CHAPTER 5

Results

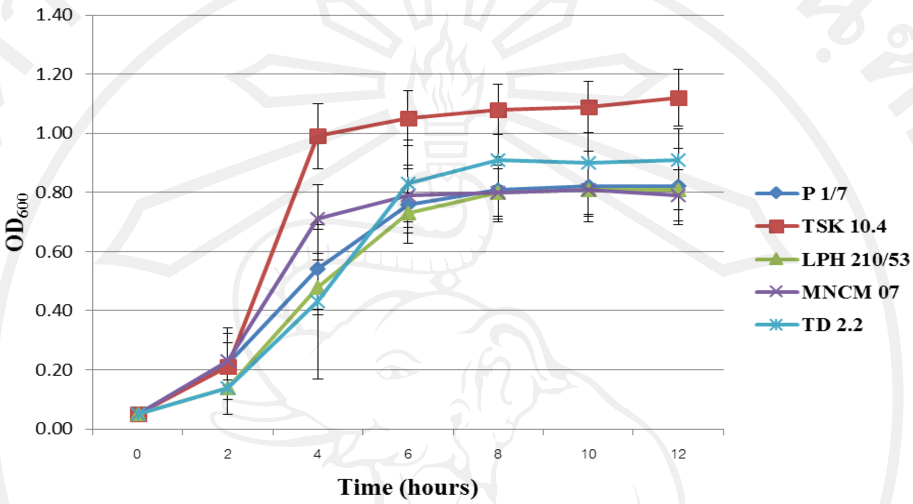
5.1 Growth of *S. suis* strains

The growth curves of *S. suis* 5 strains (serotype 2: P 1/7, TSK 10.4, LPH 210/53; serotype 14: TD 2.2, MNCM 07), which were cultured in THB broth are shown in Figure 6. The data of bacteria density and colony forming unit of 5 strains (serotype 2 and serotype 14) showed that the growth of both serotype 2 and serotype 14 had no difference, except TSK 10.4 strain (serotype 2), which grew faster than other strains. All of *S. suis* strains reached the mid-log phase after culturing in THB broth for 3 to 4 hours. The cultures were harvested as indicated (after reaching mid-log phase at an OD_{600} of 0.4) for further cell lines infection experiments.

5.2 Growth of macrophage and monocyte cell lines

To determine the growth characteristics of the macrophage cell lines, mouse macrophage cells (RAW264.7) or human macrophage cells (U 937) were seeded at the concentration of 10^5 cells per well to 24-well plates in culture media (DMEM and RPMI 1640), supplemented with 10% heat-inactivate FBS and 2 mM L-glutamine, respectively. Macrophage cells were harvested and counted for viability daily for seven consecutive days by using 0.2% Trypan blue. The viable cells were colourless and dead cells were stained blue. The growth rate of RAW264.7 cells in DMEM-C was gradually increased and the cells number started from 9.46×10^4 cells at day 0, 1.33×10^5 cells at day 1, 2.67×10^5 cells at day 2 to 9.31×10^5 cells at day 3, respectively. Whereas the growth rate of U937 cells in RPMI-C started at 2.78×10^5 cells at day 0, 6.53×10^5 cells at day 1, 1.53×10^6 cells at day 2 to 1.72×10^6 cells at day 3 respectively. It was found that the growth of RAW264.7 cells were slower than U 937 cells. At 3 days, RAW 264.7 cells grew to approximately 10^6 cells, whereas U 937 cells grew to approximately 10^6 cells at 2 days. The optimal time for cultivation of both cells were selected to use in further study (Figure 7).

(a)



(b)

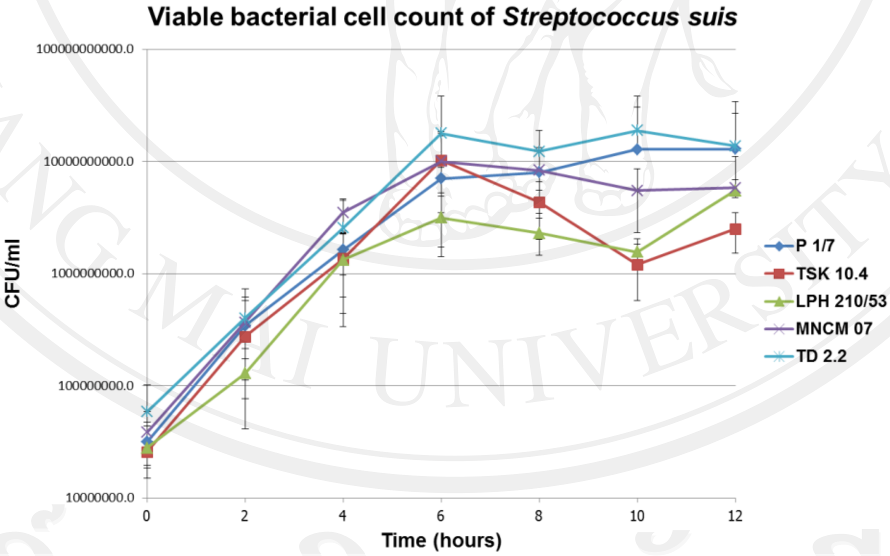
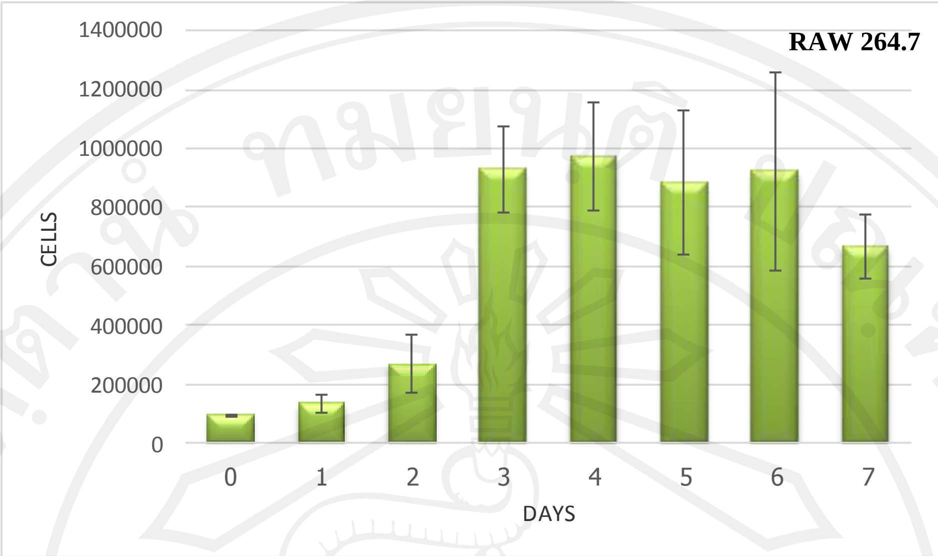


Figure 6 Growth of *S. suis* serotype 2 (P1/7, TSK 10.4, LPH210/53) and serotype 14 (MNCM07, TD 2.2). *S. suis* were grown in Todd-Hewitt broth, supernatants were harvested every 2 hours for 12 hours. (a) Optical density at 600 nm (OD_{600}), (b) Viable bacterial cell count (CFU/ml).

