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Trigger factor of *Streptococcus suis* is involved in stress tolerance and virulence

Tao Wu [a,b,1], Zhanqin Zhao [b,d,1], Lin Zhang [c,1], Hongwei Ma [a], Ka Lu [a], Wen Ren [a], Zhengya Liu [a], Haitao Chang [b], Weicheng Bei [b], Yinsheng Qiu [a], Huanchun Chen [a,b,*]

[a] *Hubei Key Laboratory of Animal Nutrition and Feed Science, School of Animal Science and Nutritional Engineering, Wuhan Polytechnic University, Wuhan 430023, PR China*
[b] *Division of Animal Pathogens, State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, PR China*
[c] *Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Science, Jingzhou 434000, PR China*
[d] *Laboratory of Animal Infectious Diseases and Microbiology, College of Animal Science & Technology, Henan University of Science and Techology, Luoyang 471003, PR China*

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ABSTRACT

Streptococcus suis serotype 2 is an important zoonotic pathogen that causes serious diseases such as meningitis, septicemia, endocarditis, arthritis and septic shock in pigs and humans. Little is known about the regulation of virulence gene expression in *S. suis serotype 2*. In this study, we cloned and deleted the entire *tig* gene from the chromosome of *S. suis serotype 2* SC21 strain, and constructed a mutant strain (Δtig) and a complementation strain (CΔtig). The results demonstrated that the *tig* gene, encoding trigger factor from *S. suis serotype 2* SC21, affects the stress tolerance and the expression of a few virulence genes of *S. suis serotype 2*. Deletion of the *tig* gene of *S. suis serotype 2* resulted in mutant strain, ΔTig, which exhibited a significant decrease in adherence to cell line HEp-2, and lacked hemolytic activity. Tig deficiency diminishes stresses tolerance of *S. suis serotype 2* such as survive thermal, oxidative and acid stresses. Quantification of expression levels of known *S. suis serotype 2* SC21 virulence genes by real-time polymerase chain reaction *in vitro* revealed that trigger factor influences the expression of epf, cps, adh, rpob, fbps, hyl, sly, mrp and hrcA virulence-associated genes. ΔTig was shown to be attenuated in a LD50 assay and bacteriology, indicating that trigger factor plays an important part in the pathogenesis and stress tolerance of *.S. suis serotype 2* infection. Mutant ΔTig was 100% defective in virulence in CD1 mice at up to 107 CFU, and provided 100% protection when challenged with 107 CFU of the SC21 strain.

1. Introduction

Trigger factor is a ribosome-associated peptidyl-prolyl cis-trans isomerase which is a molecular chaperone that is highly conserved in most bacteria [10–12,22] and is present in all species of eubacteria. It is generally believed that trigger factor is central to protein biogenesis and bacterial survival of environmental insult. It binds to the ribosomal 50S subunit near the translation exit tunnel and is thought to be the first protein to interact with nascent polypeptides emerging from the ribosome [10–12,19,38]. Although the full spectrum of activities of this protein in bacterial physiology and virulence expression is not yet fully appreciated. But it verified that the chaperone has a peptidyl-prolyl cis-trans isomerase (PPIase) activity that catalyzes the rate-limiting proline isomerization in the protein-folding process [10–12].

The trigger factor protein was originally found assisting in the maintenance of the translocation-competent conformation of the precursor protein proOmpA (outer membrane protein A) *in vitro* in the gram-negative bacteria [6]. The trigger factor in *Escherichia coli* is associated with the 50S ribosomal subunit, binds polypeptides as they emerge from the ribosome and functions in cooperation with the DnaK chaperone complex in folding of newly synthesized proteins [40]. At the same time trigger factor is demonstrated that it can protect cells against low temperature [14,17]. Trigger factor also interacts with GroEL, greatly promoting the affinity and binding capacity of GroEL for various denatured proteins [14–17].

