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Research paper

Genome comparisons of two Taiwanese community-associated methicillin-resistant *Staphylococcus aureus* ST59 clones support the multi-origin theory of CA-MRSA

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ABSTRACT

Sequence type (ST) 59 is an epidemic lineage of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) in Asia. Two ST59 clones are prevalent in Taiwan: the Taiwan clone (TW) causes severe infections, whereas the Asian-Pacific clone (AP) is usually commensal. In this study, we sequenced the genome and transcriptome of the representative strains of these two clones and found their differences to focus on three mobile genetic elements: TW carries SCC*mec* Type V_T, Panton-Valentine leucocidin (PVL)-encoding prophage ΦSa2, whereas AP carries SCC*mec* Type IV and staphylokinase (SAK)-encoding prophage ΦSa3. The anti-virulent role of SAK was confirmed using murine skin and bloodstream infection models. ΦSa3 usually integrates into the *hlb* gene, but in AP was found to be integrated at the genomic island νSaβ. The mutation of the *attB* site "TGTATCCAAACTGG" to "TGTATCCGAATTGG" led to a failure in the integration of ΦSa3 in *hlb*, prompting atypical integration at other sites. The *sak* gene possessed remarkably different patterns of distribution among the different STs of *S. aureus*. We conclude that the atypical integration of ΦSa3 may help *S. aureus* adapt to the human host habitat and that the subsequent loss of ΦSa3 contributes toward the development of a virulent CA-MRSA lineage for wider horizontal transmission.

1. Introduction

During the past two decades, community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) has become a successful pathogen that poses serious threats to public health worldwide (David and Daum, 2010). As an example, two ST59 clones of CA-MRSA are currently circulating in Taiwan's community: the "Taiwan" clone (TW) can elicit sepsis and accounts for nearly 70% of the CA-MRSA infections in children (Huang et al., 2008), whereas the "Asian-Pacific" clone (AP) is usually commensal and responsible for 80% of the colonizing isolates sampled from the noses of healthy individuals (Huang et al., 2005). The respective abundance of these two related clones identified from the same geographical region and isolated in patients with distinct clinical symptoms provides an excellent opportunity to explore the factors implicated in CA-MRSA pathogenesis.

Genomic comparisons employing microarray-based DNA hybridization have revealed that TW carries the staphylococcal chromosomal cassette *mec* (SCC*mec*) Type VT and prophage ΦSa2, but that AP carries SCC*mec* Type IV and prophage ΦSa3 (Chen et al., 2013). Because SCC*mec*, the central determinant for broad-spectrum-lactam resistance, is not directly associated with pathogenicity, the differential virulence traits probably result from the remaining two prophages. ΦSa2 encodes Panton-Valentine leukocidin (PVL), a β-pore-forming cytotoxin that can facilitate necrotizing skin infections by allowing the bacteria to escape from endosomes, replicate intracellularly, and induce apoptosis (Chi et al., 2014). As specifically found in the majority of CA-MRSA isolates, PVL is often believed to be a key characteristic determinant of CA-MRSA (Otto, 2010). In contrast, ΦSa3 has been identified in most lineages of *S. aureus* at a common integration site within the *hlb* gene, which encodes β-haemolysin (Pantucek et al., 2004; van Wamel et al., 2006). One of its products, staphylokinase (SAK), is able to form a complex with human plasminogen and thus promote its conversion into plasmin (Collen and Lijnen, 1994; Okada et al., 2000). While SAK enhances the bacterial colonization capacity in human hosts, it might meanwhile decrease these microorganisms' virulence potential due to the plasmin-mediated degradation of the extracellular

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matrix depriving bacteria of a necessary adherent surface (Kwiecinski et al., 2013, 2010).

Although the anti-virulent role of SAK has been proposed by the above studies, it has not yet been confirmed whether a lack of SAK would cause an increase in the invasiveness in ST59 CA-MRSA, particularly in the TW clone. If so, what are the underlying genetic mechanisms for the specific presence of the SAK-encoding ΦSa3 in AP but not in TW? What is the impact of this event on the evolution of CA-MRSA in Taiwan? To address these issues, we applied multiple approaches, including genome and transcriptome sequencing, congenic mutant construction, and animal experiments to reach our final conclusion that SAK may contribute to the appearance of the endemic CA-MRSA clone TW in Taiwan.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains used in this study, i.e., SA957, SA44, SA40 and SA266, were collected from Chang Gung Memorial Hospital, a tertiary hospital in Taiwan. The former two strains were isolated from patients with disseminated *S. aureus* infections and were classified as TW; the latter two strains were isolated from the noses of healthy individuals and classified as AP. In particular, SA957 and SA40 are the representative strains for TW and AP, respectively, in terms of their PFGE bands, clinical manifestations and antimicrobial resistance profiles (Chen et al., 2013; Huang et al., 2008).

2.2. Genome sequencing and analyses

The bacterial genomic DNA was extracted via QIAamp DNA Mini Kit (Qiagen, CA, USA). The genome of SA957 was sequenced by the Illumina Hiseq2000 sequencer (Illumina, CA, USA) and was de novo assembled utilizing the Velvet assembler v1.0 (Zerbino et al., 2009); gaps between the contigs were closed by PCR amplification and sequencing. The SA40 genome was sequenced using the PacBio RS II sequencer (Pacific Biosciences, CA, USA), and the de novo assembly was performed with the Hierarchical Genome Assembly Process (HGAP) workflow, available in SMRT Analysis v2.3.0 (Pacific Biosciences, CA, USA). The genomes of SA957 and SA40 have been deposited into the NCBI GenBank database, with their accession numbers being CP003603 and CP013182, respectively.

