune evasion of *S. aureus*.

The genome of FORC_062 has various related genes (FORC62_0141-FORC62_0156) in a gene cluster. The FORC_062 genome has a type VII secretion system in a gene cluster containing *esxA* (FORC62_0274), *esaA* (FORC62_0275), *essA* (FORC62_0276), *esaB* (FORC62_0277), *essB* (FORC62_0278), *essC* (FORC62_0279), *esaC* (FORC62_0280), and *esxB* (FORC62_0281). The membrane and cytoplasmic proteins cooperate to export the effector proteins during host infection, and the virulence pathway plays a key role in promoting bacterial survival and pathogenesis (Lopez et al., 2017). In this strain, various toxin-related genes were detected in the genome, encoding hemolysin, staphylococcal scalded skin syndrome-related protein, and toxic shock syndrome-related protein. For hemolysin, several genes encoding α-*hly/hla* FORC62_1190 and FORC62_1191), β-*hlb* (FORC62_2026), δ-*hld* (FORC62_2057a), γ-*hlgA* (FORC62_2428), *hlgC* (FORC62_2429), and *hlgB* (FORC62_2430) were found. In particular, the staphylococcal scalded skin syndrome-related gene *eta* (FORC62_1200) and the toxic shock syndrome-related gene *tsst* (FORC62_2034) were also found in the genome. Therefore, these virulence factors may contribute to the host pathogenicity and virulence as pathogenic features of FORC_062.

3.3. Comparative genome analysis of S. aureus clinical isolates and their different antibiotic resistance activities

To figure out the evolutionary relationship of FORC_062 and other *S. aureus* strains at the genomic level, comparative ANI tree analysis with clinical isolates was conducted, showing that it belongs to a separate ANI phylogenetic tree group containing N315, Mu3, MU50, JH1, and JH9 (Fig. 2). All strains in this group are MRSA. Interestingly,

