CHAPTER 6

Discussion

Streptococcus suis is an important pathogen in swine and a zoonotic agent in human. It causes many pathological conditions in pigs such as arthritis, endocarditis, meningitis and septicemia. In addition, this bacterium represents a health risk for people working in close contact with swine or swine-derived products, causing streptococcal toxic shock syndrome (STSS), meningitis and permanent hearing loss. *S. suis* infection is a major cause of sudden death of pigs and increasingly becoming human health concern due to its zoonotic transmission capabilities. Thus, the identification and characterization of putative virulence factors and other infection-related proteins may lead to more understanding in pathogenesis and the strategies in prevention and control of *S. suis* infection.

In this study, the comparative proteomic profiles of *S. suis* serotype 2 (3 strains) and serotype 14 (2 strains) were performed using the strains isolated from 3 sources; diseased pig (SS2: P 1/7) which is a reference strain, healthy pigs (SS2: TSK 10.4; SS14: TD 2.2) and human patients (SS2: LPH 210/53; SS14: MNCM 07). The optimal MOI for RAW 264.7 cells infection by 5 strains of *S. suis* were 200, which were the highest MOI used in this study. This result indicated that at the MOI 200 that were used in cytotoxicity test, all the 5 strains of *S. suis* could hardly invade the mouse macrophages. This result was in accordance with the previous study which investigated the interactions of strains of *S. suis* to various types of cells. In their study, several epithelial cell lines such as A549, HeLa, MDCK, PK(15), LLC-PK1 were infected with *S. suis* strains S735, 31533, AAH4 and uncapsulated mutant 2A at the MOI of 100 to 0.001 bacteria per cell. Their results indicated that all strains of *S. suis* serotype 2 were not able to invade all epithelial cells but adheres to epithelial cells (Lalonde *et al*., 2000). In according to the study on interactions of *S. suis* serotype 2 strains with the mouse macrophage cell line J774, *S. suis*

was able to resist uptake by this cell line due to the production of capsular polysaccharide (Segura and Gottschalk, 2002).

However, the study in the mouse macrophage model might not adequately reflect human infection. Despite the inability of *S. suis* to invade mouse macrophages, all strains were able to invade human macrophages with the higher number of intracellular bacteria at the optimal MOI of 50. These results indicated that human macrophages were more susceptible to *S. suis* invasion and the ability of *S. suis* to invade and survive within the cells confirmed that these phagocytic cells could play a role in pathogenesis of *S. suis* as suggested previously by Gottschalk and Segura (Gottschalk and Segura, 2010).

The protein profiles of *S. suis* serotype 2 and serotype 14 were investigated by proteomic analysis (LC-MS/MS). After exposure to the intramacrophagic condition of RAW 264.7 cells, the total of 261 proteins were differentially expressed and could be categorized into 19 functions. Most of the proteins detected (16%) were proteins-relating to stress response. Some of the proteins in this group such as chaperone protein DnaJ, phosphoesterase PA-phosphatase-like protein, superoxide dismutase, 8-OXO-dGTPase domain (mutT domain), benzaldehyde lyase, LysR substrate binding domain protein were also found to express when cultured *S. suis* serotype 2 isolates (from China) in Todd-Hewitt broth (Jing *et al*., 2008).

In *Streptococcus pneumoniae*, Cai and colleagues suggested that several ribosomal proteins could also interact with DnaJ, indicating the diverse roles for DnaJ in protein synthesis in pneumococci. Therefore, DnaJ may be an important virulence factor which confers fitness for pneumococci to adapt host stress by interacting with the ribosomal proteins (Cai *et al*., 2013). In *Escherichia coli*, *dnaJ* deficient mutants did not grow as well as the wild type at temperatures above 30^{o}C (Sell *et al*., 1990). Moreover, Takaya and colleagues reported that DnaJ was essential for *Salmonella enterica* serovar Typhimurium invasion of epithelial cells and survival within macrophages (Takaya *et al*., 2004). The second group of proteins that were expressed including transport-relating proteins, for example, ABC superfamily ATP binding cassette transporter ABC protein, ABC transporter integral membrane subunit, UvrD/REP helicase, peptide ABC transporter, ATP-binding protein.