The trigger factor is founded in the gram-positive bacteria too, such as in *Streptococcus pyogenes*, an apparent trigger factor homologue, RopA, has been shown to be essential for secretion and maturation of the secreted cysteine protease SpeB [21,22]. Trigger factor of S. mutans was found to be up-regulated in response to deficiency of LuxS (Z. T. Wen and R. A. Burne, unpublished data), which was shown to affect acid and oxidative stress tolerance and biofilm formation [41]. The expression of trigger factor was also

* Corresponding author. Hubei Key Laboratory of Animal Nutrition and Feed Science, School of Animal Science and Nutritional Engineering, Wuhan Polytechnic University, Wuhan 430023, PR China. Tel.:+86 27 87288629; fax: +86 27 87281795.
E-mail address: chenhch@mail.hzau.edu.cn (H. Chen).
[1] Equally contributed in this work.

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increased in cells stimulated by the synthetic competence-stimulating peptide (CSP) (G. Svensater, personal communication) and in populations that were grown in biofilms [32]. that is trigger Factor in *Streptococcus* mutans is involved in stress tolerance, competence development, and biofilm formation.

Streptococcus suis serotype 2 is an important swine pathogen mainly associated with arthritis, endocarditis, meningitis, pneumonia, and septicemia [20,31]. It is also a major zoonotic agent for humans in contact with colonized, otherwise healthy pigs or their by-products, causing life-threatening infections. In 2005 an unprecedented epidemic in China which resulted in 204 human cases [36]. During the past decade, many virulence factors of *S. suis serotype 2* involved in the survival, spread and persistence of the bacterium within the host have been identified. These factors include muramidase-release proteins (MRP) [29] and extracellular factor (EF), which are required for resistance to phagocytosis [39]; suilysin (SLY) which can damage host cells and increase cell permeability[13]; capsular polysaccharide (CPS) [30], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [4], fibrinogen binding protein (FBPS) [1,42] hyaluronate lyase (HYL) [25], highly immunogenic proteins such as surface antigen one (Sao) [18] and serum opacity factor(Ofs), whose exact role in virulence is undetermined [2]. It is known that expression of these genes contributes to the virulence of *S. suis serotype 2*, but production of the disease requires temporal and coordinated expression of a series of genes that allow the prospective pathogen to shift to its pathogenic state and adapt to a hostile environment in the host. Many important virulence factors are environmentally regulated and induced at specific stages of the infection process [23]. Environmentally regulated genes of *S. suis serotype 2* were therefore selected to identify more putative virulence factors [28].

The genomic sequences of the *S. suis serotype 2* strain 05ZYH33 was published in 2007. Analysis of sequence data revealed several putative virulence genes, including genes for putative bacterial surface proteins and virulence regulators. The *tig* gene of trigger factor that was selected *in vivo* showed similarity in the database to trigger factor of *Streptococcus* mutans and *S. pyogenes* [5]. Trigger factor of *S. pyogenes* is associated with its stress tolerance [21,22]. This indicated that tig may been involved in the pathogenesis of *S. suis serotype 2* infection in piglets. In this study, we cloned and deleted the entire *tig* gene from the chromosome of *S. suis serotype 2* SC21 strain (wide type), and constructed a mutant strain (Δtig) and a complementation strain (CΔtig). The results demonstrated that the *tig* gene, encoding trigger factor from *S. suis serotype 2* SC21, affects the stress tolerance and the expression of a few genes from *S. suis serotype 2* by quantitative real-time polymerase chain reaction (qRT-PCR), including those involving in virulence, adhesion for bacteria. The level of *tig* gene expression was restored in mutant Δtig strain by plasmid-mediated expression of *tig* gene, confirming trigger factor deficiency as the cause for the observed changes in virulence gene expression and stress tolerance in *S. suis serotype 2*.

