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ORIGINAL ARTICLE

DNA microarray analysis of acid-responsive genes of *Streptococcus suis* serotype 2

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Abstract An acidic environment is frequently found in phagocytes, while the intracellular survival and growth of *Streptococcus suis* serotype 2 (*S. suis* S2) in phagocytes is an essential part of the infection cycle of this pathogen. In this study, we used DNA microarrays to analyze the gene expression profile of *S. suis* S2 in response to acidic treatment. A total of 196 genes were differentially regulated when *S. suis* S2 was grown at pH 5.8 relative to at pH 7.2, especially including the inducible transcription of genes that encoded two-component regulatory systems, protection and repair functions, and intracellular pH homeostasis. The data showed that *S. suis* S2 is capable of employing diverse responsive mechanisms to protect against or adapt to acidic stress.

Keywords *Streptococcus suis* serotype 2 · Expression profile · Acid stress · Microarray

Introduction

Streptococcus suis is an important zoonotic pathogen causing a variety of life-threatening infections including meningitis, arthritis, septicemia and even sudden death in pigs and humans (Staats et al. 1997). Among the known 35 serotypes, *S. suis* S2 is the most virulent and the most frequently isolated clinical serotype. This serotype caused the two recent large-scale outbreaks of human *S. suis* S2 epidemic in China that was characterized by a toxic shock-like syndrome (Lun et al. 2007). Despite increasing research into *S. suis* in recent years, knowledge of virulence determinants and the pathogenesis of this pathogen remains limited.

The major ecological niche employed by *S. suis* is the epithelium of the host upper respiratory tract (Clifton-Hadley and Alexander 1980; Gottschalk and Segura 2000). Critical events in the development of disease include bacterial invasion from mucosal surface into deeper tissues and blood circulation, survival in blood, and invasion from blood to the central nervous system (Gottschalk and Segura 2000). On the other hand, as a major component of host innate immunity, phagocytes are able to take up, and contribute to the clearance of, internalized bacteria.

Streptococcus suis must develop specific adaptation strategies to enhance survival and limit bacterial killing in the host organism. A sequence of events including uptake of bacteria by monocytes, intracellular survival and invasion of the central nervous system by the "Trojan horse theory" has been proposed (Chabot-Roy et al. 2006). An acidic environment is frequently found in phagocytes, and *S. suis* has to display rapid responses to this adverse condition. In the present work, DNA microarrays were employed to investigate the genome-wide

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transcriptional alterations of this bacterium upon acid treatment.

Materials and methods

Bacterial growth

The *S. suis* S2 strain 05ZYH33 was used in this study. 05ZYH33 (MRP^+, EF^+, SLY^+ and $89K^+$) was isolated from a patient who died from streptococcal toxic shock syndrome (STSS) in an outbreak in Sichuan, China, in 2005 (Geng et al. 2008; Yu et al. 2006). Bacteria were maintained on Columbia blood agar (bioMérieux, Marcy l'Etoile, France) supplemented with 5% sheep blood. For microarray analysis, bacteria were cultured in Todd-Hewitt broth (THB) (BD, San Jose, CA) at 37°C to an OD_{600} value of about 1.1 in stationary phase, and then 1:100 diluted into fresh THB at pH 5.8 (test) or 7.2 (reference) for cultivation at 37°C. Bacterial cells were harvested at the mid-exponential phase (A_{600} of about 0.6 and 0.3 for pH 5.8 and 7.2, respectively; see Fig. 1).

RNA isolation

Total cellular RNA was extracted using TRizol Reagent (Invitrogen, Carlsbad, CA) without the DNA-removing step (for RT-PCR) or by using MasterPureTM RNA Purification kit (Epicenter, Singapore) removing contaminating DNA according to the manufacturer's instructions (for microarray) as previously described (Han et al. 2004; Zeng et al. 2010). RNA quality was monitored by agarose gel electrophoresis and RNA quantity was measured by spectrophotometer.

