Functional and structural characterization of the anti-phagocytic properties of a novel transglutaminase from Streptococcus suis

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**Key words:** SsTGase; homodimer; anti-phagocytosis; virulence factor

Background: SsTGase was a newly identified secreted immunogenic protein of S. suis 2.
Results: Anti-phagocytic ability of SsTGase-N was dependent on its TGase activity, and its crystal structure revealed that dimerization was crucial for maintaining functional activities.

Conclusion: SsTGase was a novel virulence factor of Ss2 by acting as a TGase in dimer form.

Significance: The presented research suggested that SsTGase could serve as a new therapeutic target.

Abstract

Streptococcus suis serotype 2 (S. suis 2, Ss2) is an important swine and human zoonotic pathogen. In the present study, we identified a novel secreted immunogenic protein, SsTGase, contained a highly conserved eukaryotic-like transglutaminase (TGase) domain at the N terminus. We found that inactivation of SsTGase significantly reduced the virulence of Ss2 in a pig infection model and impaired its anti-phagocytosis in human blood. We further solved the crystal structure of the N-terminal portion of the protein in homodimer form at 2.1 Å. Structure based mutagenesis and biochemical studies suggested that disruption of the homodimer directly resulted in the loss of its TGase activity and anti-phagocytic ability. Characterization of SsTGase as a novel virulence factor of Ss2 by acting as a TGase would be beneficial for developing new therapeutic agents against Ss2 infections.

Streptococcus suis serotype 2 is an important swine and human zoonotic pathogen, causing septicemia, arthritis, endocarditis, meningitis, and even acute death in pigs and human beings (1,2). Although several virulence factors have been identified, including capsule polysaccharide (CPS), extra-cellular protein factor (EF), suilysin (SLY), factor H binding surface protein (Fhb) and adenosine synthase, etc (3-9), the underlying mechanism of Ss2 pathogenesis remains unclear. In our previous study, we identified a new secreted protein from Ss2 culture supernatant encoded by SSU05_1815 locus with a high immunogenicity through an immunoproteomic approach (10). However, genetic studies and sequence analyses revealed that this protein was a transmembrane protein with a single transmembrane segment at the C terminus, suggesting this protein was released into culture supernatant. Moreover, a highly conserved eukaryotic-like transglutaminase (TGase) domain (residues from 247 to 348) was found at the N terminus. The TGase domain belongs to the TGase-like superfamily (PF01841 in the PFAM database) (11), which contains a highly conserved catalytic triad Cys302-His333-Asp348. Therefore, we named this protein as SsTGase in Ss2. TGases, also named protein-glutamine γ-glutamyltransferase, constitute a large superfamily of enzymes, widely distributed in eukaryotes and prokaryotes and have been extensively studied since they were first extracted from animal liver (12-15). The enzyme catalyzes an acyl–transfer reaction between glutamine residues and lysine, or other primary amine, leading to inter- or intramolecular cross-linking and polymerization of the proteins (16-20). The catalytic reaction of TGases is based on a highly conserved catalytic center: a Cys-His-Asp triad or, less frequently, a Cys-His dyad. TGases are involved in regulation of a myriad of physiological processes by acting as biological glues, such as blood clotting, wound healing, epidermal keratinization, neoplastic diseases and curing membranes (21-24). What is more, the enzyme has been applied in food, cosmetic and textile industries, serving as a biocatalyst (25).

To date, several crystal structures of TGases have been resolved in mammals (26-31), including human factor XIII, fish-derived TGases (FTG) and human transglutaminase 2 (TG2) (28,31). Previous studies have mainly focused on the biochemical characteristics of TGases in mammals, but the roles of TGases played in microorganisms remain largely unknown. Among microorganisms, only TGases from Streptoverticillium mobaraense (MTG) and Phytophthora have been studied, which represented a completely different structure fold compared with those in mammals (27,29). Therefore, bacterial TGases of the PF01841 superfamily are currently largely unknown for their structural features and
specific activities.

In the present study, we showed that the SsTGase was secreted by Ss2 and developed strong anti-phagocytic activity. Inactivation of SsTGase significantly reduced virulence in a pig infection model and impaired anti-phagocytic resistance of Ss2 in human blood. To further investigate the molecular mechanism underlying the pathogenesis of SsTGase in Ss2, we determined the crystal structure of the N-terminal portion of SsTGase (residues from 38 to 437, referred to as SsTGase-N hereafter) that also included the TGase domain at 2.1 Å. The structure reveals that although C-terminal domain of SsTGase-N contains a catalytic core region similar to other TGases, its N-terminal domain displays a new structural fold. The overall folding of the SsTGase-N homodimer was novel and different from other known structures of TGases. Inactivation of the protein directly resulted in the loss of its anti-phagocytic ability, indicating anti-phagocytic ability of SsTGase-N was dependent on its TGase activity. Furthermore, structure based mutagenesis and biochemical studies suggested that dimerization of the protein was critical for its activation and anti-phagocytic ability. These observations provide a novel insight into the activation mechanism and functions of SsTGase, which would be valuable for the development of novel antibiotic strategies targeting SsTGase.