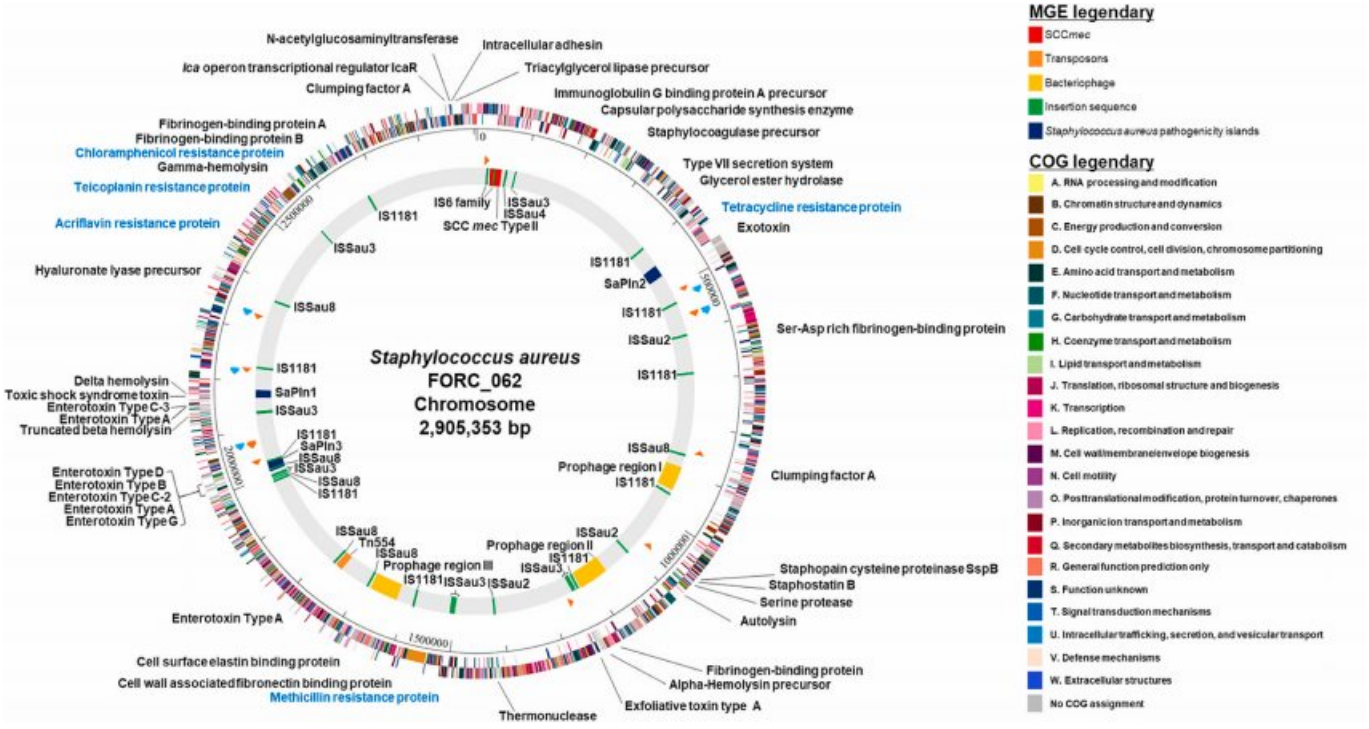

Fig. 1. Genome map of *S. aureus* FORC_062. The outer circle indicates the locations of all annotated ORFs, and the inner circle with the red peaks indicates the GC content. Between these circles, the sky blue arrows indicate the rRNA operons, and the orange arrows indicate tRNAs. The GC skew (C–G)/(C + G) were calculated in the innermost circle (purple and green). All annotated ORFs are colored differently according to the Clusters of Orthologous Groups (COG) assignments. Genes with specialized functions are labeled with different colors as follows: resistance-related genes (blue) and virulence-related genes (black). The mobile genetic elements (MGE) were indicated on the base of the genome map of FORC_062. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Detected virulence factors of *S. aureus* FORC_062 against the Antibiotic Resistance Genes Database (ARDB)[a] and the Virulence Factor Database (VFDB)[b].

Virulence factor	Annotation	Location (Locus_tag)	Function
Antibiotic			
gyrA	DNA gyrase subunit A	6488–9157 (FORC62_0007)	Fluoroquinolone resistant
mecI, mecR1, mecA	Methicillin resistant	36,958–41,192 (FORC62_0030 - FORC62_0032)	Methicillin resistant PBP2
ermA	rRNA adenine N-6-methyltransferase	50,773–51,504 (FORC62_0044) 1,833,576–1,834,307 (FORC62_1747)	Erm 23 S ribosomal RNA methyltransferase
tet38	Tetracycline resistance protein	146,405–147,763 (FORC62_0129)	Major facilitator superfamily (MFS) antibiotic efflux pump
mepR, mepA	Multidrug and toxic compound extrusion (MATE) transporter	374,219–376,100 (FORC62_0323 - FORC62_0325)	Multidrug and toxic compound extrusion (MATE) transporter
tetM	Tetracycline resistance protein TetM	434,141–436,060 (FORC62_0390)	Tetracycline-resistant ribosomal protection protein
fusA	Translation elongation factor G	609,998–612,079 (FORC62_0540)	Antibiotic resistant fusA
mgrA	Organic hydroperoxide resistance transcriptional regulator	756,851–757,294 (FORC62_0676)	ATP-binding cassette (ABC) antibiotic efflux pump
ileS	Isoleucyl-tRNA synthetase	1,274,260–1,277,013 (FORC62_1220)	Antibiotic resistant isoleucyl-tRNA synthetase
parC	Prephenate dehydrogenase	1,459,268–1,461,670 (FORC62_1389)	Fluoroquinolone resistant
arlS, arlR	Major facilitator superfamily (MFS) antibiotic efflux pump	1,525,189–1,527,200 (FORC62_1448 - FORC62_1449)	Major facilitator superfamily (MFS) antibiotic efflux pump
Adherence			
atl	Autolysin	1,084,991–1,088,737 (FORC62_1014)	Autolysin
ebh	Extracellular matrix-binding protein	1,561,143–1,572,815 (FORC62_1468) 1,540,941–1,561,082 (FORC62_1467)	Fibronectin binding