A periplasmic ABC transport protein had been reported to be a new immunoreactive protein of *S. suis* (Zhang *et al*., 2011) and ABC transporters are associated with virulence in many pathogenic bacteria. They import various nutrients required for survival in different niches and export substrances toxic to the cell (Garmory and Titball, 2004). In *S. suis*, protein of ABC transporter is a substrate binding protein for uptake of multiple metal ions. These data imply that protein of ABC transporter maybe plays an adaptive role *in vivo*, in response to specific environmental conditions (Zheng *et al*., 2011).

The third most differentially expressed were proteins involved in transcription, for example, ATP-dependent RNA helicase HrpB, transcriptional regulator, GntR family/aminotransferase, transcriptional regulatory protein OmpR, PaaX family transcriptional regulator, N-acetyltransferase GCN5. These proteins had been previously reported to express when cultured *S. suis* in THY medium as well (Jing *et al*., 2008; Wu *et al*., 2011). Many secretory proteins of virulent strain of SS2 were detected such as chaperone protein DnaK (heat shock protein 70), ABC transporter ATP-binding protein, DNA-directed RNA polymerase alpha chain, Lys protein (LysR, LysM), 30S ribosomal protein (S1, S2 and S10) and 50S ribosomal protein (L1, L5 and L22) (Jing *et al*., 2008; Yang *et al*., 2013). The expression of these protein groups are up-regulated during exposure to mouse macrophages but not increased during growth in culture medium. This suggests that up-regulation of these proteins when *S. suis* were in mouse macrophages may help this bacterium to be able to adapt and survive in intracellular environment.

The analysis of protein profiles of *S. suis* during the exposure to human macrophages U 937 could identify 118 proteins with 11 different functions. Most of proteins up-regulated were proteins-relating to translation, carbohydrate metabolism, DNA replication, transport, protein metabolism. Interestingly, the proteins involved in translation were differentially expressed at the highest proportion (48%). Examples of those proteins were ribosomal protein subunits (e.g. 30S ribosomal proteins S3, S7, S8 and S15, and 50S ribosomal proteins L1, L3, L17, L17/L12, L19, L20, L24, L29, L30, L33) and other translation proteins such as UPF0374 protein SSU05_0445, argininosuccinate lyase, translation initiation factor IF-2, MutS2 protein, peptide deformylase, UPF0348 protein SSU98_0368, glutamate-tRNA ligase, elongation factor G. Other proteins play role in carbohydrate metabolism were observed, for example,

galactokinase, phosphoglycerate kinase, 6-phosphofrutokinase, and phosphoenol pyruvate carboxylase. These proteins had also found to express in other virulent strains such as SS2 strain HA9801 (isolated from a pig with septicemia) (Zhang and Lu, 2007), SS2 strain ZYS (isolated from diseased piglet) (Zhang *et al.*, 2008; Chen *et al*., 2011), SS2 strain 98012 (originated from clinically infected patients) (Jing *et al*., 2008), SS2 strain 05ZYH33 (isolated from Sichuan China) (Han *et al*., 2012).

In *Staphylococcus aureus*, EF-Tu and EF-G are known to perform a variety of function, depending on their cytoplasmic or surface localization. This factors support the persistence of bacteria in host tissues (Papa *et al*., 2013). In addition, elongation factor G had been reported to be a novel immunogenic proteins of SS9, which are encoded by genes that are conserved among SS9 strains. This protein may be developed as antigen for further study of SS9 vaccine (Wu *et al*., 2011). Interestingly, the proteins-relating to translation of *S. suis* were also found to specially express in other conditions such as in infected human (detected by *in vivo* induced antigen technology) as well as when *S. suis* were exposed to stress such as in the presence of licochalcone A (an antimicrobial substance). Taken together, expression of some virulence associated genes are often turned off when cultured in the medium but up-regulated when *S. suis* exposed to the stress conditions.

This suggests that *S. suis* remodels its proteome when exposes to the condition within macrophage cells by specific adaptation to the stress condition such as intracellular environment, in infected host or the presence of growth inhibitor. Accordingly, Li and colleagues suggested that the protein expression of organisms is usually adjusted in response to the change in environmental stimuli. After invade into host, bacteria sense *in vivo* circumstance, and in response, induce or repress the expression of some specific genes to benefit their growing *in vivo* (Li, *et al*., 2013). The genes up-regulated or specially expressed *in vivo* usually contributed to the *in vivo* survival and pathogenicity of pathogen (Shelburne *et al*., 2004; Li *et al*., 2013). Inside macrophage cells, *S. suis* may be adapt itself by up-regulated or down-regulated the expression of some specific proteins for the survival within the stress condition macrophage cells.