**Experimental Procedures**

*Generation of the mutant strain ΔSsTGase and the complemented strain CΔSsTGase*—The ΔSsTGase mutant was obtained from the 05ZYH33 WT by in-frame deletion of the *sstgase* gene (SSU05_1815) as our previously described (9). Briefly, DNA fragments corresponding to the upstream and downstream regions of the *sstgase* gene were amplified using primer pairs SsTGaseKOP1/SsTGaseKOP2 and SsTGase KOP5/SsTGase KOP6, respectively (Table 1). The chloramphenicol (Cm) cassette was amplified from plasmid pSET1 with primers CM-F and CM-R (Table 1). The primer pairs SsTGase KOP2/CM-F and CM-R/SsTGaseKOP5 were designed to be fused as an intact fragment by overlap extension PCR. PCR amplicons were cloned into the temperature-sensitive *S. suis*- *E. coli* shuttle vector pSET4s, giving rise to the knockout vector pSET4s:: *sstgase*. The procedures for the selection of mutants by double crossover were described previously (32). The resulting mutant strain was verified by PCR using three pairs of primers, SsTGaseIN1/SsTGaseIN2, SsTGase -F/SsTGase-R, and SsTGaseKOP1/SsTGaseKOP6 (Table 1), and direct DNA sequencing analysis of the mutation sites using genomic DNA as the template. For complementation assays, a DNA fragment containing the entire *sstgase* gene and its upstream promoter was amplified using primers CΔsTGase-F and CΔsTGase-R. The amplicon was subsequently cloned into the *E. coli*- *S. suis* shuttle vector pAT18 (33), resulting in the recombinant plasmid pAT18:: *sstgase*. This plasmid was transformed into the ΔSsTGase mutant, and the complemented ΔSsTGase strain was screened on THB agar with selective pressure of erythromycin (Em). Reverse transcription-PCR (RT-PCR) analyses of the CΔsTGase, 05ZYH33, and ΔSsTGase strains were used to further identify the transcription of the gene *sstgase* in CΔsTGase.

*Western blot*—A rabbit SsTGase antibody was generated against recombinant SsTGase-N protein. 05ZYH33, ΔSsTGase and CΔSsTGase samples were separated on 12% (v/v) polyacrylamide vertical slab gel with a 5% (v/v) stacking gel. Then the proteins were electro-transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare Life Sciences). The membrane was blocked with 5% skimmed milk, followed by incubation with 1:200 diluted pre-immune rabbit sera / rabbit anti-SsTGase-N sera at room temperature for 1 h. The membrane was then washed with TBST buffer containing 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20 and incubated with goat anti-rabbit IgG (H+L)-HRP (1:8000) (Santa Cruz Biotechnology) at room temperature for 1 h. After washing, the membrane was developed in SuperSignal West Pico.
Chemiluminescent Substrate (Pierce).

**Blood survival assay**—Diluted strains of the wild-type (05ZYH33), ΔSsTGase and CΔSsTGase (50 µL, 2×10^4 CFU (colony forming units) /ml) were added to fresh human blood (450 µL), and the mixtures were rotated at 20 rpm at 37 °C under 5% CO₂, after which aliquots were incubated with a final concentration of 0.1% (w/v) saponin on ice for 15 min to lyse cells. Viable bacterial counts were determined by plating diluted samples onto THB agar. The percentage of live bacteria was subsequently calculated as (CFU on plate/CFU in original inoculum) ×100%.

**Polymorphonuclear leukocyte (PMN) killing assay**—After being isolated from heparinized venous blood, PMNs were infected with *S. suis* serotype 2 at a multiplicity of infection (MOI) of 1:15 in 50% nonimmune human serum at 37 °C under 5% CO₂, respectively. Samples were taken at time points and analyzed immediately. Colonies were counted, and the percentage of surviving bacteria was calculated as follows: (CFU<sub>PMN+</sub>/CFU<sub>PMN−</sub>) ×100%. The data were presented as means ± standard deviations (SD) from three or four separate experiments.

**Experimental infections of piglets**—To evaluate the effects of deletion of *sstgase* on the virulence of 05ZYH33, specific-pathogen-free (SPF) piglets (4-week-old, 6 piglets/group) were challenged with 05ZYH33, ΔSsTGase, CΔsTGase strains and an avirulent strain 1330 (dose of 2×10^8 CFU/piglet), respectively. Survival time, clinical signs, and bacterial loads in blood and tissue samples were recorded for 12 days post-inoculation. To have a better understanding of the difference between the 05ZYH33 and ΔSsTGase, and to reduce the individual differences in infection, groups of four SPF-piglets were inoculated intravenously with a 1:1 mixture of 05ZYH33/ΔSsTGase (dose of 10^8 CFU/piglet), respectively. When the infected piglets showed *Ss*2 typical infection symptoms, the capability of 05ZYH33/ΔSsTGase surviving in blood and colonizing the various tissues of piglets were analyzed by plating them on the plates without antibiotics or with chloramphenicol (Cm) resistance. All animal experiments were performed in a biosafety level 3 (BSL3) facility and were approved by the local ethics committee.

**Ethics statement**—The healthy donors who provide the blood in this study provided written informed consent in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the 307 hospital. This research was approved by ethics committee on Animal Experimentation of the Chinese Association for the Accreditation of Laboratory Animals Care (CAALAC), including the relevant local animal welfare bodies in China. In addition, the permit number of all animal work was SCXK-(JUN) 2013-008 approved by the animal ethics committee of Beijing Institute of Microbiology and Epidemiology. All efforts were made to minimize suffering of animals employed in this study.