(a)



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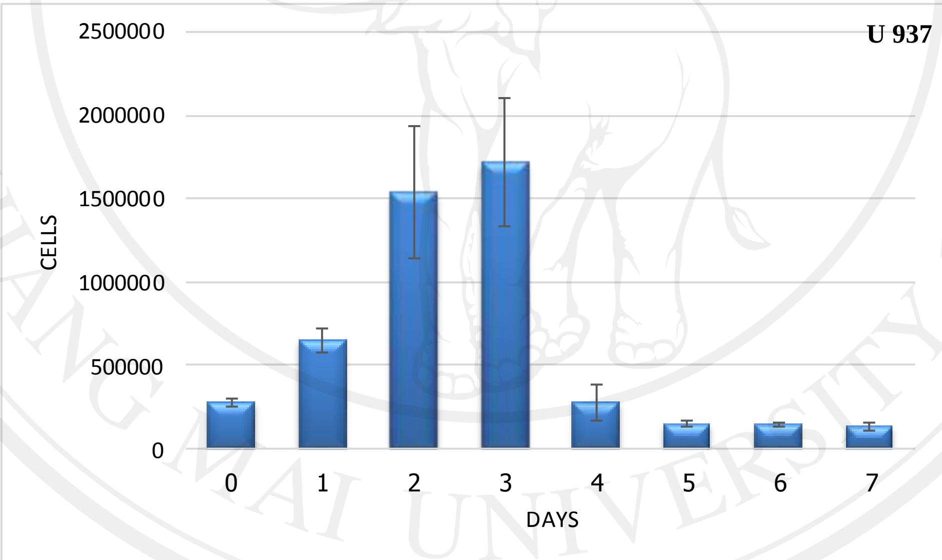

Figure 7 Growth of macrophage/monocyte cell lines. (a) The growth of mouse macrophage (RAW 264.7) cells when cultured in DMEM-C medium. (b) The growth of human macrophage (U 937) cells in RPMI-C medium without PMA stimulation.

5.3 *S. suis* invasion assay

This study showed all strains of *S. suis* had the capacity to invade the mouse macrophage (RAW 264.7) and human macrophage cells (U 937) tested. Before using the standard conditions for invasion assays (10^5 cells per well, 2 hours invasion time), many different variables had been tested in order to find the optimal experimental conditions that promote *S. suis* invasion. Different multiplicity of infection (MOI) including 1, 10, 50, 100, 200 and exponential phase of all 5 bacterial strains were used to invade mouse and human macrophage cell lines. The results of optimal MOI for the infection to both macrophage cells were shown in the Table 7 and Table 8. The number of intracellular bacteria of RAW 264.7 cells started from 0, 3.5×10^1 to 1.62×10^2 cfu/ml whereas the number of intracellular bacteria of U 937 cells started at 4.5×10^1 to 4.00×10^2 cfu/ml, respectively. It was found that the optimal condition to infect RAW 264.7 and U 937 cells with 5 strains of *S. suis* at 2 hr were at MOI of 200 and MOI of 50, respectively. At the MOI of 200 and 50, the numbers of intracellular bacteria were highest and the viability of both macrophage cells was above 80% (data shown in appendix B) when compared with other MOI.

As a comparison, all of *S. suis* 5 strains were able to invade both types of PMA-stimulated cells. TSK 10.4 strain (serotype 2, isolated from healthy pig) invaded the mouse macrophages better than other strains. Moreover, *S. suis* strains isolates from patients LPH 210.53 and MNCM 07 (serotype 2 and 14 respectively), were able to invade the human macrophage at higher degree than other 3 strains as shown in Figures 8 and 9.

Table 7 Intracellular bacteria recovered from RAW 264.7 cells

MOI	Number of colony post-invasion (CFU/ml)				
	P 1/7	**TSK 10.4**	**LPH 210/53**	**MNCM 07**	**TD2.2**
1	0	0	0	0	0
10	0	35 ± 0.05	0	8.3 ± 0.29	0
50	8.3 ± 0.28	56 ± 1.04	15 ± 0.5	11.6 ± 0.29	1.6 ± 0.29
100	15 ± 0.50	136 ± 2.02	18.3 ± 0.29	23 ± 1.04	13 ± 0.76
200	45 ± 0.50	162 ± 1.53	38.3 ± 1.26	153 ± 0.76	65 ± 2.18

Table 8 Intracellular bacteria recovered from U 937 cells

MOI	Number of colony post-invasion (CFU/ml)				
	P 1/7	**TSK 10.4**	**LPH 210/53**	**MNCM 07**	**TD2.2**
1	45.17 ± 8.61	53.83 ± 2.25	133.5 ± 20.79	46.67 ± 2.93	128.17 ± 12.39
10	282.67 ± 11.18	142.5 ± 19.31	282.83 ± 97.44	332.16 ± 14.00	167.67 ± 29.51
50	304.17 ± 9.57	341.67 ± 18.61	400 ± 15.88	334.17 ± 10.96	291.5 ± 22.42
100	298.5 ± 19.29	303.5 ± 67.77	357.17 ± 5.86	318.83 ± 13.66	173.5 ± 44.25
200	230.5 ± 6.24	262.83 ± 108.16	360.83 ± 68.70	284.5 ± 9.17	243.83 ± 13.97