clfA, clfB	Clumping factors	869,632–872,079 (FORC62_0778), 2,808,317–2,810,950 (FORC62_2638)	Fibrinogen binding
ebp	Cell surface elastin binding protein	1,621,805–1,623,265 (FORC62_1512)	Elastin binding protein
efb	Fibrinogen-binding protein	1,238,713–1,239,210 (FORC62_1184)	Fibrinogen binding proteins
fnbA, fnbB	Fibronectin-binding protein	2,665,057–2,671,739 (FORC62_2508 - FORC62_2509)	Host cell attachment
icaA, icaB, icaC, icaD, icaR	Intracellular adhesin	2,859,795–2,863,934 (FORC62_2673 - FORC62_2677)	Intracellular adhesin
sdrC, sdrD, sdrE	Ser-Asp repeat protein	626,721–637,657 (FORC62_0554 - FORC62_0556)	Attachment
spa	Staphylococcal protein A	117,420–118,772 (FORC62_0104)	Staphylococcal protein A
Exoenzyme			
geh	Glycerol ester hydrolase	359,702–361,777 (FORC62_0310)	Lipase
hysA	Hyaluronate lyase	2,370,123–2,372,552 (FORC62_2218)	Hyaluronate lyase
	Serine protease		Autolysis
splA, splB, splC, splD, SplF		2,008,777–2,012,825 (FORC62_1896 - FORC62_1900)	
sspA	Serine protease	1,080,297–1,081,325 (FORC62_1010)	Autolytic
sspB	SspB	1,079,034–1,080,215 (FORC62_1009)	Pathogenesis
sspC	Staphostatin B	1,078,667–1,078,996 (FORC62_1008)	Cysteine protease
coa	Staphylocoagulase	259,445–261,421 (FORC62_0221)	Promotes pathogenesis
nuc	Thermonuclease	1,424,373–1,424,906 (FORC62_1356)	Thermonuclease
lip	Triacylglycerol lipase	2,864,269–2,866,314 (FORC62_2678)	Lipase
Host Immune evasion			
-	Capsular polysaccharide synthesis	162,180–178,748 (FORC62_0141 - FORC62_0156)	Antiphagocytosis:
Secretion system			
esxA, esaA, essA, esaB, essB, essC, esaC, esxB	Type VII secretion system	321,827–332,464 (FORC62_0274 - FORC62_0281)	Secretion
Toxin			
hla	Alpha hemolysin	1,243,513–1,244,471 (FORC62_1190 - FORC62_1191)	Cellular damage
hlb	Beta hemolysin	2,153,026–2,153,849 (FORC62_2026)	Beta hemolysin
hld	Delta hemolysin	2,182,522–2,182,656 (FORC62_2057a)	Lysing erythrocytes
sec, seg, sei, set7, set7, set6, set7, set8, set9, set10, set11, set12, set13, set14, set15	Exotoxin	461,137–474,861 (FORC62_0415 - FORC_0426), 2,161,498–2,162,298 (FORC62_2032), 2,024,018–2,024,794 (FORC62_1911), 2,026,796–2,027,524 (FORC62_1915)	Food-poisoning
yent1, yent2, sell, selm, seln, selo	Enterotoxin	2,025,077–2,029,341 (FORC62_1912 - FORC62_1917), 2,160,607–2,161,329 (FORC62_2031)	Food poisoning
Eta	Exfoliative toxin type A	1,253,292–1,254,239 (FORC62_1200)	Exfoliative toxin
hlgA, hlgB, hlgC	Gamma hemolysin	2574646–2,578,068 (FORC62_2428 - FORC62_2430)	Pore formation
lukD, lukE	Leukotoxin	2,016,955–2,018,875 (FORC62_1905 - FORC62_1906)	Pore formation
Tsst	Toxic shock syndrome toxin	2,164,524–2,165,228 (FORC62_2034)	Toxic shock syndrome