**Measurement of transglutaminase activity**—Transglutaminase activity (TGase; EC 2.3.2.13) was assayed by a Transglutaminase Colorimetric Microassay Kit (TCM kit; Covalab) which uses immobilized N-carbobenzoxy (CBZ) -Gln-Gly as amine acceptor and biotin-conjugated cadaverine as amine donor. Protein samples were incubated in a 96-well microtiter plate coated with CBZ-Gln-Gly at 37 °C for 15 min with calcium, DTT and biotinylated cadaverine, both in the presence and the absence of EDTA supplied in the kit. The wells were washed three times with phosphate buffer containing 0.1% Tween 20. To assay the formation of cadaverine covalently linked to CBZ -Gln-Gly (γ-glutamyl-cadaverine-biotin) by TGase, the wells were filled with streptavidin-labelled horseradish peroxidase (HRP) and incubated for 15 min at 37 °C. Following with washing three times with phosphate buffer containing 0.1% Tween 20, the wells were filled with H₂O₂ as the substrate and tetramethyl benzidine as the electron acceptor (chromogen). After incubation for 10 min at room temperature, 50 µL of reaction blocking reagent was added and the
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mixture was quantified by measuring OD_{450}. As references for the TGase activity, the kit includes the purified guinea pig TGase with a specific activity of 0.1 U/mg. By definition, 1 U of TGase catalyzes the formation of 1 µmole of hydroxamate per minute at pH 6.0 at 37 °C, using L-glutamic acid γ-monohydroxamate as the standard.

Circular dichroism (CD) spectroscopic analysis—For CD spectroscopic analysis, the purified proteins from peak1 and peak2 solubilized in buffer containing 20 mM Hepes pH 7.5, 200 mM NaCl, were concentrated to 1mg/ml. CD spectroscopy was carried out using an Applied Photophysics Chirascan Plus spectropolarimeter with a 10 mm path-length cell and a bandwidth of 1.0 nm. Spectra were recorded from 260 to 180 nm at an interval of 1 nm and were repeated three times. All resultant spectra were obtained by subtraction of the spectrum of the buffer.

Cloning, expression and purification of SsTGase-N—The coding sequence of sstgase-N (residues from 39 to 437) was cloned into the pGEX6p-1 vector (Novagen), generating an N-terminal PreScission Protease Cleavage site following the GST-tag, which was confirmed by DNA sequencing. Overexpression of SsTGase-N was induced in E. coli BL21 (DE3) strain by 0.5 mM isopropyl-β-D-thiogalactoside when the cell density reached OD_{600nm} = 1.2. After growth for 6 hours at 30 °C, the cells were collected, resuspended in buffer containing 25 mM Tris-HCl pH 8.0, 200 mM NaCl and lysed by sonication. Recombinant GST-tagged protein was purified by glutathione affinity chromatography and gel-filtration chromatography with buffer containing 20 mM Hepes pH 7.5, 200 mM NaCl, 2 mM DTT. Notably, the GST tag of purified homodimer was cleaved off by PreScission protease (Amersham-GE) before gel-filtration. The selenomethionine (Se-Met)-substituted SsTGase-N derivative was expressed in E. coli B834 strain, grown in selenomethionine medium (Molecular Dimensions Limited) and purified similarly (34).

According to previously described procedures (35), point mutations (C302S, H333S, D348S, T215A and R311A) of SsTGase-N were generated by two-step PCR and confirmed by DNA sequencing. All the mutants were purified in the same way as wild-type protein.

Crystallization, Data Collection and Structure Determination — Crystals of SsTGase-N were generated by mixing 1 µL of protein solution with 1 µL of well buffer using the hanging drop vapor diffusion method at 18 °C. Crystals appeared after two weeks in the reservoir solution containing 0.2 M sodium chloride, 0.1 M Hepes pH 7.2 and 20% (w/v) polyethylene glycol 4000. The crystals were cryo-protected in reservoir solution plus 15%-20% (v/v) glycerol and flash-frozen in liquid nitrogen prior to data collection.

All the data were collected at the Shanghai Synchrotron Radiation Facility (SSRF) BL17U beamline, integrated and scaled using the HKL2000 package (36). Further processing was carried out using programs from the CCP4 suite (37). Data collection statistics are summarized in Table 2. The selenium sites were located using SHELXD from Bijvoet differences in the Se-SAD data (38). Heavy atom positions were defined and the phases were calculated with PHASER’s SAD experimental phasing module (39). The real-space constraints were applied to the electron density map in DM. The final model rebuilding was performed with COOT (40) and the protein structure was refined with PHENIX (41) using NCS and stereochemistry information as restraints. Structural figures were generated in PyMOL (42). The structure factors of SsTGase-N have been deposited in the Protein Data Bank (accession code 4XZ7).