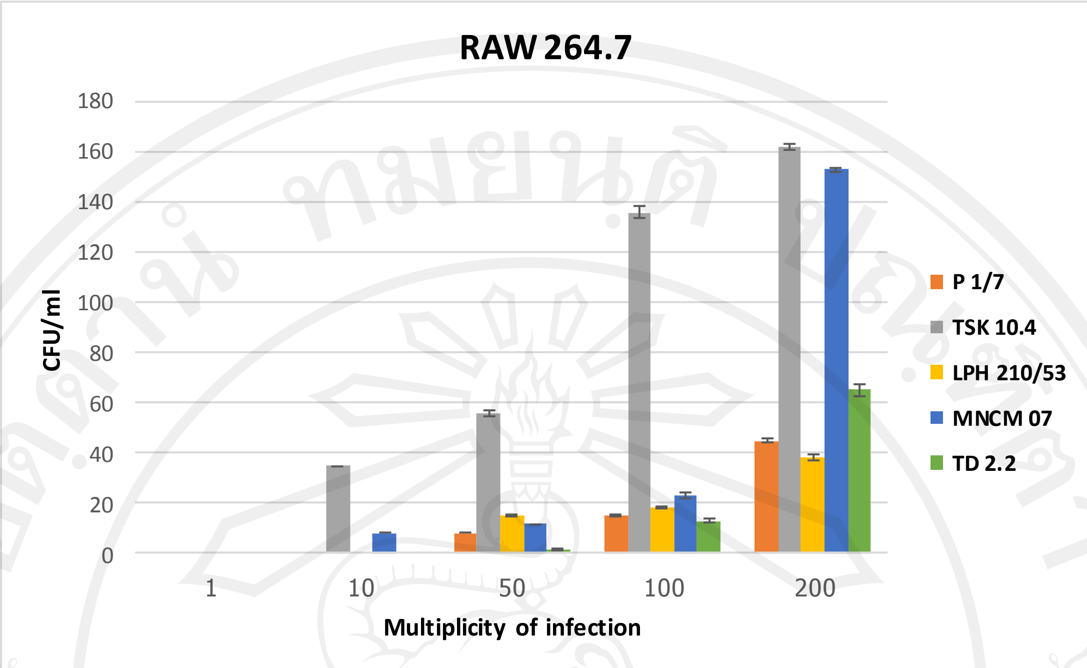

Figure 8 Invasion of RAW 264.7 cells by *S. suis* serotype 2 (■ P1/7, ■ TSK 10.4, ■ LPH210/53) and serotype 14 (■ MNCM07, ■ TD 2.2) at MOI of 1, 10, 50, 100, 200

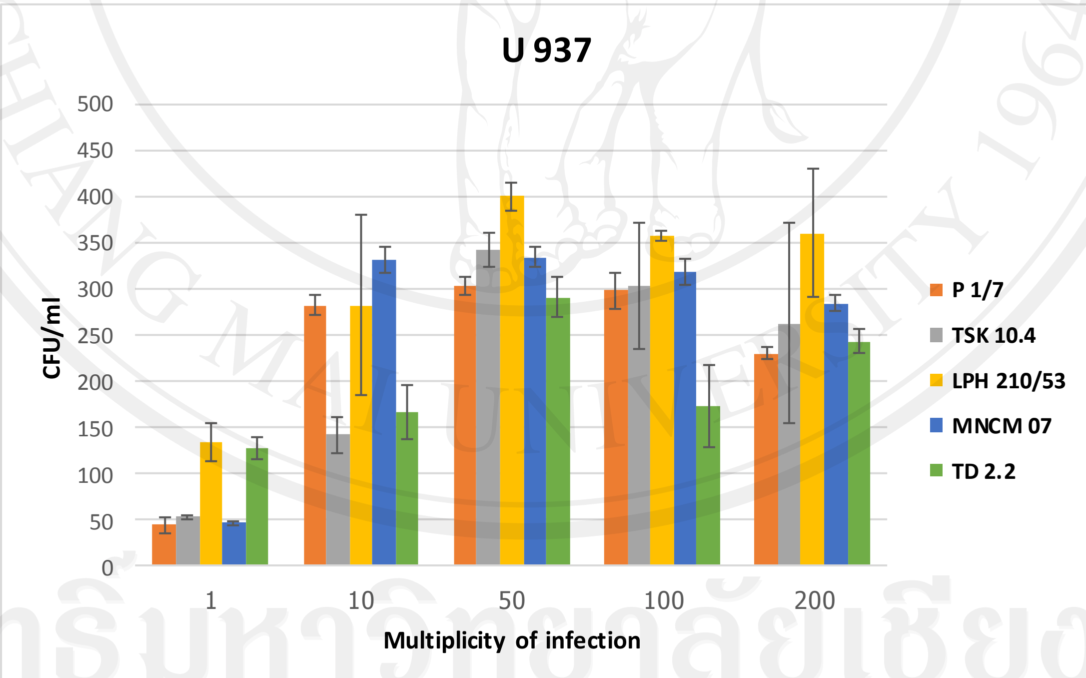

Figure 9 Invasion of U 937 cells by *S. suis* serotype 2 (■ P1/7, ■ TSK 10.4, ■ LPH210/53) and serotype 14 (■ MNCM07, ■ TD 2.2) at MOI of 1, 10, 50, 100, 200

5.4 Proteomic analysis of *S. suis* serotypes 2 and 14

In this study, the LC-MS/MS was used to detect protein expression patterns of *S. suis* 5 strains including P1/7 (diseased pig), TSK 10.4 (healthy pig), LPH 210/53 (patient) TD 2.2 (healthy pig) and MNCM 07 (patient). After *S. suis* strains were exposed to mouse macrophage (RAW 264.7) for 4 hours and macrophage cells were disrupted by distilled water. Proteins in cell lysate of *S. suis* were digested with trypsin before injecting to LC-MS/MS. Peptides were relatively quantified and identified by DecyderMS and Mascot programs, respectively.

5.4.1 Proteins of *S. suis* with differential expression during exposure to mouse macrophages (RAW 264.7)

At MOI 200, the intracellular bacteria harvested were few in numbers and insufficient in protein concentration. Therefore, the in-solution digestion was performed instead of SDS-PAGE and in-gel digestion. The results showed that *S. suis* 5 strains differentially expressed 261 proteins (Table 33 in appendix B) that could be divided into 19 functions such as response to stress 16 %, transcription 15 %, transport 15 %, signal 11 %, protein metabolism 10 %, carbohydrate metabolism 6 %, lipid metabolism 5 %, respiration 5 %, translation 4 %, DNA replication 4 %, DNA metabolism 2 %, nucleotide metabolism 2 %, cell adhesion 1 %, cell development 1 %, cytoskeleton 1 %, immune response 1 % (Figure 10).

Comparison of the proteins amongst the *S. suis* serotype 2 and serotype 14 indicated that 3 proteins including glycine hydroxymethyl transferase, peptidase M48 and flagellar biosynthesis protein FlhA specifically up-regulated in P 1/7 as shown in Table 10. There were 13 proteins that were found up-regulated only in TSK 10.4 strain including fatty oxidation complex, alpha subunit Fad, peptidase U62 modulator of DNA gyrase, 2-isopropylmalate synthase/homocitrate synthase family protein, pyridine nucleotide-disulphide oxidoreductase dimerisation region, benzaldehyde lyase, 8-OXO-dGTPase domain (mutT domain), alcohol dehydrogenase, methionine sulfoxide reductase B, gatC gene product, polar amino acid ABC transporter ATPase, translocation protein in type III secretion, inner-membrane translocator and secretion system-associated protein type

VI (Table 11). Whereas, it was not found differential proteins of LPH 210/53, MNCM 07 and TD 2.2 strains during exposure to mouse macrophages.