[a] , Liu, B., Pop, M., 2009. ARDB–Antibiotic Resistance Genes Database. Nucleic Acids Res. 37, D443-D447.

[b] , Liu, B., Zheng, D., Jin, Q., Chen, L., Yang, J., 2019. VFDB 2019: a comparative pathogenomic platform with an interactive web interface. Nucleic Acids Res. 47, D687–D692.

comparative genome analysis revealed that group-specific staphylococcal cassette chromosome *mec* (SCC*mec*) type II (2 A), and other MRSA strains in the ANI tree belong to SCC*mec* type I (1 B), type IVa (2 B), or type IVc (2 B). Subsequent comparative analysis of SCC*mec* gene clusters in this group showed that they have *mecI* (transcriptional repressor), *mecR1* (transcriptional regulator), and *mecA* (low affinity penicillin-binding protein) for resistance activity against methicillin and

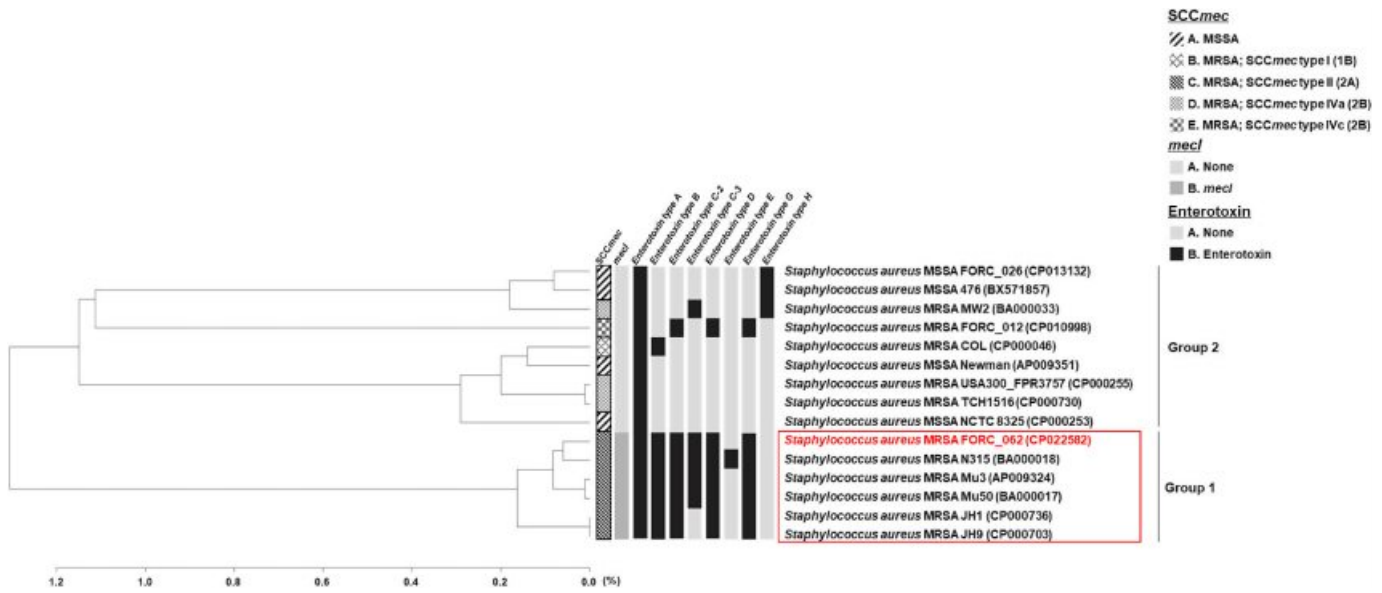

Fig. 2. Genome tree of completely genome sequenced *S. aureus* strains was obtained based on the average nucleotide identity (ANI) values. The ANI values were calculated using JSpecies, with nucleotide fragment length of 1020 bp, based on the BLAST algorithm. The tree was constructed using R software. Scale bar estimated substitutions per site.

oxacillin. However, SCC*mec* gene clusters in other strains have only *mecR1* and *mecA* (Fig. 2). Therefore, presence of *mecI* may be a key component for this group. Furthermore, comparative genome analysis of MRSA strains in this group as well as other clinical isolates showed that the group strains have many different types of enterotoxin genes. Notably, all strains in this group have enterotoxin type A, type B, type C-2, type D, and type G, suggesting their high toxicity. However, other strains have only enterotoxin type A. Interestingly, enterotoxin type H is present only in MSSA strains FORC_026, MSSA475, and MW2 in another group (Fig. 2). Therefore, the presence of multiple types of enterotoxin genes or the presence of enterotoxin type H may be important key components for differentiation of this group from other strains. Consequently, these three properties, type of SCC*mec, mecI* and multiple types of enterotoxin genes may be characteristic features of this group.

Among them, N315 is the most closely related strain to FORC_062 with a 99.94% ANI value. The methicillin-resistant *S. aureus* (MRSA) N315 is a clinical isolate from a pharyngeal smear of a Japanese patient (Kuroda et al., 2001). Comparative genome analysis between FORC_062 and N315 showed two major unique regions ranging from positions 430, 346 to 455,700 nt (FORC62_0385 to FORC62_0408) and from 1,639,530 to 1,675,318 nt (FORC62_1531 to FORC62_1583) in the genome of FORC_062 (Fig. S3). These unique regions contain tetracycline resistance gene *tetM* (FORC62_0390), indicating antibiotic resistance activities against tetracycline and minocycline (Warsa et al., 1996), and an additional type II TA system (FORC62_1582), associated with the reduction of β-lactam susceptibility of *S. aureus* (Schuster et al., 2015), respectively. To confirm the antibiotic resistance activities of FORC_062 in comparison with those of N315, their antibiotic susceptibility tests were performed and compared using the Kirby-Bauer Disk method (Hudzicki, 2009). Surprisingly, FORC_062 is more resistant to tetracycline and various β-lactam antibiotics (amoxicillin, cefazoline, cefotaxime, and ceftazidime) (Fig. S4).