Results

SsTGase was secreted by Ss2 and possessed strong anti-phagocytic ability — SsTGase is encoded by SSU05_1815 locus of China pathogenic strain 05ZYH33 (NC_009442.1) isolated from a deceased Streptococcal Toxic Shock Syndrome (STSS) patient. To investigate the specific roles of the
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protein, we constructed a mutant strain ΔSsTGase and a complemented strain CΔSsTGase derived from wild type strain 05ZYH33 (WT). First, we identified that SsTGase was secreted by Ss2 through western blotting, despite the fact it contained a predicted transmembrane segment (Figs. 1A and 1B). This secreted form was possibly produced through proteolysis of the full-length protein during biological processes. The results from the polymorphonuclear leukocyte (PMN) killing assay suggested that the survival rates of the ΔSsTGase strain were much lower than those of the WT and CΔSsTGase strains by 1~3 hours (Fig. 1C). Furthermore, to investigate the anti-phagocytic activity of SsTGase, we carried out the blood survival assay with the culture supernatant of the WT, CΔSsTGase and ΔSsTGase strains. The results showed that culture supernatant of WT and CΔSsTGase rather than that of ΔSsTGase evidently improved the survival rates of three strains of Ss2 (WT, ΔSsTGase and CΔSsTGase) in human blood (Fig. 2A). In addition, the survival rate of ΔSsTGase strain could also be improved significantly after incubating the strain with different amount of SsTGase-N (residues 39-437) protein in human blood (Fig. 2B), which suggested that both SsTGase and SsTGase-N possessed significant anti-phagocytic activity.

SsTGase was a new virulence factor of Ss2—To determine whether SsTGase is a potential virulence factor of Ss2, a piglet infection model was performed to test the virulence of the WT, ΔSsTGase and CΔSsTGase strains, with the North-American avirulent strain 1330 considered as a negative control. Each piglet was injected with 2×10⁸ CFU (colony forming unit) of bacteria, and the survival rates of infected piglets were measured over 12 days. All the piglets infected with the WT strain died within 6 days after infection, in contrast to a survival rate of 83.33% in the group infected by the ΔSsTGase strain, 33.33% in the group infected by the CΔSsTGase strain and 100% in the group infected by the 1330 strain (Fig. 2C). Severe symptoms such as high fever, limping, swollen joints, shivering and central nervous system failure were observed among the groups infected by the WT and CΔSsTGase strains, whereas only mild symptoms were observed in the groups infected by the ΔSsTGase strain. Additionally, the efficiencies of colonization by the WT and CΔSsTGase strains in blood were much higher than that of the ΔSsTGase strain from 12 to 132 hours (Fig. 2D).

To avoid the individual differences in piglets, the competitive-infection assay was adopted to further compare the virulence of the WT and ΔSsTGase strains, in which a group of 4 piglets were challenged with a 1:1 mixture of the WT and ΔSsTGase strains. Consequently, the cell numbers of the WT bacteria in blood and various tissue samples (heart, liver, kidney, spleen, lung, tonsil and lymph) were much higher than those of the ΔSsTGase bacteria (Figs. 2E and 2F). Taken together, these data indicated that SsTGase was a new virulence factor of Ss2 with anti-phagocytic activity.

Anti-phagocytic ability of SsTGase-N was dependent on its TGase activity—SsTGase-N corresponds to an active form with a high TGase activity (0.72971 units/mg) compared with TGase from guinea pig liver (0.10789 units/mg), while no activity could be detected after mixing it with monodansylcadaverine (MDC), a potent inhibitor of TGases (Fig. 3A). Similar with other TGases from microorganisms, the SsTGase-N enzyme activity is Ca²⁺-independent (Fig. 3A). In addition, we generated three point mutations of the catalytic residues containing C302A/H333A/D348A to study the effect of each single active residue. All of the three point mutations displayed dramatically decreasing TGase activity, suggesting that these three residues played an important role in catalyzing TGase reaction (Fig. 3C).

Notably, anti-phagocytic abilities of the three point mutations also decreased to an extremely low level (Fig. 3B). Moreover, survival rate of WT strain dropped dramatically after incubation with MDC (Fig. 1C). These results indicated that the
anti-phagocytic ability of SsTGase-N was dependent on its TGase activity.

Overall structure of SsTGase-N — To better understand the biological functions of SsTGase, we solved the crystal structure of SsTGase-N that also included a TGase domain (residues 247 to 348). The structure reveals that SsTGase-N forms a homodimer in an anti-parallel manner (Fig. 4A). In each protomer, the region spanning residues from 353 to 363 is invisible, probably due to intrinsic flexibility (Fig. 4B). Each protomer forms an elongated and twisted dumbbell-like fold, which could be divided into three regions: an N-terminal domain (residues 38-208, referred to as NTD hereafter), a C-terminal TGase-like domain (residues 221-437, referred to as CTD hereafter) and a connecting helix (α5 helix, residues from 209 to 220). The NTD consists of two antiparallel β-sheets in the center, with four and three β-strands respectively. Either of the two β-sheets is flanked by two helices. Similar to other solved structures of TGases, a deep cleft is generated at the edge of the CTD where catalytic residues Cys302, His333 and Asp348 are located (Fig. 5B). Clusters of helices formed in the amino terminus of the CTD are composed of α6, α6′, α7 and α7′, followed by an antiparallel β-sheet comprising of three β-strands (β8, β9 and β10). In the C terminus of CTD, two parallel β-strands and two α-helices wrap around the whole domain. The two protomers are compacted together and the CTD from one protomer lies opposite to the NTD of the other (Fig. 4A).