Moreover, comparison of proteins between 5 strains of serotype 2 and serotype 14 showed that 74 proteins of all strains were similary expressed (Table 9) such as UDP-N-acetylglucosamine pyrophosphorylase protein, glycosyl transferase family protein, tyrosine recombinase XerC, lipoprotein lppf, aldehyde dehydrogenase, pts system eiibc component, fumarate hydratase, class I, sulfur oxidation V protein, hydrolase-alpha/beta fold family protein, TonB-dependent receptor, transcriptional regulator-GntR, PAS/PAC sensor hybrid histidine kinase and lytic transglycosylase. Most of the proteins that were similary expressed between these 2 serotypes were categorized as carbohydrate metabolism, cell development, DNA metabolism, lipid metabolism, nucleotide metabolism, protein metsbolism, respiration, response to stress, signal transduction, transcription, translation and transport, respectively.

Interestingly, when compared the protein profiles between serotype of *S. suis*, 6 proteins (Table 12) were similary expressed in all serotype 2 strains (such as DNA protecting protein (DprA), tail protein (tape measure), UvrD/REP helicase, multidrug resistance protein vceB, DNA polymerase iii alpha chain protein and FIST C domain-containing protein). Whereas in serotype 14, alkaline serine protease-subtilase family was the only protein expressed in both strains of serotype 14 (Table 13). Finally, when analyzed the protein profiles between origin of isolates (clinical and animal), 11 proteins of healthy pig isolates (Table 14) were similary expressed (such as phosphomethylpyrimidine kinase, ABC transporter-like protein, dephospho-CoA kinase, cyclopropane-fatty-acyl-phospholipid synthase, mannosyl transferase/ glycosyl transferase, imidazole glycerol phosphate synthase subunit hisH, glycosyltransferase, periplasmic binding protein/LacI transcriptional regulator, A/G-specific adenine glycosylase, glycogen debranching enzyme GlgX and preprotein translocase SecA subunit). Amongst the clinical isolates, 2 proteins of isolates from patients (such as toxin-antitoxin system (Xre family) and cytochrome-c oxidase chain I) were similary expressed (Table 15). The results showed that when compared between serotype (2 and 14) or between origin of isolates, various proteins with different functions were observed with no specificity to serotype or origin.

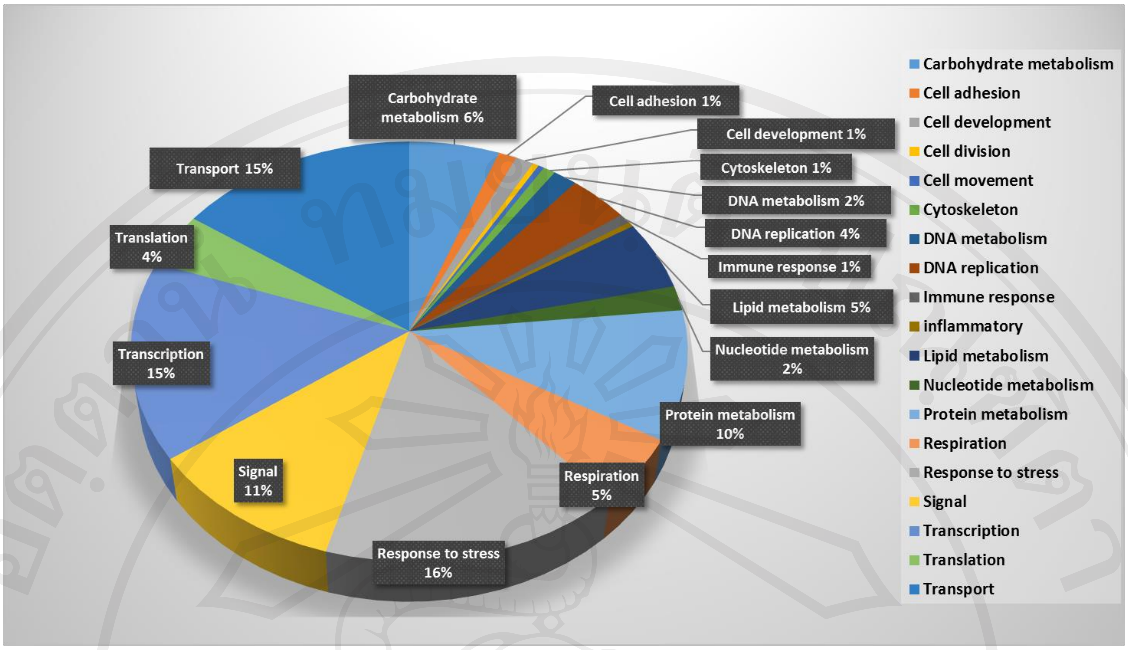

Figure 10 Functional categorization of significantly differential protein expression of *S. suis* after exposure to the mouse macrophage (RAW 264.7)

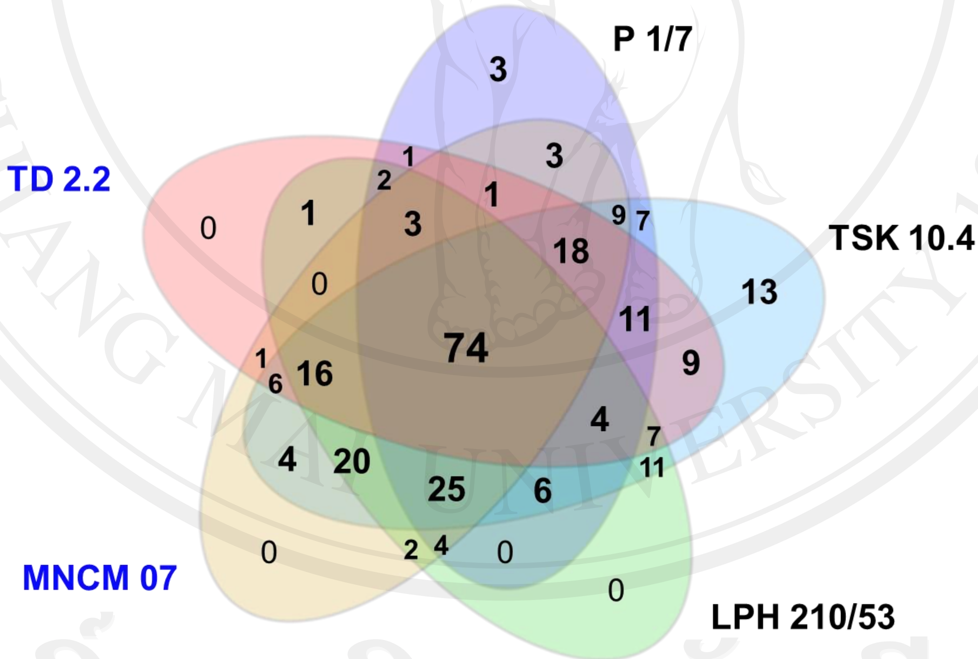

Figure 11 The number of differentially expressed proteins of *S. suis* serotypes 2 and 14 during exposure to mouse macrophages.