In this study, the results also indicated the up-regulation of enolase expression of strains P1/7, TSK 10.4 and TD 2.2. This protein is in the particular interest because it had been proposed to be a putative virulence-associated factors of *S. suis* serotype 2. Enolase of *S. suis* was found to binds human plasminogen and fibronectin (Pancholi *et al*., 2001) and induces the production of antibodies in infected pigs *in vivo* (Zhang *et al*., 2009; Esgleas *et al*., 2009). Up-regulation of enolase expression in both isolates from pig in human macrophages suggested the possibility to cause diseases of these strains.

The virulence-associated genes profiling of 5 *S. suis* strains used in this study are indicated in Table 4. The selected genes were the hemolysin suilysin (SLY, *sly*), the extracellular protein factor (EPF, *epf*) and the muramidase-released protein (MRP, *mrp*). All 5 strains could be divided into 2 clades based on the study of Zhu and colleagues in China (2013). Four strains of *S. suis* including P1/7 (SS2), LPH 210/53 (SS2), TD 2.2 (SS14) and MNCM07 (SS14) with the genotype *sly*+/*epf*+/*mrp*+ or *sly*+/*epf**+/*mrp*+ were in clade 2, which, by the study of Zhang, was associated with higher virulence. Whereas *S. suis* strain TSK 10.4 (SS2) with the genotype *sly*-/*epf*-/*mrp*+ was clade 1, which was associated with lower virulence (Zhu *et al*., 2013).

Besides those 5 strains, the distribution study of virulence-associated genes was also performed using 50 strains of *S. suis* isolated in Northern part of Thailand. The virulence profiles of *S. suis* isolated multiplex PCR (MP-PCR) had shown that there were 5 genotypes amongst the 50 strains used. Those genotypes included *sly*+/*epf*+/*mrp*+ [7 (14%) of the isolates], *sly*+/*epf*-/*mrp*- [11 (22%)], *sly*-/*epf*-/*mrp*+ [21 (42%)], *sly*+/*epf*-/*mrp*+ [3 (6%)] and *sly*-/*epf*-/*mrp*- [8 (16%)]. These results indicated that the genotype *sly*-/*epf*-/*mrp*+ was the most prevalent genotype of the strains used. Based on Zhang study, most of the strains isolated in Northern part of Thailand were lower virulence, with 44% in pigs and 40% in human.

Previously, Phiwpan and colleagues reported that the most prevalent virulence-associated genes of *S. suis* serotype 2 (23 strains) isolated from patients were *cps2*/*sly*+/*epf*+/*mrp*+ (91.3%) and *cps2*/*sly*+/*epf*-/*mrp*- (23.7%), respectively (Phiwpan *et al*., 2010). These virulence-associated gene prevalence was found to be similar to those of *S. suis* isolates in Germany, the Netherlands and Austria (Silva *et al*., 2006). In contrary, in this study, most of the isolates from patients in northern part of Thailand were *sly*-/*epf*-/*mrp*+ (40%). Moreover, this result indicates that isolates without *sly* and *epf* could be

pathogenic to human and infection caused by *S. suis* in Thailand, particularly northern part, differs from those previously reported.

Despite the fact that serotype 2 is the most frequently serotype isolated from pigs with other clinical manifestations worldwide, serotype 3 (29%) and 4 (21%) were the most prevalent serotype in Korea. In their study, all the *S. suis* isolates carried *sly*, 33% and 4% of the isolates carried *mrp* and *epf*, respectively (Kim *et al*., 2010), whereas the genotypes *sly-epf-mrp+* and *sly-epf-mrp-* were the most prevalent in United States and France (Berthelot-Herault *et al*., 2000; Fittipaldi *et al*., 2009). These are similar to the results from this study, in which the most prevalent isolates in healthy pigs were *sly-/epf-/mrp+* (44%). In contrary, the genotypes *sly+epf+mrp+* and *sly+epf+mrp-* were the most prevalent in China (Wei *et al*., 2009). Therefore, the distribution of virulence-associated genes of *S. suis* isolated from healthy pigs and human patients in this study appears to differ greatly from that reported for *S. suis* infection with other clinical manifestation in different countries.