3.4. Identification of differentially expressed genes of FORC_062 under exposure to raw chicken breast

Chicken breast has lots of proteins and lipids but a very low amount of carbohydrate (Murphy et al., 1998). In this nutritional condition, it is necessary to understand how *S. aureus* FORC_062 adapts to chicken breast for survival after contamination. Interestingly, the growth rate of a test group with raw chicken breast was much faster than that of a control group without raw chicken breast, suggesting that it has many nutrients required in the growth of *S. aureus* (Fig. S5). To demonstrate this, global gene expression changes of FORC_062 were monitored and compared at the transcriptome level using RNA-Seq analysis, when it was exposed to raw chicken breast, and the results of RNA sequencing were re-confirmed using qRT-PCR (Fig. S6). The comparative RNA-Seq analysis revealed upregulation of amino acid/dipeptide transporters, deamination of amino acids, carbohydrate metabolism, lipid metabolism, enterotoxin production, and antibiotic resistance, as well as the downregulation of amino acid biosynthesis (Fig. 3). Among protein-associated transporters, gene expressions regarding only dipeptide or amino acid transporters were upregulated, suggesting that *S. aureus* may selectively uptake only amino acids or small peptides from the raw chicken breast, and not large molecules of polypeptides or proteins. Probably due to the uptake of various amino acids, gene expressions regarding biosynthesis of amino acids might be suppressed. After uptake, they may be deaminated and converted to short carbohydrates, and then gene expression regarding carbohydrate metabolism was upregulated, and probably associated with energy production and cell growth. In addition, in the lipid metabolism, gene expressions regarding long-chain and short-chain lipid degradations were upregulated, suggesting that lipids may be degraded to glycerol and fatty acids, which could be utilized in carbohydrate metabolism. Unexpectedly, during the incubation of FORC_062 in the raw chicken breast, gene expressions regarding enterotoxins, type VII secretion system, and tetracycline resistance protein were highly upregulated, suggesting that contamination of *S. aureus* to the raw chicken breast could cause foodborne outbreaks by toxin production and antibiotic resistance.

4. Discussion

So far, pathogenesis and toxicity of *S. aureus* as a clinical or foodborne pathogen have been performed *in vitro* in the laboratories. Although hundreds of complete or draft genome sequences of *S. aureus* are available in public databases, its molecular mechanisms for infection, pathogenicity, and cytotoxicity remain unknown. According to the rapid development of next-generation sequencing (NGS) technologies and their broad applications, it is now possible to extend our knowledge on its host infection, molecular response, and interaction mechanisms

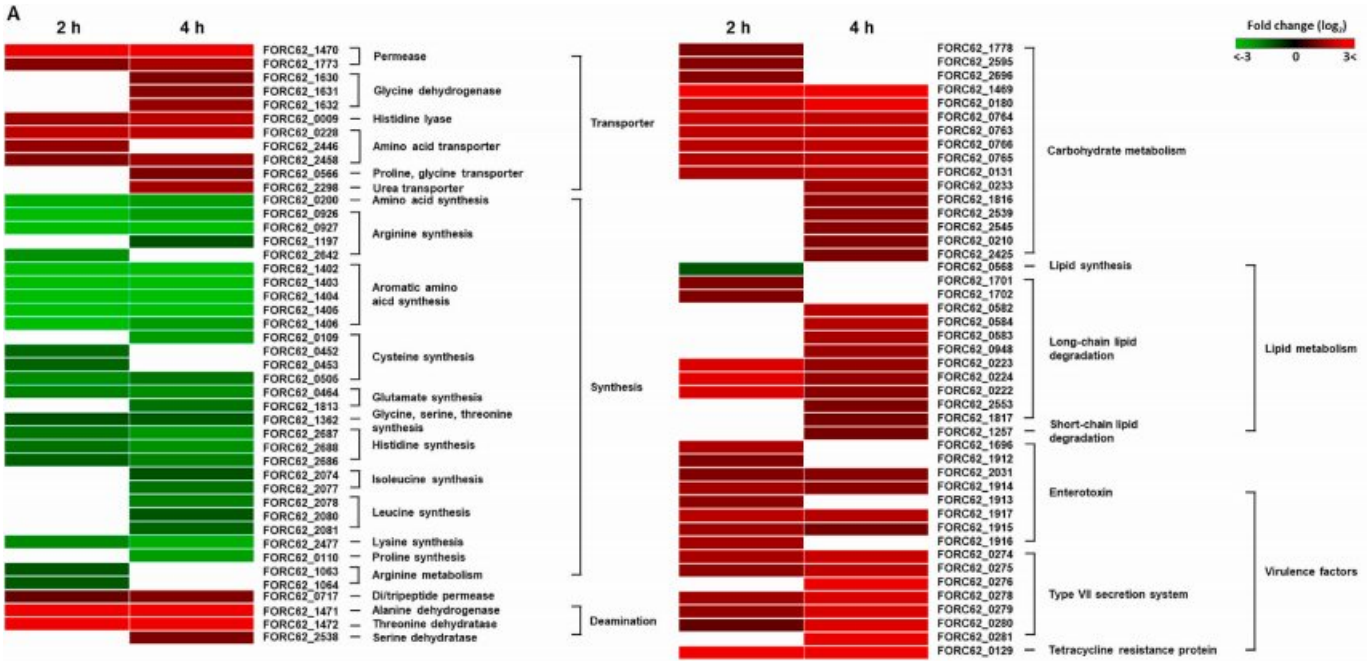

Fig. 3. (A) Heat map of DEGs under exposure to raw chicken breast. The results of RNA-Seq analysis were depicted using the heat map. The red bars indicated the up-regulated genes, and the green bars indicated the down-regulated genes. The genes were categorized according to their functions. The results of both 2 h infection and 4 h infection were shown. The scale bar is in the upper right of the page. The heat map was constructed using Gitools (Perez-Llamas and Lopez-Bigas, 2011). In addition to the heat map, amino acid biosynthesis and metabolism-associated gene expression profiles for 2 h infection (B) and 4 h infection (C) were depicted in the overall metabolic pathways of *S. aureus* using the iPath program ver. 3 with the clusters of orthologous groups (COGs) category of FORC_062. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

