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Identification and characterization of two novel superantigens among *Staphylococcus aureus* complex

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ABSTRACT

Staphylococcal enterotoxins (SEs), also known as superantigens, play a very important role in infections and food poisoning caused by *Staphylococcus aureus*. Recently, *S. argenteus* and *S. schweitzeri* were recognized as novel species closely related to *S. aureus*. In this study of these three species, it was found that two putative SE genes were located upstream of some *v*Saβ pathogenicity islands and the deduced amino acid sequences showed < 65.3% identity with those of known SEs. The related proteins, designated staphylococcal enterotoxin-like toxin 26 (SE*l*26) and 27 (SE*l*27), were identified and characterized among the three species. The mRNAs encoding SE*l*26 and SE*l*27 were expressed during all the growth phases. Recombinant SE*l*26 and SE*l*27 exhibited superantigenic activity in human peripheral blood mononuclear cells and mouse splenocytes by examining cell proliferation and cytokine production. Interestingly, these two genes were present universally in *S. argenteus* sequence type 2250 with clinical importance. Meanwhile, SE*l*27 variants from different species showed differential sensitivity to human peripheral blood mononuclear cells, which corresponded to the primary bacterial species hosts. It was demonstrated from these results that SE*l*26 and SE*l*27 were characterized to be two novel SE toxins and some SEs evolved along with the bacteria when the organisms adapted the hosts' immune systems.

1. Introduction

Bacterial superantigens (Sags) are secreted protein toxins produced mainly by *Staphylococcus aureus* and several Streptococci (Fraser and Proft, 2008). Systemic intoxication by a Sag can lead to the life-threatening condition toxic shock syndrome (TSS) or streptococcal TSS (STSS), caused by a sudden cytokine storm when large numbers of T cells are stimulated by the Sag cross-linking major histocompatibility complex (MHC) class II antigens and T-cell receptors (TCR). The cytokines including (but not limited to) interleukin-1 (IL-1), IL-2, interferon-γ (IFN-γ), tumor necrosis factor *α* (TNF-*α*) and TNF-*β*, are generally believed to be the main factors inducing toxicity (Jupin et al., 1988; McCormick et al., 2001). A recent study showed that a population of innate-like T cells, called mucosa-associated invariant T (MAIT) cells, launch a rapid, robust and distinct hyperinflammatory response to Sags, and quickly acquire an anergic phenotype that impedes their cognate antimicrobial function (Shaler et al., 2017). Since all Sags are associated with one or more illnesses caused by *S. aureus* or group A streptococci, including serious capillary leak syndromes (in the form of TSS/STSS, sepsis, infective endocarditis, pneumonia, and osteomyelitis), guttate psoriasis, atopic dermatitis, severe nasal polyposis, acute glomerulonephritis and so on (Fraser and Proft, 2008; Spaulding et al., 2013), these toxins are considered as contributors to serious illnesses.

Except toxic shock syndrome toxin (TSST), Sags produced by *Staphylococcus* are also well known as staphylococcal enterotoxins (SEs) and SE-like toxins, since SEs are demonstrated to have emetic activity in primates and are a common causative agent of food poisoning while SE-lile toxins do not have the activity in primates or have not been tested (Lina et al., 2004; Spaulding et al., 2013). A recent study suggested that new SE-like toxins (also named SE in the following text), including SE*l*K, SE*l*L, SE*l*M, SE*l*N, SE*l*O, SE*l*P, and SE*l*Q, also have the potential to induce emetic reactions in monkeys and may play a role in staphylococcal food poisoning, although a higher dose of 100 μg/kg was needed and significantly smaller numbers of monkeys were affected when these new SEs were compared to classical SEA or SEB (Omoe et al., 2013). Therefore, the "*l*" designation should be removed from the

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nomenclature of these SEs, according to the previously proposed standard (Lina et al., 2004). Variants were observed within most SEs (Zhang et al., 2017) and those of SEC and SE*l*X possess a similar superantigenic activity (Fraser and Proft, 2008; Wilson et al., 2011). SEs were previously considered to be mainly produced by *S. aureus*, in spite of detection in coagulase negative staphylococci (CoNS), such as *S. delphini* and *S. intermedius* (Gharsa et al., 2015; Lina et al., 2004). We recently demonstrated that most of the SEs were also present in *S. argenteus* and *S. schweitzeri* (Zhang et al., 2017). These two species (coagulase positive) are closely related to *S. aureus* (Tong et al., 2015), and the gene sequences of some of their SEs were divergent among the three species (termed *S. aureus* complex, SAC) (Zhang et al., 2017). However, unlike *S. argenteus* and *S. aureus*, *S. schweitzeri* is not considered to be a human pathogen (Schaumburg et al., 2015; Tong et al., 2015; Zhang et al., 2017).

Superantigenic activities of some SEs variants were determined within *S. aureus* (Fraser and Proft, 2008; Wilson et al., 2011), but variants from different species have not been characterized for comparison. Meanwhile, it is hypothesized that superantigen production can interfere with normal immune function of the host, increasing the chances of survival and transmission of the organism (Spaulding et al., 2013). Here, we report two novel SEs, designated staphylococcal enterotoxin-like toxin 26 (SE*l*26) and 27 (SE*l*27), present in *S. aureus*, *S. argenteus* and *S. schweitzeri* and the variants from different SAC species were identified and characterized to provide insight into the roles of SEs during SAC speciation.

2. Material and methods

2.1. Bioinformatic analyses

The SE gene sequences were aligned by ClustalX software (Thompson et al., 1997). The maximum likelihood phylogenetic tree was constructed and assessed using MEGA 6.06 (Tamura et al., 2013). The genetic locations and surroundings of *sel26* and *sel27* were determined according to the genome annotation and the previous pan-genome definition (Zhang et al., 2017). The SE genotype was retrieved from our previous work (Zhang et al., 2017). Recombination events were detected among alleles using recombination detection program RDP 4.92 (Martin and Rybicki, 2000).

