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Effect of Tran on virulence through regulating metabolism and stress tolerance of *Streptococcus suis* serotype 2

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ABSTRACT

Streptococcus suis (SS) is an important zoonotic pathogen causing a variety of life-threatening infections in pigs and humans. Tran, a novel transcriptional regulator which was identified to be an infection-related factor in *S. suis* serotype 2 using suppression subtractive hybridization (SSH), has been reported by our group. In this study, a *tran* deletion mutant was constructed to compare with the wild-type ZY05719 in some biological characteristics. It is suggested that longer chains and relatively slower growth could be observed in *tran* deletion mutants. In order to identify gene transcription profiles, microarray analysis was performed. It indicated that the inactivation of Tran led to 130 differentially expressed genes spread throughout the genome. Among these, 21 genes were upregulated, and 109 genes were downregulated. Most of the differentially expressed genes were involved in bacterial metabolism, such as the phosphotransferase system (PTS), and heat shock proteins. In the case of glucose scarcity, the growth characteristics of *tran* deletion mutants were impacted significantly, meanwhile Δ*tran* mutant was highly sensitive to environmental stresses. Moreover, cell adherence decreased by 22.2%, and virulence in zebrafish declined to more than five times in Δ*tran* mutants. These data demonstrate the role of Tran in regulation in *S. suis* serotype 2, that is affect bacterial virulence by influencing bacterial metabolism and stress tolerance of external environment.

1. Introduction

Streptococcus suis (*S. suis*) is a major swine pathogen responsible for a wide range of diseases (Gottschalk and Segura, 2000). Notably, two large-scale streptococcosis outbreaks were caused by *S. suis* serotype 2 (SS2) in 1998 and 2005 in China. The outbreaks led to severe economic loss in swine industry and posed great public health concerns worldwide (Tang et al., 2006).

The capsule of *S. suis* serotype 2 has been shown to play an essential role in pathogenesis (Smith et al., 1999). Suilysin (Jiang et al., 2009), adhesins (Tikkanen et al., 1996), and fibronectin (Cockerill et al., 1996), etc. have been described as virulence associated factors. However, virulent strains lacking virulence factors have been isolated from diseased pigs (Gottschalk and Segura, 2000), indicating that unknown virulence factors still exist.

Expression of these factors is controlled by transcriptional regulators, which in turn respond to environmental signals (Kreikemeyer et al., 2003). Such responses typically involve genome wide changes in transcriptional activation or repression of specific genes through interactions among multiple regulators. In recent years, thorough research and analyses of global regulatory networks in *Streptococcus pyogenes* have been made (Finney, 1985). However, the corresponding knowledge regarding SS2 is relatively limited (Benjamini et al., 2001).

According to previous studies, Tran is a transcriptional regulator (accession number: JX465717), and was identified to be an infection-related factor by suppression subtractive hybridization (SSH) in our group (Jiang et al., 2009). Transcriptional control of gene expression requires protein DNA interactions at the proximal promoter (Roman et al., 2008). Primary sequence analyses of Tran showed an unusual modular organization with an N-terminal region homologous to members of the XRE family of transcriptional regulators and a C-terminal region similar to the toxin–antitoxin system and the toxin component domain protein. Tran has been shown to be highly conserved in *streptococcus*, through blast analysis. For example, the sequence similarity between Tran and transcriptional regulators in *S. suis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Streptococcus sanguinis*, *Streptococcus agalactiae* was 99%, 58%, 40%, 57%, 33%, respectively. However, whether

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Table 1
Summary of bacterial strains and plasmids used in this study.

Strain and plasmid	Relevant characteristics	Source of references
Bacterial strains		
ZY05719	Virulent strain isolated from a dead patient with STSS	Collected in our laboratory
Δ*tran* mutant	Isogenic *tran* deletion mutant of strain ZY05719	This study
CΔ*tran* mutant	Complemented *tran* mutant; Spc^r	This study
E. coli DH5α	Cloning host for recombinant vector	Invitrogen
Plasmids		
pMD19-T vector	Clone vector; Amp^r	Takara
pSET4s vector	*S. suis* thermosensitive suicide vector; Spc^r	Takamatsu et al. (2001)
pSET2 vector	*E. coli*–*S. suis* shuttle vector; Spc^r	Takamatsu et al. (2001)

Amp^r, ampicillin resistant; Spc^r, spectinomycin resistant.

this transcriptional regulator is related to virulence, and which gene expressions are influenced by Tran in SS2 was still unclear.

In this report, the role of this selected transcription regulator (Tran) in the pathogenesis of an *S. suis* infection was studied. Microarray analysis was performed to identify gene transcription profiles using *tran* knock-out mutant compared to the wild-type strain ZY05719, which was originally isolated from SS2 infected pigs in Sichuan province by our group in 2005 (Chen et al., 2007). Our data confirmed that SS2 Tran protein is a transcriptional regulator that contributes to bacterial metabolism control, and stress tolerance. Moreover, the experimental infection in zebrafish suggested that the virulence of *tran* knock-out mutant was attenuated compared to the wild-type strain, indicating that Tran was indeed involved in the pathogenesis of *S. suis*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains, plasmids and growth conditions used are listed in Table 1. SS2 strains were grown in Todd Hewitt broth (THB) (Difco Laboratories, Detroit, MI) or plated on THB agar supplemented with 5% (vol/vol) sheep blood. *Escherichia coli* DH5α was cultured in Luria Broth (LB) liquid medium or plated on LB agar at 37 °C. Antibiotics (Sigma) were supplemented to culture media when necessary, at the following concentrations: ampicillin, 50 μg/mL for *E. coli*; spectinomycin (Spc), 100 μg/mL for *S. suis*, and 50 μg/mL for *E. coli*.