The interactions between the two protomers are distributed in two regions, mainly composed of hydrogen-bonding interactions and water-mediated interactions. In one region, Thr215 from one protomer interacts with Ser218 of the other through hydrogen-bonding interactions, stabilizing the interactions between the two α5 helices in an anti-parallel orientation (Fig. 6A). In the other region, water-mediated interactions could be observed among the main-chain carbonyl group of Leu176 located at α4 helix (residues from 172 to 179), the side-chain guanidine group of Arg311 and the carbonyl group of Leu412 (Fig. 6A). Notably, Arg311 sits on the same α helix where the catalytic residue Cys302 is located.

CTD of SsTGase-N contains a conserved catalytic core region — Although SsTGase-N shares low primary sequence similarity with known TGases from mammals or bacteria, it possesses an active region consisting of consensus sequence motif of thiol proteases (Fig. 5A). Cys302 sits on the amino terminus of α7 helix and is known to supply a thiolate ion for nucleophilic assault. The sulphydryl group of Cys302 forms a hydrogen bond with His333 at a loop between β8 and β9 (Fig. 5B). Additionally, the imidazole ring of His333 forms a hydrogen bond with Asp348, which is in a loop connecting β10 strand and α8 helix (Fig. 5B). Thr350 and Tyr377 also participate in the hydrogen-bonding pattern in the active cavity (Fig. 5B). Specifically, both of Tyr377 and Thr350 form hydrogen bonds with Asp348, while Tyr560 in human factor XIII suppresses enzyme activity by forming a hydrogen bond with active residue Cys314 (31). Hydrogen bonds formed by Cys302, His333, Asp348, Tyr377 and Thr350 could enhance the stability of the entire active cavity, indicating that substrates approaching the enzyme might disrupt the stable state of active cavity by breaking the hydrogen bonds mentioned above.

Structural alignment of the entire TGase domain of SsTGase-N with the corresponding region of human factor XIII and FTG, which are representatives of TGases from mammals, reveals that overall folding of the active site region of SsTGase-N adopts a similar fold with human factor XIII and FTG, with root mean square deviations (RMSDs) 3.24 Å and 0.517 Å, respectively (Fig. 5C). In addition, the three active residues of SsTGase superimpose well with the catalytic triad of human factor XIII and FTG (28,31) (Fig. 5C). Therefore, CTD of SsTGase-N has a conserved catalytic core region similar to other TGases.

Interestingly, a Dali search with the NTD of SsTGase-N only returned entries with low Z-scores.
of 4.1-2.0, suggesting that no known structure was identified to share significant homology with this domain (43). That is, NTD of SsTGase-N likely represents a newly identified structural fold. Moreover, the overall folding of how SsTGase-N packs into homodimer is novel and differs from other known structures of TGases.

A new activation mechanism of SsTGase-N in solution environment — Thezymogen forms of TGases require proteolytic activation or the presence of Ca^{2+} to gain their activities. For instance, MTG is secreted from the cytoplasmic membrane as azymogen and is activated by proteolytic processing (44). During the purification of the recombinant protein, two peaks (peak1 and peak2) of SsTGase-N emerged in the gel filtration profile, corresponding to the dimer and monomer forms respectively, deduced from the peak positions in the gel filtration assay (Fig. 6B). The protein from peak1 rerun on gel filtration assay remained a single peak, while the protein from peak2 rerun on gel filtration resulted in a shift into peak1 position (Fig. 6B). Therefore, there is a dynamic equilibrium between these two forms and the dimer form possibly represents the more stable state. Notably, although we have conducted crystallization experiments with proteins of both conformations, only the dimer form could be crystallized. Importantly, circular dichroism results showed that the protein from peak2 shared a similar spectrogram profile with the protein from peak1, indicating that monomer form was also properly folded (Fig. 6C). Unexpectedly, the monomer form displayed extremely low TGase enzyme activity and anti-phagocytic capacity compared with the dimer form (Figs. 3B and 3C). All the protein we used for the activity assay, unless otherwise specified, came from peak1. Thus, we proposed that dimerization of the protein could promote activation of the protein. To test that, we set out to examine the interface between the monomers. Since our structure indicated that interactions between the two monomers are mainly mediated by hydrogen-bonding interactions between Thr^{215} from one monomer and Ser^{218} of the other as well as water-mediated interactions between Leu^{412} and Arg^{311} from one monomer and Leu^{176} of the other, we generated two point mutations T215A and R311A. No conformational changes of the protein appeared after mutating Thr^{215} to Ala, and the protein from peak1 and peak2 displayed a similar TGase activity and anti-phagocytic ability with the wild-type (Figs. 6B, 3B and 3C). Interestingly, the SsTGase-N^{R311A} protein only displayed a single peak at the monomer position in the gel filtration profile, implying that the conformation of the protein changed into a monomer completely and Arg^{311} played an important role in stabilizing the dimerization of the protein (Fig. 6B). TGase activity and anti-phagocytic ability of SsTGase-N^{R311A} decreased to a quite low level (Figs. 3B and 3C), suggesting that dimerization of the protein is crucial for maintaining functional activities. Structural analysis revealed that Arg^{311} and catalytic amino acid Cys^{302} from one monomer sit on the same α helix (α7' helix), which can be stabilized by water-mediated interactions mediated by α4 helix of the other. To our knowledge, similar interactions have not been observed in other solved structures, such as human factor XIII and human transglutaminase 3 (TGase 3) (27,29,31). Taken together, we proposed that the SsTGase-N monomer was not stable enough to catalyze the reaction in solution environment, while dimerization of the protein could promote its activation by stabilizing the architecture of catalytic cavity.