(○ P1/7, ○ TSK 10.4, ○ LPH 210/53 and ○ MNCM07, ○ TD 2.2).

Table 9 Proteins of *S. suis* serotype 2 and serotype 14 that were similarly up-regulated during exposure to mouse macrophages

Protein identification	Peptide sequences	ID score	Function
UDP-N-acetylgluco samine pyrophospho rylase protein	HVEDTTT	15.39	Carbohydrate metabolism
Glycosyl transferase family protein	EIGGFR	22.06	Carbohydrate metabolism
diaminopimelate epimerase	LGPTSLVSMGN	18.38	Carbohydrate metabolism
glycoside hydrolase family 5	ANTGAG	8.08	Carbohydrate metabolism
chorismate mutase	GAMPRV	15.32	Carbohydrate metabolism
alpha-L-arabinofuranosidase	GDKAPATLR	25.18	Carbohydrate metabolism
cadherin	DINDGSV	3.82	Cell adhesion
tyrosine recombinase XerC	NTAAADGGPM	19.53	Cell development
S-layer protein	GGFTINP	10.22	Cell development
cell division protein FtsH	AMARAM	13.45	Cell division
lateral flagellar hook basal body protein, LfiE	EDVVGAMI	21.84	Cell movement
ROK family protein	SAEGMAGIIGD	20.31	Cytoskeleton
Endonuclease	KVQVLNNLVEL YG	25.03	DNA metabolism
Mycobacteriophage excisionase	AISTIPPIS	17.64	DNA repair
DNA polymerase I	AAAVTAKV	12.55	DNA replication
hydrolase/ autolysin	SPNPATP	5.1	inflammatory

Table 9 (Continued)

Protein identification	Peptide sequences	ID score	Function
lipoprotein lppf	ESRGAGTME	7.78	Lipid metabolism
3-oxoacyl-(acyl-carrier-protein) synthase 2	AAITPMSVA	35.4	Lipid metabolism
Aldehyde Dehydrogenase	PQIIMPD	25.45	Lipid metabolism
salicylyl-CoA 5-hydroxylase	GIVASGR	9.83	Lipid metabolism
AMP-dependent synthetase and ligase	TVGAAIEGT	19.62	Nucleotide metabolism
purple acid phosphatase/fibronectin domain-containing protein	AHIGG	14.39	Protein metabolism
uroporphyrin-III C-methyltransferase	IRPLTVLPV	10.13	Protein metabolism
pts system eiibc component	DVGGI	14.79	Protein metabolism
asparagine synthase	VFAKS	10.85	Protein metabolism
N-acetyl-gamma-glutamyl-phosphate reductase	GKGSAG	5.63	Protein metabolism
FKBP-type peptidylprolyl isomerase	QESLAAAQAFMA	16.56	Protein metabolism
fumarate hydratase, class I	LFMAKGGGSANKTMLFQETKAL	27.5	Protein metabolism
indolepyruvate oxidoreductase subunit B	LVDLAEQNGY	13.23	Respiration

Table 9 (Continued)

Protein identification	Peptide sequences	ID score	Function
citrate synthase type II	VGALSAFYHDST DINNP	19.13	Respiration
ferrochelatase	SCSTVGAVW	36.4	Stress response
sulfur oxidation V protein	IATDSVNVIA	45.36	Stress response
thiol:disulfide interchange protein DsbA precursor	EKIMMP	13.97	Stress response
antibiotic-resistance protein	APEPR	9.98	Stress response
(S)-2-hydroxy-acid oxidase subunit D	GGIILIFR	48.37	Stress response
DnaJ domain protein	TPEAEGKP	17.41	Stress response
hydrolase, alpha/beta fold family protein	QGHGRTN	16.44	Stress response
beta-lactamase domain-containing protein	NIESGCIII	57.2	Signal transduction
DUF224 cysteine-rich region domain protein	AISEU	9.62	Signal transduction
serine/threonine protein kinase	HSAMEA	6.31	Signal transduction
phosphoglycerate kinase	KMGLDIGSD	17.46	Signal transduction
TonB-dependent receptor	MDVPTGS	31.91	Signal transduction
acetoin expression regulatory protein	TLSGETTRA	15	Signal transduction
histidine kinase	ELADNSIEPSPELY L	24.53	Signal transduction

Table 9 (Continued)

Protein identification	Peptide sequences	ID score	Function
Flagellar biosynthesis pathway component FliR	IGLSMAQMNDP	11.41	Signal transduction
hydrogenase accessory protein HypB	AHGVRA	11.85	Signal transduction
internalin	ADTISKS	35.73	Transcription
hydrogenase 3 and formate hydrogenase complex, HycG subunit	ASMKAS	7.81	Transcription
acetyltransferase	EIAILIGRD	14.27	Transcription
transcriptional regulator, GntR family/ amino transferase, classes I and II	LIYAITGNGTFV APNA	24.02	Transcription
transcriptional modulator of MazE/toxin MazF	LLLMSSSMVD	13.44	Transcription
PAS/PAC sensor-containing diguanylate cyclase	CPGSDAE	4.81	Transcription
integrase catalytic subunit	SSGVVA	10.65	Transcription
RpiR family phosphosugar-binding transcriptional regulator	KATNTGDAT	13.97	Transcription
yecA family protein	GGHEP	5.09	Transcription
transposase IS3518	EQGMGYTAAAN A	21.44	Transcription
PAS/PAC and Chase sensor-containing diguanylate cyclase/phosphodiesterase	AKAGFAELETIQ	33.04	transcription
single-stranded-DNA-specific exonuclease RecJ	GKGHIK	16.72	Transcription

Table 9 (Continued)

Protein identification	Peptide sequences	ID score	Function
PAS/PAC sensor hybrid histidine kinase	EQVAAGFAG	14.06	Transcription
WD-40 repeat-containing protein	RPTGGPFGP	10.65	Transcription
30S ribosomal protein S2	AAIDGILR	24.69	Translation
50S ribosomal protein L5	FRVREGMPMGA KVT	19.51	Translation
large membrane protein	DTYPV	6.47	Transport
integral membrane protein	GTVEPRGEISDE LM	15.06	Transport
Mn2+/Fe2+ transporter	AHIIII	41.19	Transport
major facilitator family transporter	MVMVGLYVRL KLEETPVF	18.27	Transport
heavy metal efflux pump CzcA	SAMKDMPDG	15.24	Transport
lytic transglycosylase	AAYNGGSGNVS	31.3	Transport
hydrophobe/amphiphile efflux-1 (HAE1) family protein	CMCAA	10.22	Transport
Na+/H+ antiporter NhaC	AEWMTLIQNGY NSGA	22.4	Transport
histidine ammonia-lyase	VSQPGVNSG	18.02	Transport
chromate transporter permease	IPATVS	10.38	Transport
flagellar hook-basal body complex protein	QAAGGAAGPAT	24.63	Transport
bacteriochlorophyll synthase 44.5 kDa chain	AAASMVWI	27.27	Transport