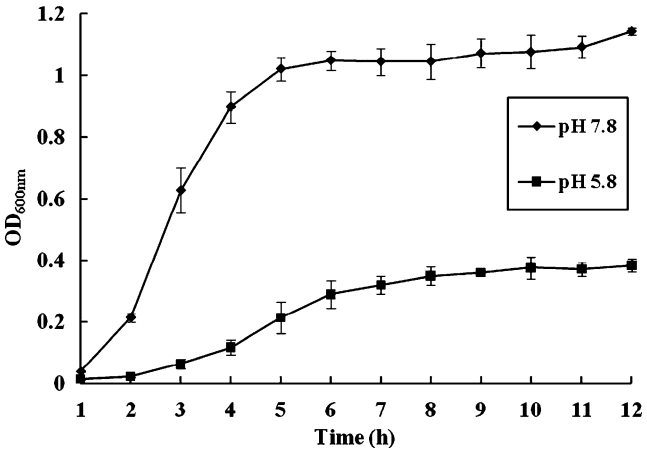


Fig 1 Growth curve of *Streptococcus suis* S2. Bacteria were cultured in Todd-Hewitt broth (THB) at 37°C to an OD_{600} value of about 1.1 in the stationary phase, and then 1:100 diluted into 20 ml fresh THB at pH 5.8 or 7.2 for cultivation at 37°C. The optical densities (OD_{600}) were monitored for each culture at 1-h intervals until the cultures reached stationary phase. Experiments were performed in triplicate

Microarray experiments

Microarray experiments were performed as previously described (Han et al. 2004; Zeng et al. 2010). Total RNA was used to synthesize cDNA in the presence of aminoallyl-dUTP and random hexamer primers. The aminoallyl modified cDNA was then labelled with Cy5 or Cy3 dye. The dual-fluorescently (Cy3 or Cy5 dye) labeled cDNA probes, for which the incorporated dye was reversed, were synthesized from the RNA samples of four biological replicates, and then hybridized to four separated microarray slides, respectively. A ratio of mRNA levels was calculated for each gene. Significant changes in gene expression were identified using SAM software (Tusher et al. 2001). After SAM analysis, only genes with at least 2-fold changes in expression were collected for further analysis.

Real-time quantitative PCR analysis

Gene-specific primers were designed to produce an amplicon of 150–200 bp for each gene tested. The contaminating DNA in RNA samples was removed by using Amibion's DNA-free™ Kit (Applied Biosystems, Foster City, CA). cDNAs were generated by using 5 μg RNA and 3 μg random hexamer primers. Using three independent cultures and RNA preparations, real-time RT-PCR was performed in triplicate as described previously using the LightCycler system (Roche, Pleasanton, CA) together with the SYBR Green master mix. On the basis of the standard curves of 16S rRNA expression, the relative mRNA level was determined by calculating the threshold cycle (ΔCt) of each gene using the classic ΔCt method. Negative controls were performed by using 'cDNA' generated without reverse transcriptase as templates. Reactions containing primer pairs without template were also included as blank controls. The 16S rRNA gene was used as an internal control to normalize all the other genes. A mean ratio of two was taken as the cutoff for statistical significance.

Results and discussion

Overview of microarray data

Streptococcus suis S2 could not grow at pH 4.0 (data not shown), and exhibited restricted growth at pH 5.8 relative to pH 7.2 in THB medium (Fig. 1). The gene expression profiles of *S. suis* S2 continuously grown under pH 5.8 (acidic stress) and 7.2, respectively, were compared. Of the 1,756 genes whose mRNA expression was detected by