with the related microbiota or given environments. In particular, epidemiological studies of *S. aureus* infection via food consumption do not provide enough information on proliferation and toxin production of *S. aureus* in food. It is important to understand where cell proliferation and toxin production happen after infection of *S. aureus* in food or in a human host. To clarify this, further omics studies for *S. aureus* contamination to food and its clinical infection to the host at the genome level and the transcriptome level need to be conducted. For the extended omics study, *S. aureus* FORC_062 was initially isolated from a human blood sample in South Korea and its genome was completely sequenced and analyzed with bioinformatics tools.

It is well-known that *S. aureus* has various mobile genetic elements (MGEs) in its genome, which may be closely related to bacterial evolution, probably due to genome plasticity and acquisition of virulence factors (Alibayov et al., 2014). FORC_062 contains several MGEs, including type II SCC*mec* (staphylococcal cassette chromosome *mec*; FORC62_0030 - FORC62_0054), transposons/IS elements, prophage, or pathogenicity island (PI) in the genome. The genome of FORC_062 also has one complete transposable element (Tn*554*) and many other mobile elements (IS3, ISL3, IS1182, and IS6), which is probably associated with genome plasticity and adaptation via gene transfer, acquisition, or deletion (Alibayov et al., 2014; Kaya et al., 2018). In addition, a prophage has a type II toxin-antitoxin (TA) system, suggesting specific gene transfer and integration in the genome (Deghorain et al., 2012; Kwan et al., 2005). The presence of three pathogenicity islands (PIs) of SaPIn1, SaPIn2, and SaPIn3, which encode various kinds of virulence factors.

Among the *S. aureus* strains, MRSA is the critical antibiotic-resistant strain, which needs to be controlled for safety. Antibiotic resistance has been known to be associated with the presence of a SCC*mec* mobile genetic element including *mecA* gene (a penicillin binding protein 2 A) and *ccr* genes (cassette chromosome recombinase), providing the beta-lactam antibiotic resistance activity and the mobility of SCC*mec* genetic element, respectively. The *mec* gene complex consists of *mecA, mecR1,* and *mecI*. Based on this combination, the *mec* gene complex is divided into three classes: Class A (*mecA, mecR1, mecI*, and IS*431* downstream of *mecA*); Class B (*mecA,* truncated *mecR1,* and IS*431* downstream of *mecA*); and Class C (*mecA* and truncated *mecR1* by IS*431*). Class A and Class B are accompanied with *ccr* gene complex of *ccrA* and *ccrB*. However, Class C is accompanied with only *ccrC*. The SCC*mec* of FORC_062 belongs to the type II SCC*mec* element containing the Class A *mec* gene complex with *ccrA* and *ccrB* like N315 (Ito et al., 2009). According to the recent classification of SCC*mec* elements (Mitsumoto-Kaseida et al., 2017), they could be divided into health care-associated MRSA (HA-MRSA) for types I, II, and III, and community-associated MRSA (CA-MRSA) for types IV and V. Therefore, the SCC*mec* of FORC_062 also belongs to the HA-MRSA group. Interestingly, it was suggested in a recent paper that type II MRSA (HA-MRSA) might be more pathogenic and resistant to β-lactam antibiotics than type IV MRSA (CA-MRSA), supporting pathogenesis of FORC_062 as a clinical isolate (Mitsumoto-Kaseida et al., 2017). In addition, the presence of a *tetM* gene in the chromosome and experimental confirmation showed the phenotype of tetracycline resistance activity (Fig. S4).

Toxins of *S. aureus* include the membrane-damaging toxins (receptor-mediated and non-receptor-mediated toxins), receptor function-interfering toxins, and toxin-like enzymes. Membrane-damaging toxins cause pore formation in the membrane for loss of vital molecules and metabolites, indicating cytolytic toxins (Otto, 2014). This cytolysis can be categorized into two subgroups: receptor-mediated and receptor-independent. According to the association with the receptor, target cell specificities of the cytolysis-related toxins are determined. Receptor-mediated toxins such as α-toxin, γ-toxin, and leukotoxin recognize and bind to the toxin-specific receptor in the membrane and then the toxins produce pores for cytolysis (Oliveira et al., 2018). FORC_062 has α-toxins (FORC62_1190–1191), γ-toxins (FORC62_2428–2430), and leukotoxins (FORC62_1905–1906),