Discussion

In this study, we identified SsTGase as a new virulence factor in the infection process with anti-phagocytic function, which was dependent on its TGase activity. Structural analyses show that SsTGase-N shares a common feature of active site cavity with eukaryotic TGases, but with a novel activation mechanism. Due to ubiquitous distribution nature of TGases, it played important roles in physiological and pathological processes by post-translational modifications of substrates. For instance, glycoprotein gp42 presented in the cell
wall of *Phytophthora sojae* could induce plenty of defense mechanisms, bringing out a hypersensitive response, resulting in death of the infected cells (21,45). Moreover, some bacterial toxins, including the cytotoxic factor 1 of *E. coli*, act as TGase (46). Thus, we proposed that SsTGase was secreted from cytoplasmic membrane and activated by disruptions of the physiological homoeostatic environment after its invading, and then the matured SsTGase could modify the surface proteins of *Ss* 2 and/or host to avoid phagocytosis. Obviously, the detailed information of how SsTGase functions as a virulence factor in the infection process of *Ss* 2 need further investigation.

Interestingly, mapping the electrostatic potential of SsTGase-N onto its surface revealed that the active site was mainly surrounded by highly negatively charged residues (Fig. 7). Indeed, a similar situation was also observed in the structure of GP42, of which a strong negative potential delineates a groove adjacent to the active site (29). To our knowledge, identification of the target proteins by TGase has been very challenging, due to the highly cross-linked property and the insolubility of the product (47). Therefore, to date, no substrates of SsTGase-N have been identified. This structural feature prompted us to propose that it was likely that the enzyme tended to interact with positively charged substrates.

Structural directed mutagenesis studies revealed that dimerization was required for the enzymatic activity of SsTGase-N. Based on this, we proposed a new activation mechanism of SsTGase-N. Notably, human transglutaminase 2 (TG2) undergoes a large conformational change upon activation, while overall structures of MTGase zymogen and mature MTGase are essentially the same (30,48). Therefore, structural changes of SsTGase-N upon activation still await the determination of the structure of SsTGase-N in a monomer state. Detailed description of the activated mechanism will clearly require more biochemical characterizations.

The characterization of SsTGase as a novel virulence factor of *Ss* 2 by acting as a new TGase in TGase-like superfamily (PF01841) will facilitate the development of new therapeutic agents capable of efficiently interfering with *Ss* 2 infection. In addition, compared with the high cost of transglutaminase of animal origin (25,49), SsTGase might have the potential to be applied as a new biocatalysts in the biomedical and biotechnology fields.

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**Conflict of interest**

The authors declare that they have no conflicts of interest with the contents of this article.

**Author Contributions**

M.Y., Y.J., and Y.Y. designed the study and wrote the paper. J.Y. purified and crystallized SsTGase-N protein. J.Ge. determined X-ray structure of SsTGase-N. J.Y. and Y.P. designed and constructed vectors for expression of the mutant protein and analyzed anti-phagocytic ability and TGase activity of the mutant protein. Y.P., J.Gu., Y.Z., H.J. and H.H. performed blood survival assay, polymorphonuclear leukocyte (PMN) killing assay, infections of piglets experiment and western blot. All authors analyzed the results and approved the final version of the manuscript.
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**Footnotes**

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Functional and structural characterization of a novel transglutaminase

Figure Legends

FIGURE 1. SsTGase developed strong anti-phagocytic ability in the supernatant of Ss2.

A. Domain organization of SsTGase-N. The numbers of the amino acid residues identifying the boundaries between adjacent domains are indicated below. S, signal sequence; TGase, transglutaminase-like domain; TM, transmembrane segment. The region spanning residues 38 to 437 were used in crystallization of SsTGase-N.

B. SsTGase presented in supernatant was detected by western blotting after incubation with pre-immune rabbit sera (left) and anti-SsTGase-N sera (right). Each of the lane is labeled above the line. Sup: supernatant.

C. Decreased resistance of SsTGase to PMN-mediated killing. The wild-type strain 05ZYH33, the mutant strain ΔSsTGase, the complemented strain CΔSsTGase and 05ZYH33 plus MDC were co-incubated with human PMNs at an MOI of 1:15 in 50% non-immune human serum at 37 °C under 5% CO₂. Samples were taken at time points and analyzed immediately. Colonies were counted, and the data were presented as means ± standard deviations (SD) from three separate experiments. **P < 0.01.

FIGURE 2. SsTGase was a new virulence factor of Ss2.

A. SsTGase developed strong anti-phagocytic ability. The culture supernatant of the WT and CΔSsTGase strains markedly enhanced Ss2 survival in human blood. Samples of culture supernatant of WT, ΔSsTGase and CΔSsTGase strains, and PBS were incubated with the bacteria of WT, ΔSsTGase or CΔSsTGase in fresh human blood for 1 h, respectively. The survived bacteria were counted on THB plates. Each bar showed the average and standard deviation of three to four independent measurements. ***P < 0.001.

B. SsTGase-N strongly improved the ΔSsTGase strain survival in human blood. Different amounts of SsTGase-N (1, 5, 10, 20, 50 µg) were incubated with culture supernatant of the ΔSsTGase strain for 30 min, and then mixed with fresh human blood and ΔSsTGase strain for 1 h. Each bar showed the average and standard deviation of three to four independent measurements.