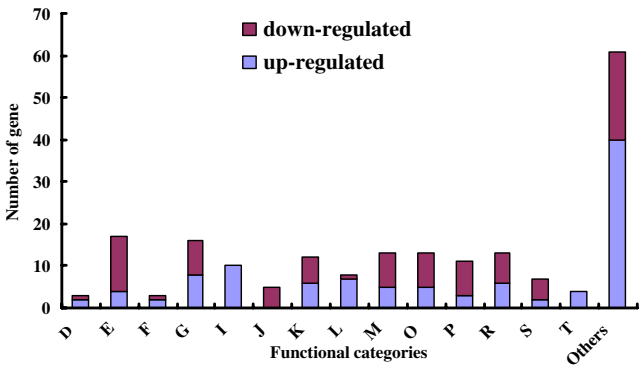
microarray, 196 were differentially regulated upon acidic stress, including 92 (47%) down-regulated and 104 (53%) up-regulated genes (Supplemental Table S1). The 196 differentially regulated genes could be assigned into 15 function categories (COG) based on the 05ZYH33 genome annotation as shown in Fig. 2, which included many central biological functions such as metabolism, transcription, translation and DNA replication.

A total of 16 genes were selected arbitrarily for test by quantitative RT-PCR, and the RT-PCR and microarray data for these 16 genes gave a correlation coefficient (r) of 0.96 (Fig. 3), indicating the reliability of microarray data.

Two-component gene regulatory systems

To sense and respond to changes in external conditions, bacteria often use specialized protein pairs, e.g., sensor kinase/response regulator, referred to as a two-component regulatory system. Changes in the environment are detected by the membrane-localized sensor kinase, which transduces the signal to a receiver protein that is active as a response regulator to mediate altered gene expression (Quach et al. 2009). The current microarray analysis revealed two two-component regulatory systems, NisK/R (05SSU0906-0907) and CiaR/H (05SSU1094-1095), which might play roles in acid tolerance in *S. suis* S2.

nisK/R genes were up-regulated 2.4-fold at pH 5.8 relative to pH 7.2. *nisk/r* genes are located in the 89K pathogenicity island, and act to initiate the infection cycle through adapting to different environments (Chen et al. 2007). The 89K island appears to be specific to STSS-causing *S. suis* S2 strains (Tang et al. 2006). Besides general elements such as transposases and palindrome sequences, 89K possesses several virulence-associated components including ABC-transporters, two-component gene regulatory systems and a type IV secretary system (Chen et al. 2007; Li et al. 2008).



D: Cell division and chromosome partitioning
E: Amino acid transport and metabolism
F: Nucleotide transport and metabolism
G: Carbohydrate transport and metabolism
I: Lipid metabolism
J: Translation, ribosomal structure and biogenesis
K: Transcription
L: DNA replication, recombination and repair
M: Cell envelope biogenesis, outer membrane
O: Posttranslational modification, protein turnover, chaperones
P: Inorganic ion transport and metabolism
R: General function prediction only
S: Function unknown
T: Signal transduction mechanisms

Fig 2 Differentially regulated genes grouped by functional classification. The differentially regulated genes on the chromosome were divided into 15 categories according to 05ZYH33 genome annotation. The number of genes up-regulated and down-regulated for each functional group is represented

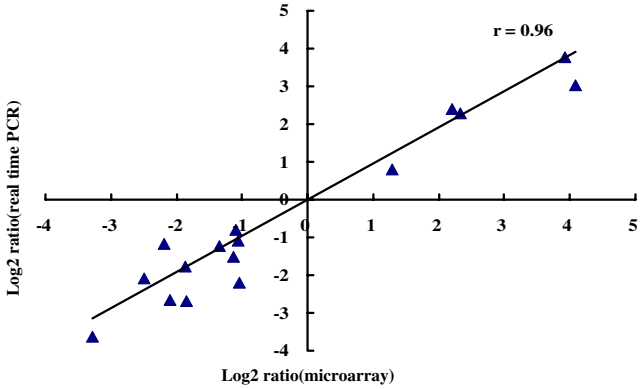


Fig 3 Comparison of microarray and RT-PCR data. The relative transcriptional levels of the 16 genes listed in Supplementary Table S2 were determined by microarray and real-time RT-PCR. The fold changes in gene transcription in response to pH value alteration (pH 5.8 versus 7.2) were logarithm-transformed in base 2. The real-time RT-PCR $\log_2$ values were plotted against the microarray data $\log_2$ values