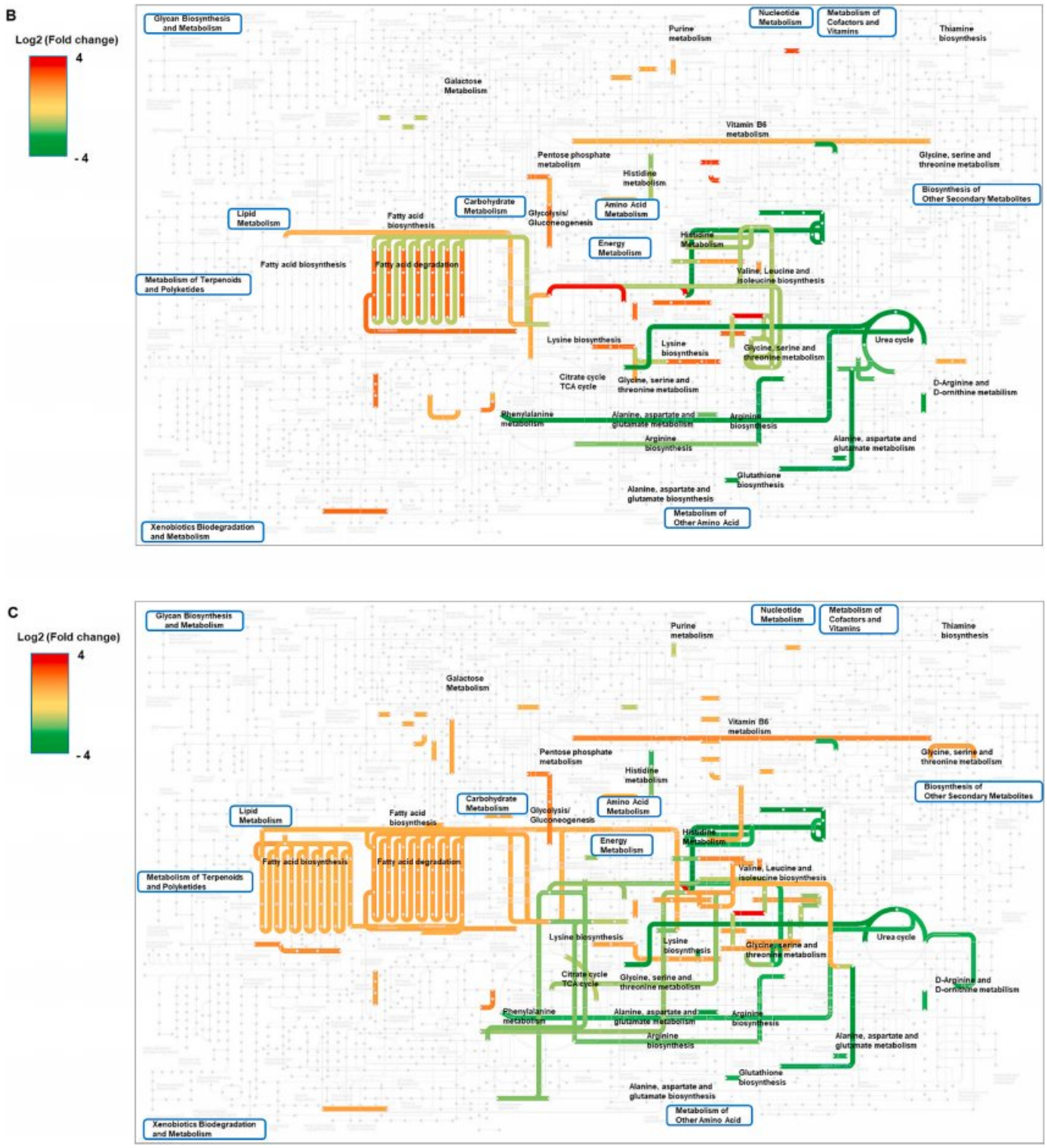

Fig. 3. (*continued*).

indicating that this strain has the capability of pore formation. In addition, receptor-independent toxins such as phenol-soluble modulins (PSMs) and λ-toxins bind to the membrane, and then play a role in the disruption of the membranes for transient pore formation without receptor interaction (Wang et al., 2007). FORC_062 has only λ-toxin (FOR62_2057a), but not PSMs in the genome, suggesting that λ-toxins may contribute to the formation of transient pore formation with low specificity. The receptor-interfering toxins contain *S. aureus* enterotoxins and enterotoxin-like toxins, which may be responsible for the interference with receptor functions in the intestine, causing emesis and diarrhea (Hennekinne et al., 2012). While it its known that *S. aureus* has ~20 enterotoxins, their mechanisms are not fully understood. However, it was suggested that they might be associated with the activation of cytokine release and cell death by apoptosis (Lin et al., 2010). FORC_062 has enterotoxin A (FORC62_1696–1697, 1912, 2031), B (FORC62_1914), C (FORC62_1913, 2032), D (FORC62_1917), and G (FORC62_1911). Among them, staphylococcal enterotoxin B was suggested to be a biological weapon and enterotoxin C to promote infective