C. Survival curves of piglets after challenged with Ss2. Piglets inoculated with the 05ZYH33 (solid-dots line), ΔSsTGase (hollow-dots line), CΔSsTGase strains (solid inverted-triangle line) and avirulent strain 1330 (hollow-triangle line) respectively. Bacteria (2×10⁸ CFU) were administered to each animal (n=6 piglets per group) by intravenous injections.

D. Bacterial growth in blood after intravenous challenge. Results from individual piglets were shown as log 10 of bacterial counts (CFU/ml). 05ZYH33, ΔSsTGase and CΔSsTGase strains were represented as black solid-dots, white hollow-dots, black solid-box respectively. Horizontal lines indicated the mean for each group.

E. Bacterial growth in blood after intravenous challenge by competitive-infection assay. Groups of four SPF-piglets were inoculated intravenously with a 1:1 mixture of 05ZYH33/ΔSsTGase strains (dose of 10⁸ CFU/piglet), respectively. Results from individual piglets were shown as log 10 of bacterial counts (CFU/ml). 05ZYH33 and ΔSsTGase strains were shown as solid-dots, hollow-boxes respectively. Horizontal lines indicated the mean for each group.

F. Bacterial counts in various organs by competitive-infection assay. Groups of four SPF-piglets were inoculated intravenously with a 1:1 mixture of 05ZYH33/ΔSsTGase strains (dose of 10⁸ CFU/piglet), respectively. Results from individual piglets are shown as log 10 of bacterial counts (CFU/0.5g). 05ZYH33 and ΔSsTGase strains were shown as solid-dots, hollow-boxes respectively. Horizontal lines indicated the
mean for each group.

**FIGURE 3.** Anti-phagocytic ability of SsTGase-N was dependent on its TGase activity.

A. Transglutaminase activity of SsTGase-N was Ca\(^{2+}\)-independent. Colorimetric assay of transglutaminase activity of purified SsTGase-N by Transglutaminase Colorimetric Microassay Kit (TCM kit; Covalab). TCM kit uses immobilized N-carbobenzyo (CBZ) -Gln-Gly as the amine acceptor and biotin-conjugated cadaverine as the amine donor. As a reference for TGase activity, purified guinea pig TGase (gpTGase) with specific activity of 0.1 U/mg were incubated under the same conditions. Representative of each lane was labeled. MDC, a potent inhibitor of TGases. Each bar showed the average and standard deviation of three to four independent measurements.

B. Anti-phagocytic abilities of SsTGase-N as well as mutants were demonstrated by blood survival assay. Each mutation was labeled above the line. Each bar showed the average and standard deviation of three to four independent measurements. ***P < 0.001.

C. Colorimetric assay of transglutaminase activity of purified SsTGase-N as well as mutants by Transglutaminase Colorimetric Microassay Kit (TCM kit; Covalab). Each mutation was labeled above the line. Each bar showed the average and standard deviation of three to four independent measurements. ***P < 0.001.

**FIGURE 4.** Overall structure of SsTGase-N.

A. Ribbon representation of the structure of SsTGase-N with its N and C terminus indicated. NTDs with α5 helices are shown as blue and magenta, and CTDs are shown as red and cyan.

B. The topology of SsTGase-N. NTD and α5 helix are depicted in pink, and CTD is depicted in cyan. The regions where catalytic residues located are shown in green color. The missing region between β10 strand and α8 helix (residues 353 to 363) is represented as a dotted line.

**FIGURE 5.** Common feature of the active site in TGase-like family.

A. Sequence alignment between SsTGase-N and homologous functional TG domains of human coagulation Factor XIII (Factor XIII), fish-derived transglutaminase (FTG), human transglutaminase3 (TGase3) and WbmE protein from *B. bronchiseptica*. Conserved residues of catalytic triad are indicated by red arrows.

B. Graphic representation of the catalytic active cavity of SsTGase-N. The color scheme is the same as in Fig. 4A. Catalytic active residues and residues involved in stabilizing catalytic active cavity are shown in yellow and magenta sticks respectively. Hydrogen bonds are represented as red dashed lines. Enlarged views of catalytic active cavity in the left panel are shown in the right panel.

C. Stereo views of structural alignment of the catalytic region of SsTGase-N (cyan), human coagulation Factor XIII (pink, Protein Data Bank ID 1GGT) and fish-derived transglutaminase (orange, Protein Data Bank ID 1G0D). Detailed views of the superposition of catalytic residues in the left panel are shown in the right panel.

**FIGURE 6.** Dimerization of the protein could promote activation of the protein by stabilizing the architecture of catalytic cavity.

A. Graphic representation of the interface between two monomers. The color scheme is the same as in Fig. 4A. Residues involved in interactions are indicated and shown in yellow sticks. Hydrogen bonds are represented as red dashed lines. Enlarged views of the parts in the black box in the left panel are shown in the right panel.
B. The mutant proteins used in the measurement of anti-phagocytic abilities and transglutaminase activities fold as well as wild-type protein. The purification profiles of the wild-type, mutant proteins, protein from peak1 and the protein from peak2 are shown after a gel filtration. The sizes of the molecular markers are marked on top of the peaks.