Table 10 Proteins up-regulated in *S. suis* strain P1/7 during exposure to mouse macrophages

Protein identification	Peptide sequences	ID score	Function
glycine hydroxymethyl transferase	SGTTANM	6.34	Protein metabolism
peptidase M48, Ste24p	AASHM	3.48	Protein metabolism
flagellar biosynthesis protein FlhA	MAVSTGMI	1.73	Signal transduction

Table 11 Proteins up-regulated in *S. suis* strain TSK 10.4 during exposure to mouse macrophages

Protein identification	Peptide sequences	ID score	Function
fatty oxidation complex, alpha subunit Fad	HIAAKAAR	18.91	Lipid metabolism
peptidase U62 modulator of DNA gyrase	AAKMQSP	14.68	Protein metabolism
2-isopropylmalate synthase/homocitrate synthase family protein	MNAGTAA	13.68	Protein metabolism
pyridine nucleotide-disulphide oxidoreductase dimerisation region	GVDIKTIGEQ	22.5	Respiration
benzaldehyde lyase	GDGALGFHLQ	53.91	Response to stress
8-OXO-dGTPase domain (mutT domain)	CNAYELPQAG	30.02	Response to stress
alcohol dehydrogenase	AMSFIPS	12.66	Response to stress
methionine sulfoxide reductase B	KPATTG	9.07	Response to stress
gatC gene product	DVKGV	8.17	Transcription

Table 11 (Continued)

Protein identification	Peptide sequences	ID score	Function
polar amino acid ABC transporter ATPase	ILKGVDLTIEK	33.72	Transport
translocation protein in type III secretion	SGGGFIAFM	22.34	Transport
inner-membrane translocator	SAAFMAVGAFA AYNFHL	19.72	Transport
secretion system-associated protein type VI	ASGHATLW	15.72	Transport

Table 12 Proteins up-regulated in all strains of *S. suis* serotype 2 during exposure to mouse macrophages

Protein identification	Peptide sequences	ID score	Function
DNA protecting protein DprA	VVQLPGKH	13.44	DNA replication
DNA polymerase iii alpha chain protein	PGTANGVVF	10.29	DNA replication
tail protein (tape measure)	SGTGGASSEFSR	10.07	Cytoskeleton
UvrD/REP helicase	CLCETKDKMEK	36.93	Transport
multidrug resistance protein vceB	AFGMFM	16.08	Response to stress
FIST C domain-containing protein	GHGTMGGW	20	Transcription

Table 13 Proteins up-regulated in both strains of *S. suis* serotype 14 during exposure to mouse macrophages

Protein identification	Peptide sequences	ID score	Function
alkaline serine protease, subtilase family	RDMLGDDPAQD	13.55	Protein metabolism

Table 14 Proteins up-regulated in *S. suis* isolates from pigs both serotype 2 and serotype 14

Protein identification	Peptide sequences	ID score	Function
ABC transporter-like protein	DVTMAV	15.8	Transport
preprotein translocase, SecA subunit	TLKYKQAESLLKEGSW	32.86	Transport
periplasmic binding protein/LacI transcriptional regulator	EQNAAAA	10.88	Transcription
dephospho-CoA kinase	SAVALMMR	12.74	Lipid metabolism
cyclopropane-fatty-acyl-phospholipid synthase	AGPASKP	2.83	Lipid metabolism
imidazole glycerol phosphate synthase subunit hisH	VVAAVCNYG	23.73	Response to stress
mannosyltransferase/glycosyltransferase	IIPRGLTV	17.88	Carbohydrate metabolism
glycosyltransferase	LQIPFQSDSEIKQ	48.19	Carbohydrate metabolism
glycogen debranching enzyme GlgX	AAGMYQVGAFPGMAW	19.76	Carbohydrate metabolism

Table 14 (Continued)

Protein identification	Peptide sequences	ID score	Function
A/G-specific adenine glycosylase	KKNPAA	8.36	Nucleotide metabolism
phosphomethylpyrimidine kinase	AAGGYGMCIP	32.69	Signal transduction

Table 15 Proteins up-regulated in *S. suis* isolates from patients both serotype 2 and serotype 14

Protein identification	Peptide sequences	ID score	Function
toxin-antitoxin system, antitoxin component, Xre family	IRSITSGRTLPSMSEF	18.5	Signal
cytochrome-c oxidase chain I	GMPGPAVDL	14.35	Respiration

Protein expression pattern of *S. suis* during exposure to mouse macrophages

The expression pattern of 261 proteins expressed in *S. suis* serotype 2 and serotype 14 during exposure to mouse macrophages were investigated using Multi Experiment Viewer (MeV). The biggest group was proteins-related to stress response (42 proteins) and the second most expressed were protein involved in transcription (40 proteins) and transport (40 proteins). Of 42 proteins of stress response group, 3 proteins were similary expressed in 4 strains (TD 2.2, TSK 10.4, LPH 210/53 and MNCM 07 strains) such as hydrolase α/β fold family protein, chaperone protein DnaJ and sulfur oxidation V protein. There were 3 proteins specific to LPH 210/53 (such as aerobic-type carbon monoxide, dehydrogenase large subunit and superoxide dismutase), 4 proteins expressed only in TSK 10.4 strain (such as β-carbonic anhydrase, 8-OXO-dGTPase domain, methionine sulfoxide reductase B and benzaldehyde lyase) and 1 protein expressed only in TD 2.2 (as cupin 2 domain-containing protein) (Figure 12). In the transcription group, 8 proteins were similary expressed in TD 2.2, TSK 10.4, LPH 210/53 and MNCM 07 strains such as integrase catalytic subunit, hydrogenase 3, yecA family protein and internalin. There was 1 protein specifically expressed only in TD 2.2 strain (as modulator of DNA) and 1 protein expressed only in TSK 10.4 strain (as PAS/PAC sensor hybrid histidine kinase) (Figure 13). Besides, 4 proteins of the transport group (integral membrane protein, YjgP/YjgQ family permease, chromate transporter permease and Na+/H+ antiporter NhaC) were similary expressed in 4 strains (TD 2.2, TSK 10.4, LPH 210/53 and MNCM 07 strains), whereas 7 proteins were specifically expressed only in TSK 10.4 strain (such as secretion system-associated protein type VI, ABC transporter ATPase, alanine racemase) and 3 proteins expressed only in TD 2.2 (as radical SAM protein, preprotein translocase, SecA subunit and gluconate transporter) (Figure 14).