endocarditis sepsis as well as kidney failure (Salgado-Pabon et al., 2013; Zapor and Fishbain, 2004), indicating the toxicity of *S. aureus* FORC_062. In addition, this strain has a toxic shock syndrome toxin TSST (FORC62_2034), which stimulates the release of pro-inflammatory cytokines (IL-1, IL-2, and TNF-α) (Wilson et al., 2011). The toxin-like enzymes contain proteases (aureolysin, glutamyl endopeptidase SspA, and staphopain cysteine proteinase SspB), staphylokinase, coagulases (staphylocoagulase and von Willebrand factor (vWF)), β-toxin, lipases, and nucleases (Brinkmann and Zychlinsky, 2012; Jusko et al., 2013; Kwiecinski et al., 2013; Novick et al., 2001; Thomer et al., 2013). FORC_062 has SspA (FORC62_1010), SspB (FORC62_1009), staphylocoagulase (FORC62_0221), vWFs (FORC62_0104, 0779, 0781), β-toxin (FORC62_2026), lipases (FORC62_0310, 2678), and thermonuclease (FORC62_1356), indicating additional toxicity of FORC_062. Consequently, a clinical isolate of *S. aureus* FORC_062 has a variety of toxins and toxin-like enzymes, substantiating its pathogenesis and toxicity to the host cells.

The toxin–antitoxin (TA) system is a small genetic element consisting of a stable toxin gene and its unstable cognate antitoxin for programmed cell death (PCD) (Engelberg-Kulka et al., 2005). Among the six types of TA systems, the type II TA system MazEF is a well-known bacterial PCD system, consisting of a MazF stable toxin and a MazE labile antitoxin. MazF is an mRNA endonuclease for the initiation of the PCD pathway by various stresses and MazE interacts with MazF to block the initiation of the PCD pathway. Therefore, the MazEF-mediated PCD pathway can be a defense mechanism over bacteriophage infection. In addition, a previous study reported that the deletion mutant of MazEF was more susceptible to β-lactam antibiotics, suggesting that this system may be involved in antibiotic resistance (Schuster et al., 2015). FORC_062 has one copy of MazEF (FORC62_2089–2090) in the chromosome, suggesting that FORC_062 has a regulation capability of the PCD pathway and probably the enhancement of antibiotic resistance.

To elucidate the interaction between MRSA FORC_062 and a specific food sample, transcriptome analysis of total mRNA on the surface of the raw chicken breast was performed. The environment of the raw chicken breast is a nutritionally rich condition, containing proteins, lipids, carbohydrates, and water (Marchi et al., 2012). Transcriptome analysis revealed specific gene expression profiles, and the upregulation of metabolisms of carbohydrates, lipids, and long-chain fatty acids, but the downregulation of amino acid biosynthesis and metabolism (Fig. 3). These results suggest that this strain imports and utilizes amino acids from the raw chicken breast, but does not synthesizes them, while it utilizes other nutrients, carbohydrates and lipids from this nutrient reservoir. Interestingly, the productions of virulence factors such as enterotoxins, tetracycline resistance protein, and the type VII secretion system were enhanced during its contact to the raw chicken breast, suggesting that clinical MRSA infection to human via specific contaminated food may be fatal. Consequently, the roles of the contaminated food may be a nutritional reservoir as well as a specific environment enhancing for pathogenesis and toxicity of clinical MRSA for food-borne outbreaks. Therefore, it is necessary to study the interaction between clinical MRSA and chicken breast in molecular level to understand the nutrition utilization from the food and enhanced pathogenesis and toxicity from clinical MRSA. These results suggest that ingestion of the clinical MRSA-contaminated chicken breast causes severe human infections, which is the clinical MRSA-associated fatal food-borne outbreak. Finally, these results suggest that the contaminated foods contribute to clinical MRSA as a nutrition reservoir as well as enhancing factor for more pathogenesis and more toxicity in food environment. Therefore, pathogenesis and toxicity of methicillin-resistant *S. aureus* FORC_062 were investigated at the genome level and the transcriptome level. Furthermore, protection of fresh foods from infection of clinical MRSA as well as rapid detection and identification of clinical MRSA in foods before consumption may be critical for the prevention of food-borne outbreaks. These genome and transcriptome studies of *S. aureus* would be useful for extension of our understanding on its pathogenesis and toxicity at the molecular level and would provide further molecular insights on a scientific prevention method against a fatal food-borne outbreak by clinical MRSA contamination.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2020.103602.

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