C. The protein in peak2 is correctly folded as the protein in peak1. The purified proteins (1 mg/ml) from peak1 (black curve) and peak2 (red curve) solubilized in buffer containing 20 mM Hepes pH 7.5, 200 mM NaCl were subjected to circular dichroism (CD) spectra.

FIGURE 7. Electrostatic surface model of the monomer of SsTGase-N. The graphic and electrostatic surface representations are shown in the left and right panel. Catalytic region is indicated with black dash circle.
Table 1. Summary of cloning primers in generation of the mutant strain ΔSsTGase and the complemented strain CΔSsTGase

| Primers        | Sequence<sup>a</sup> (5′–3′)                     | PCR products                                                                 |
|---------------|--------------------------------------------------|-------------------------------------------------------------------------------|
| SsTGase KOP1  | GCG<sup>GGATCC</sup>TAAATCAAGGCAAGTTTG         | The SsTGase gene and its upstream flanking regions                           |
| SsTGase KOP2  | CCTCGGAACCCATCGAATTACAACCGGTTGTGATGTCCG        |                                                                               |
| CM-F          | TAAATCGGATGGTTCCGAGG                            | Chloramphenicol resistant gene                                                |
| CM-R          | CACCGAACTAGAGCTTGATG                            |                                                                               |
| SsTGase KOP5  | CATCAAGCTCTAGTTCCGGTGAGCTTGATAACAAATTGGG       | The SsTGase gene and its downstream flanking regions                         |
| SsTGase KOP6  | TGGC<sup>GAATTCC</sup>GTCAGCCTCCTAAATCTGCTG    |                                                                               |
| SsTGase IN1   | GCTGCTCCCTCTCAACAAAC                           | Internal region of SsTGase gene                                              |
| SsTGase IN2   | TTAATCCGGTGCTTCTGT                              |                                                                               |
| SPC-F         | GTTTCTCGTGAATACATGTATA                         | Spectinomycin resistant gene                                                 |
| SPC-R         | GTTTCTAAAATCTGATTACCA                         |                                                                               |
| CΔSsTGase-F   | CG<sup>GAATTCC</sup>GATTAGTTGCATTGTG          | The open reading frame of SsTGase gene and its upstream promoter             |
| CΔSsTGase-R   | CGGAT<sup>CC</sup>GCTCCTAGACAAATACCA          |                                                                               |

<sup>a</sup>The underlined sequences are the restriction sites.
Table 2. Statistics of data collection and structure refinement

| Data collection | Se-SAD       | Native       |
|-----------------|--------------|--------------|
| Space Group     | P212121      | P212121      |
| Unit Cell (Å)   | 85.503, 95.047, 106.051 | 89.133, 91.935, 102.172 |
| Wavelength (Å)  | 0.979        | 0.979        |
| Resolution (Å)  | 2.75 (2.85-2.75) | 2.10 (2.18-2.10) |
| Rsym (%)        | 11.7 (92.2)  | 8.7 (73.7)   |
| I/sigma         | 16.0 (2.5)   | 26.1 (3.25)  |
| Completeness (%)| 96.3 (77.7)  | 100 (100)    |
| Redundancy      | 8.0 (8.2)    | 10.2 (10.3)  |
| Wilson B factor (Å²) | 44.91        | 24.2         |

**Refinement**

|                  |              |              |
|------------------|--------------|--------------|
| R factor         | 0.1934       |              |
| R free           | 0.2384       |              |
| No. atoms        | 6493 protein atoms+447 water atoms |              |
| B factors        |              |              |
| Overall          | 38.1         |              |
| Main chain       | 36.3         |              |
| Side chain       | 39.9         |              |
| RMSD bond lengths| 0.008        |              |
| RMSD bond angles | 1.074        |              |
| Ramachandran plot statistics (%) | | |
| In preferred regions | 95.89    | | |
| In allowed regions | 3.85      | | |
| Outliers         | 0.27         | | |

Values in parentheses are for the highest resolution shell. \( R_{sym} = \frac{\sum_h \sum_i |I_{h,i} - I_h|}{\sum_h \sum_i I_{h,i}} \), where \( I_h \) is the mean intensity of the \( i \) observations of symmetry related reflections of \( h \). \( R = \frac{\sum |F_{obs} - F_{calc}|}{\sum F_{obs}} \), where \( F_{calc} \) is the calculated protein structure factor from the atomic model (\( R_{free} \) was calculated with 5% of the reflections).
FIGURE 1

A

SsTGase

TGate

1

38

1121

348

1097

247

437

PF01841

B

M

05ZYH33

Δ1815

CΔ1815

05ZYH33+MDC

~110KD

C

Survival rate (%)

0 20 40 60 80

1h 2h 3h

Time (hours)

**

**

**

05ZYH33

Δ1815

CΔ1815

05ZYH33+MDC

**

**
FIGURE 3

A

B

C

Functional and structural characterization of a novel transglutaminase
FIGURE 4

A

B
FIGURE 6
FIGURE 7
Functional and structural characterization of the anti-phagocytic properties of a novel transglutaminase from Streptococcus suis
Jie Yu, Yaya Pian, Jingpeng Ge, Jie Guo, Yuling Zheng, Hua Jiang, Huaijie Hao, Yuan Yuan, Yongqiang Jiang and Maojun Yang

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