ciaR/H genes were up-regulated more than 15-fold at pH 5.8. CiaR/H homologues are present in all published genomes of Group B streptococcus serotypes Ia, Ib, II, III, and V (Quach et al. 2009). *Streptococcus* CiaR promotes survival in brain endothelium and in host phagocytic cells such as neutrophils and macrophages, and confers bacterial resistance to host antimicrobial mechanisms including the release of pore-forming cationic antimicrobial peptides and reactive oxygen species (Maisey et al. 2008).

Intracellular pH homeostasis

05SSU1147 encodes an SPX domain-containing protein that was up-regulated 24-fold at pH 5.8, and 05SSU1292 encodes a phosphoglycerol transferase that was induced more than 4-fold at pH 5.8. Both these proteins are likely involved in vacuolar polyphosphate accumulation and storage in the polyphosphate granules that are defined as the acidic, calcium-rich compartments of bacteria known as acidocalcisomes (Docampo et al. 1995; Scott et al. 1997). Acidocalcisomes are electron-dense acidic organelles with a high concentration of phosphorus present as polyphosphate complexed with calcium and other elements, and link poly P inextricably to pH homeostasis (Docampo et al. 2005).

In terms of acid tolerance, membrane composition can affect proton permeability either directly or indirectly.

Direct effects involve the base permeability of the lipid bilayer to H^+ ions, whereas indirect effects involve changes in membrane lipid composition that affect the optimal activity of F_1-F_0-ATPase proton pumps (Cotter and Hill 2003). Several ATPase-encoding genes, 05SSU0389-0390 and 05SSU1385-1386, were up-regulated at pH 5.8. A sufficient capacity of intracellular ATPase is required for the extrusion of hydrogen ions (H^+) and consequently for the establishment of pH homeostasis (Cotter and Hill 2003). Generally, this is modulated by the pathogen through the prevention of proton influx by altering membrane composition and increasing proton extrusion via end-product efflux and F_1-F_0-ATPase activity (Cotter and Hill 2003). These mechanisms are thought to allow *S. suis* S2 to cope with changing pH levels and reduce the denaturing effects of an acidic cytoplasm. Furthermore, these protective functions would allow sustained bacterial growth and permit the proper functioning of enzymes and other cellular processes that would otherwise be inhibited by an acidic intracellular compartment.

Protection and repair functions

A major problem for bacteria that live in acidic environments is the potential for the acidic surroundings to acidify bacterial cytoplasm, which leads to loss of enzyme activity as well as structural damage to cell membranes, proteins and DNA. Our microarray data supported the notion that *S. suis* S2 employs diverse mechanisms to protect against acid-induced cell damages.

05SSU0952-0953 and 05SSU0903, encoding recombinases, were up-regulated above 3-fold at pH 5.8. Recombinase proteins are conserved throughout all kingdoms of life (Brendel et al. 1997), and operate as a moderator of homologous recombination, and serving a housekeeping role by repairing and restarting stalled DNA replication forks, which is particularly important during DNA replication (Cox 2007). A recombinase RecA-deficient mutant of *Streptococcus mutans* shows increased susceptibility to a killing pH of 2.5 relative to the parent strain (Quivey et al. 1995).