2.2. Bacterial strains and DNA isolation

The staphylococcal strains were grown on tryptone soya agar (TSA, Becton, Dickinson and Company, NJ, USA) medium for 12 h. The biomass was collected to extract and purify the genomic DNA using TIANamp Bacteria DNA Kit (TIANGEN Biotech, Beijing, China) following the manufacturer's instructions, with the addition of lysostaphin (Sigma-Aldrich, MO USA) for bacterial lysis. The PCR amplification was performed to detect and sequence *sel26*, *sel27* and the upstream flanking genes. The primers (Table S1) were newly designed by Primer Premier 5 software (http://www.premierbiosoft.com/primerdesign/), using the related segments of the genomes mentioned in the main text as input sequences. All PCR amplification was performed in a total reaction volume of 25.0 μL. The reaction mixture contained 1 × Taq buffer, 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.4 μM of each primer, and 1.0 U Taq DNA polymerase (Thermo Scientific, MA, USA). The PCR program was as follows: 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30–90 s (dependent on the product size, 1k bp/min); followed by 72 °C for 10 min.

2.3. Transcriptional analysis of sel26 and sel27

Bacterial cells were lysed using lysostaphin (Sigma-Aldrich, MO, USA) and total RNA was extracted using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. DNA degradation and cDNA synthesis were performed using the PrimeScriptTMRT reagent Kit with gDNA Eraser (Takara, Shiga, Japan) according to the manufacturer's instructions. The expression level of each gene was analyzed by PCR using a Mastercycler® ep realplex PCR system (Eppendorf, Hamburg, Germany). The PCR mixture was prepared using SYBR® Premix Ex Taq™ (Takara, Shiga Japan) according to the manufacturer's instructions. The primer sequences are shown in Table S1 and the *gyrB* gene was adopted as an internal control (Duquenne et al., 2010). Cycling conditions were as follows: 30 s at 95 °C, followed by 40 rounds of 95 °C for 5 s, 53 °C for 15 s, and 68 °C for 20 s. To determine the dissociation of PCR products, melting curve analysis was performed at temperatures of between 53 °C and 95 °C. Statistical analysis was performed using analysis of variance (ANOVA) followed by the Tukey test.

2.4. Cloning, expression and purification of recombinant SEs

To construct the recombinant SE expression plasmids, PCR primers including the *Nhe*I and *Xho*I sites were designed to amplify a fragment of the SE gene corresponding to the mature forms (Table S1). The N-terminal signal peptide sequences of SEs were predicted using the online signal peptide prediction software SignalP (http://www.cbs.dtu.dk/services/SignalP) (Nielsen et al., 1997). The SE gene fragments were amplified by PCR using KOD plus DNA polymerase (Toyobo, Japan), and the PCR products were digested with *Nhe*I and *Xho*I restriction enzyme (Takara, Shiga, Japan). The fragments of the SE genes were then cloned into the pET28a Histidine-Tagged (His-Tag) fusion expression vectors, and the vectors were transformed into *Escherichia coli* DH5a cells. The pET28a constructs were verified by sequencing, then isolated from DH5a using the AxyPrep™ Plasmid Miniprep kit (AXYGEN, CA, USA), and transformed into *E. coli* BL21. BL21 cells containing the pET28a plasmid constructs were cultured in Luria-broth containing 50 μg/mL kanamycin (Sigma-Aldrich, MO, USA) and induced in mid-exponential phase of growth (OD_{600} = 0.6), with 0.6 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Merck, Darmstadt, Germany) for 8 h at 20 °C. Cells were recovered by centrifugation at 8000*g* for 8 min, disrupted using an ultrasonic cell disruption system SCIENTZ-II D (SCIENTZ, Ningbo, China). His-Tag recombinant proteins were purified by affinity chromatography on a Ni-NTA nickel affinity column (Yeasen, Shanghai, China). Cleavage and removal of the His-Tag was performed using bovine thrombin (Sigma-Aldrich, MO, USA), heparin affinity column (Senhui Microsphere Tech, Suzhou, China), and Ni-NTA nickel affinity column (Yeasen, Shanghai, China). The lipopolysaccharide (LPS) was removed using an endotoxin removal affinity column (Senhui Microsphere Tech, Suzhou, China). The resulting recombinant proteins had six additional amino acid residues GSHMAS at the N-terminus.

2.5. Assays of SEs stability

The protein resistance to heating and digestive enzymes was performed as described previously (Li et al., 2011; Ono et al., 2015). Bovine serum albumin (BSA; Amresco, OH, USA) was used as a protein control. To study the stability of SEs against heat treatment, 100 μL of each toxin and BSA at 100 μg/mL in PBS was added to each 200 μL microtube and then placed into a Mastercycler pro PCR system (Eppendorf, Hamburg, Germany) maintained at 99 °C. BSA and each SEs were also incubated in the presence of pepsin (Sigma-Aldrich, MO, USA) and trypsin (Sigma-Aldrich, MO, USA) to assess the potential stability in the gastrointestinal environment. Each protein at a concentration of 100 μg/mL was incubated with pepsin (100 μg/mL in 0.1 M sodium acetate buffer, pH 4.5) or trypsin (50 μg/mL in 0.01 M Tris-HCl, pH 8.0) in a final volume of 100 μL at 37 °C. At desired time intervals ranging from 30 min to 12 h, tubes were removed, immediately put into an ice bath for 5 min to cool down, and then placed at −20 °C. Twenty microliters of treated samples were mixed in SDS-