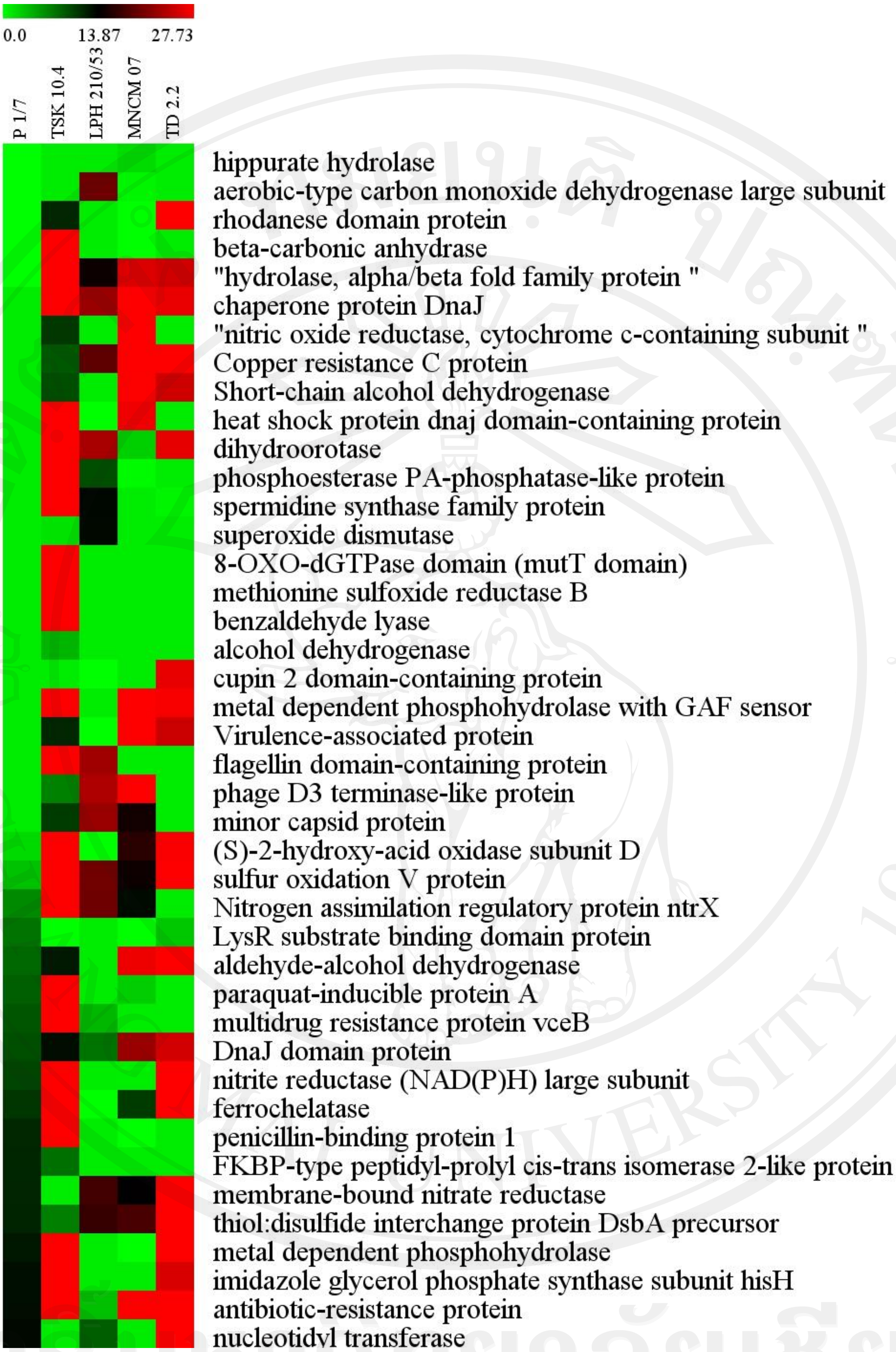

Figure 12 Heat map representation of the stress response-related proteins of *S. suis 5* strains; P 1/7, TSK 10.4, LPH 210/53, MNAM 07 and TD 2.2. Level of protein expression is marked using a color scale ranging from green (no change), dark (a little) and red (the highest) up-regulation ratio.