All the genome sequences of *S. aureus*, including both complete and draft genomes available in the GenBank database (https://www.ncbi.nlm.nih.gov/genome/genomes/154?), were downloaded. The presence of the *sak* sequence was screened using the BLASTN program in the NCBI BLAST package. Only the search hit with over 90% nucleotide identity and 99% coverage in length was considered to be SAK-positive. The determination of multi-locus sequence type (MLST) based on genome sequencing was performed using the online service BacWGSTdb (Ruan and Feng, 2016). A minimal spanning tree based on the MLST allele profiles was drawn using the software PHYLOViZ (Francisco et al., 2012). Only STs with over 20 genomes deposited in the GenBank database were included in this analysis.

2.3. Transcriptome sequencing and analyses

The bacteria were cultured in BHI broth, and the overnight culture was then diluted at 1:100 in pooled human serum and grown for 5 h at 37 °C by shaking to obtain log-phase cells. The bacteria pellet was treated with lysostaphin (0.2 mg/ml) in a TE buffer at 37 °C for 10 min. The total RNA was extracted using TRIzol (Thermo Fisher Scientific, CA, USA), and the ribosomal RNA was removed using the Ribo-Zero rRNA (Gram-positive bacteria) Removal Kit (Illumina, CA, USA) according to the manufacturer's instructions. The RNA-seq libraries were sequenced on the Illumina HiSeq 2000 platform (Illumina, CA, USA). The data obtained was processed using Cufflinks software (Ghosh and Chan, 2016). When the genes were revealed as having undergone a ≥ 2-fold change in expression with a p value of <0.01 and q value of <0.1, they were considered to be differentially expressed.

2.4. Construction of mutant strains and animal experiments

The virulence of SA957, SA40 and their congenic strains was compared using two murine models, namely, skin and bloodstream infection. The SAK complementation mutant was constructed employing the methods described previously (Nakamura et al., 2013). Briefly, the *sak* gene was amplified from SA40 genomic DNA using the forward primer 5′-CGC**GGATCC**ATGCTCAAAAGAAGTTTA-3′ (the sequence in bold, "GGATCC", is a built-in *Bam*HI site) and reverse primer 5′-CG**ACGCGT**TTATTTCTTTTCTATAAC-3′ (the "ACGCGT" sequence is a built-in *Mlu*I site). The PCR product was digested with *Bam*HI and Mlu-I and subsequently cloned into the plasmid pTXΔ. The resulting SAK-encoding plasmid was first transformed by electroporation into Strain RN4220 and then into SA957. The blank plasmid, pTXΔ16, was transformed into SA957 and served as the control.

The animal experiments were performed in accordance with the methods described previously, but with minor modifications (Chen et al., 2013; Chen et al., 2015). Briefly, bacteria were grown for 3 h in BHI broth to reach the log growth phase, and then the cells were harvested and resuspended in PBS. Six to eight week old female BALB/c mice were used for the inoculations. For the skin infection model, the mice' backs were shaven and injected subcutaneously with approximately 2×10^8 bacterial cells in 100 μL of PBS. Once a scab was formed, its length and width were measured using a caliper. The size of the lesion was calculated using the formula length × width, and compared using one-way ANOVA analysis with Bonferroni corrections. For the bloodstream infection model, the mice were injected with a dosage of 1×10^8 CFU through the tail vein. Survivals of septic mice were compared using a Mantel-Cox test. Six mice were used for each model as biological replicates.

2.5. Ethical statement

Human blood samples were obtained from healthy blood donors, who provided written informed consent for their samples' collection and subsequent analysis. All of the animal experiments were approved by the Institutional Animal Care and Use Committee, at Chang Gung University in Taiwan.

3. Results

3.1. Genomic comparison between the TW and AP clones

Firstly, we sequenced the complete genomes of two strains, SA957 and SA40, which represented TW and AP, respectively. A genome-wide comparison showed that the two strains shared 2391 genes (approximately 94% of their entire genomes), with an average nucleotide identity of 99.9%. The major genomic differences between the two strains were in line with that from the microarray-based study (Chen et al., 2013), which were located on the three mobile genetic elements: SCC*mec*, the SA957-specific ΦSa2, and the SA40-specific ΦSa3. ΦSa2 in SA957 and its counterpart in the USA300 strain FPR3757 (Accession CP000255.1) and in the USA400 strain MW2 (Accession BA000033.2) were integrated into the same site in the chromosome and shared exactly the same gene content.

Interestingly, ΦSa3 was found to have inserted into νSaβ in SA40 instead of into the usual integration site *hlb*. Being a genomic island, νSaβ shares the same position in all lineages of *S. aureus*, demonstrating that it is an ancient component of the staphylococcal genome (Feng et al., 2008). The typical gene content of νSaβ includes a type I restriction and modification system, *lukD*, *E*, and the gene clusters that encode