Molecular chaperone proteins form a multi-protein network that governs protein folding, refolding, transport, degradation and regulation (Ellis 1999). Proper functioning of the network is particularly important under stress conditions, including acid shock (Hartl and Hayer-Hartl 2002). Previous studies showed that the expression of the chaperonins DnaK, DnaJ and GrpE was inducible under acid shock in *S. mutans* (Matsui and Cvitkovitch 2010) and *Escherichia coli* (Zmijewski et al. 2004). Surprisingly, the molecular chaperone genes 05SSU0299, 05SSU0300 and 05SSU0302 (DnaJ-class molecular chaperone) as well as 05SSU0298 (transcriptional regulator of heat shock genes) were down-regulated upon acidic stress in this study. It is possible that, despite a similar mechanism of action, species-specificity of chaperones is responsible for the observed differences in gene function and regulation.

05SSU1800-1805, 05SSU1807, and 05SSU1809 were up-regulated at pH 5.8. 05SSU1803 (*fabG*) transcription was increased more than 3-fold at pH 5.8. A BLAST search indicated that they belonged to the fatty acid biosynthesis (*fab*) genes. Inducible expression of *fab* genes upon acidic stress has been observed in *Bacillus subtilis* (Ter Beek et al. 2008). Remodeling of membrane fatty acid of bacteria in response to environmental stresses has been explored extensively (Chang and Cronan 1999; McElhaney 1974).

Thirteen genes (05SSU0305, 05SSU1017, 05SSU1543-1544, 05SSU1546-1548, 05SSU1866, 05SSU1868, 05SSU1874 and 05SSU2067-2069) involved in amino acid transport and four genes (05SSU1091, 05SSU0912, 05SSU1388, 05SSU1709) responsible for amino acid biosynthesis were differentially regulated upon acidic stress. *Streptococcus suis* S2 might be able to utilize amino acid transport systems to modulate H^+ accumulation; on the other hand, it slowed down the transport of some non-essential amino acids to save energy, and meanwhile H^+ concentrations were reduced through synthesis of branched amino acids (Cotter and Hill 2003).

Virulence-related functions

Transcription of 24 genes located in the 89K pathogenicity island was induced more than 2-fold at pH 5.8 and, moreover, 05SSU1403 (*sly*) was up-regulated 5-fold at pH 5.8. Contribution of the genes in 89K to the virulence is yet unclear, with the exception that the two-component regulatory system SalK/SalR in this island was proven to be essential for full virulence of *S. suis* S2 (Li et al. 2008). SLY is a member of thiol-activated cytolysin family of hemolysins. A retrospective study correlated the presence of the suilysin gene and expression of MRP and EF with high virulence in *S. suis* S2 isolates (Staats et al. 1999). The presence of SLY enhanced epithelial invasion and cell lysis by virulent strains of *S. suis* (Norton et al. 1999). A further study speculated that SLY was involved in adherence and cell injury rather than in direct cellular invasion (Lalonde et al. 2000). Interestingly, *Listeria monocytogenes* hemolysin responds to the initial drop in pH brought about by the vacuolar ATPases to permeabilize the phagosomal membrane, and permits the bacterium to escape prior to phagosome-lysosome fusion (Beauregard et al. 1997). The differential regulation of virulence-related genes in response to alterations in the surrounding conditions, such as acidic stress, would favor the virulence of *S. suis* S2.

Conclusions

For *S. suis* S2, ecological and pathogenic success is at least partially determined by its ability to sense the environment and mount an appropriate adaptive transcriptional response. Determining the differential gene regulation of *S. suis* S2 under in vitro conditions that are representative of the in vivo environment is critical to our understanding of the contribution of transcriptional response pathways to pathogenesis. In our previous study (Zeng et al. 2010), the gene expression profiles of *S. suis* S2 grown at 29 or 37 or 40°C were determined by DNA microarray, providing a global view of responsive gene expression induced by temperature alteration. Herein, a follow-up study indicated that *S. suis* S2 had the ability to modulate the transcription of a wide set of genes upon acidic stress (pH 5.8), especially those that encode two-component gene regulatory systems, protection and repair functions, and intracellular pH homeostasis. The results provide avenues for focused hypothesis-based investigations to help delineate the roles of specific gene in the stress adaptation and pathogenicity of this deadly pathogen.

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