2.2. Construction of a Δtran-knockout mutant and functional complementation

To identify the function of Tran in *S. suis* serotype 2, a Δ*tran* knock-out mutant was constructed, using conventional methods (McKessar and Hakenbeck, 2007). DNA fragments were amplified from the chromosomal DNA of ZY05719 by PCR with two pairs of specific primers, L1/L2 and R1/R2, carrying *Pst*I/*Sal*I and *Bam*HI/*Sma*I restriction enzyme sites, respectively (Table 2). Fragments were digested with the corresponding restriction enzymes and directionally cloned into a temperature-sensitive *S. suis*–*E. coli* shuttle vector pSET4s. The resulting plasmid, pST4s-*tran*, was electroporated into ZY05719 as described previously (Cockerill et al., 1996).

The resultant strains were grown at 28 °C in the presence of Spc selection, and were then shifted to a 37 °C environment without Spc and incubated for 18–24 h. Subsequently, the cultures were spread on THB plates and incubated at 37 °C. Cultures were screened for mutants that had lost the vectors and had exchanged their wild-type allele for a genetic segment containing the *tran* gene as a consequence of homologous recombination via a double cross-over. A resulting Δ*tran* mutant strain was verified by PCR amplification with primers N1/N2, and confirmed by DNA sequencing, using the primers T1/T2.

For complementation assays, a DNA fragment containing the entire *tran* gene and promoter sequence was amplified using primers C1/C2 (shown in Table 2), which introduced *EcoRI* and *BamHI* sites, respectively. After digestion with the appropriate restriction enzymes, the resulting fragment was cloned into the *E. coli*–*S. suis* shuttle vector pSET2 to generate the *tran*-complementing plasmid pSET2-*tran*. This vector was electrotransformed directly into the Δ*tran* mutant to obtain the complementation strain CΔ*tran* using the previously reported method (Takamatsu et al., 2001).

2.3. Growth characteristics and genetic stability of mutant strains

The wild type strain ZY05719, mutant strain Δ*tran*, and the complementation strain CΔ*tran* were separately inoculated into flasks containing 100 mL THB media, and then incubated at 37 °C. Samples of culture were monitored at 1 h intervals using a spectrophotometer (Bio-Rad) at an absorbance of 600 nm, with quartz cuvettes. Uninoculated THB served as the blank control. The experiments were repeated three times.

2.3.1. DNA microarray-based analysis

The RNA samples were sent to CapitalBio Beijing, China, for microarray hybridization. Each RNA sample from different strains of the wild type strain ZY05719 and mutant strain Δ*tran* was hybridized to one Agilent *S. suis* serotype 2 ZY05719 Genome Expression Array. Briefly, double-stranded cDNA was synthesized from 6 mg of total RNA using a T7-oligo (dT) primer. Then the cDNA was purified and converted into cRNA via an *in vitro* transcription reaction. 5 μg cRNA reverse transcripted to cDNA and fragmented, then labeled with Cy3-dCTP (GE Healthcare) in the presence of Klenow. These labeled cDNA was hybridized to Agilent *S. suis* serotype 2 ZY05719 Genome Expression Array for 16 h at 42 °C. After hybridization, the GeneChips were washed and stained followed by scanning. Agilent *S. suis* serotype 2 ZY05719 Genome Expression Array contains over 15,000 probe sets, covering the whole transcripts of the *S. suis* serotype 2 ZY05719 genome.

The hybridization data were analyzed using GeneChip Operating Software (GCOS 1.4). The scanned images were first assessed by visual inspection, then analyzed to generate raw data files saved as CEL files using the default setting of GCOS 1.4. RMA (Robust Multichip Analysis) was used to normalize the different arrays. Linear models and empirical Bayes methods were used to analyze the data and find differentially expressed genes (Smyth, 2004). Statistically significant differences were defined as those with a *t* -test *P* value of less than 0.05 and Log FC (fold change) $\geq |0.5|$. The resulting *P* values were adjusted using the BH FDR algorithm (Benjamini et al., 2001). Standard deviations were compared to the median ratio for each strain.

2.3.2. RNA manipulation and real-time PCR evaluation

Real-time PCR assays were used for confirmating the microarray data in eight selected genes. Total RNA was extracted from *in vitro* late logarithmic phase (OD at 600 nm, 0.6–0.8) bacterial culture using the E.Z.N.A.TM Bacterial RNA isolation kit (OMEGA, Beijing, China) according to the manufacturer's protocol.cDNAs were reverse transcribed using the PrimeScript™ RT reagent kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The specific primers used for the various RT-PCR assays are listed in Table 2.