2. Materials and methods

2.1. Bacteria strains, growth conditions, plasmids, and primers

Bacteria strains, plasmids and oligonucleotide primers are listed in Table 1. *S. suis*, SC21 strain, is a serotype 2 clinical isolate obtained from human *S. suis* infection outbreak in June 2005 in Ziyang County, Sichuan Province, China. It was cultivated at 37 °C in Todd–Hewitt yeast broth (THY, Difco Laboratories, Detroit, MI, USA) containing 2% yeast extract. *S. suis serotype 2* clones, carrying pAT18 were selected with erythromycin (300 μg/ml). *E. coli* clones carrying the plasmid pSET4s, pAT18 or pET28a were selected in spectinomycin (100 μg/mL), erythromycin (300 μg/mL) or kanamycin (50 μg/mL).

2.2. Eukaryotic cells and media

Human laryngeal cancer cell line Hep-2 (GDC004) was obtained from China Center for Type Culture Collection, Wuhan, China. Hep-2 were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal calf serum (FCS; HyClone) with penicillin and streptomycin (100 μg/mL) at 37 °C in a humidified incubator.

2.3. General DNA techniques

Chromosomal *S. suis serotype 2* DNA was isolated according using standard procedures [24]. Plasmid DNA from *S. suis serotype 2* was isolated as previously described [34,35]. Conventional techniques for DNA manipulation, such as PCR, ligation, transformation and Southern blotting were done as described by Sambrook [26]. Restriction enzymes, T4 DNA ligase, and alkaline phosphatase were purchased from Takara Co. Ltd. (Tokyo, Japan) and used according to manufacturer instructions. Oligonucleotides were purchased from Takara Co. Ltd. (Tokyo, Japan). Southern hybridization was done as previously described [27]. Genomic DNA of *S. suis serotype 2* were digested with PvuII. Probe were labeled with digoxigenin (DIG) by using a DIG–DNA labeling and detection kit, or DIG–PCR labeling mixture according to manufacturer instructions (Boehringer GmbH, Mannheim, Germany).

2.4. Construction of a tig gene deletion mutant

The *tig* gene was deleted in the chromosome of *S. suis serotype 2* SC21 according to a previously described procedure [34,35]. In brief, two DNA fragments flanking the *tig* gene were amplified by PCR from the genome of SC21 with the primers SD1/SD2 and primers SD3/SD4. The fragments of the flanking region of the *tig* gene were digested with *Sal*I/*BamH*I and *BamH*I/*Eco*RI, respectively, mixed in equal amounts and inserted directly into *BamH*I/*Eco*RI digested and dephosphorylated thermosensitive suicide vector pSet4s, ligated, and transformed into *E. coli* DH5-α. The resulting plasmid, pST4s-*tig*, was electroporated into SC21. The resultant strains were grown at 28 °C in spectinomycin (100 μg/ml), selected and subsequently passaged at 34 °C in the absence of spectinomycin selection as previously described [34,35]. Mutant was confirmed by PCR and Southern blotting. A digoxigenin-labeled probe for the detection of deletions in Δ*tig* was obtained by PCR with the primers SD7 and SD8.

2.5. Plasmid-mediated expression of tig in S. suis serotype 2

The structural gene of the *tig* gene was amplified from chromosomal DNA of SC21 by PCR using the primers SD9 and SD10. PCR product was cut with *Xba*I/*Xho*I and ligated into the *Xba*I/*Xho*I-digested *E. coli/Streptococcus* shuttle vector pAT18 [37]. The resultant plasmid was termed pAT-*tig*. The plasmids pAT18 and pAT-*tig* were transformed by electroporation into the corresponding *S. suis serotype 2* strains with subsequent erythromycin selection. Complementation strain designed CΔTig.

2.6. Growth characteristics and hereditary stability of mutant strain

The wild-type strain SC21, mutant strain ΔTig, and complementation strain CΔTig were inoculated to THY media at the same concentration ($OD_{600} = 0.2$) and cultured in 34 °C. The OD_{600} of the culture was determined every 2 h to describe the growth curve. ΔTig