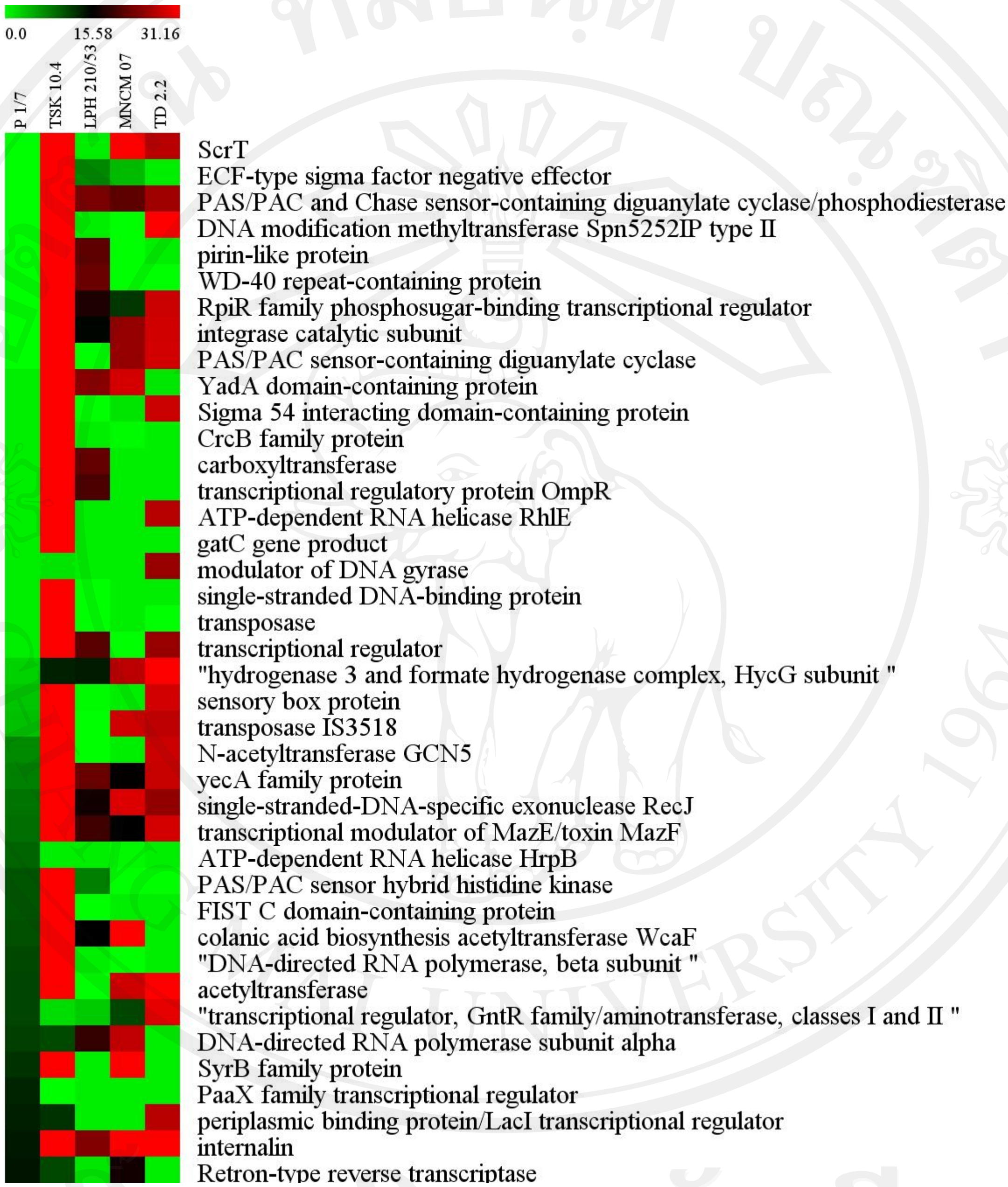

Figure 13 Heat map representation of the transcription-related proteins of *S. suis* 5 strains; P 1/7, TSK 10.4, LPH 210/53, MNAM 07 and TD 2.2. Level of protein expression is marked using a color scale ranging from green (no change), dark (a little) and red (the highest) up-regulation ratio.

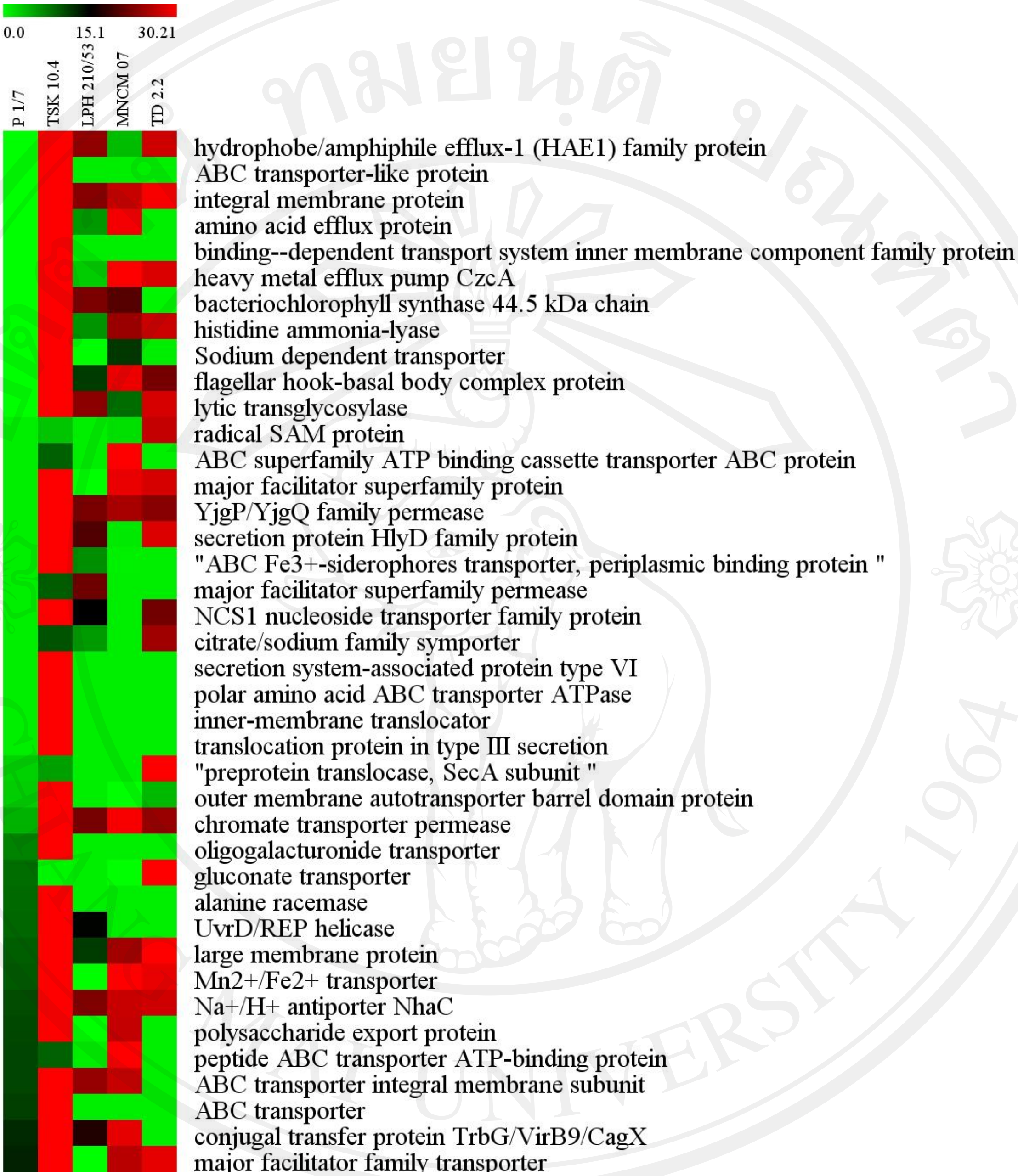

Figure 14 Heat map representation of the transport-related proteins of *S. suis 5* strains; P 1/7, TSK 10.4, LPH 210/53, MNAM 07 and TD 2.2. Level of protein expression is marked using a color scale ranging from green (no change), dark (a little) and red (the highest) up-regulation ratio.

5.4.2 Proteins of *S. suis* with differential expression during exposure to human macrophage (U 937)

Protein profiles of *S. suis* during exposure to human macrophage (U 937) were analyzed by SDS-PAGE. The protein bands on SDS-PAGE were cut into 15 ranges according to molecular weight prior to digestion and subjection into LC-MS/MS (Figure 15). The results showed that the proteins of each *S. suis* strain have differentially up-regulated during exposure to human macrophage. The total of 118 proteins could be detected. They are involved in translation (48%), carbohydrate metabolism (10%), DNA replication (10%), transport (7%), protein metabolism (6%), DNA metabolism (4%), transcription (4%), stress response (4%), lipid metabolism (3%), signal (3%) and unknown (1%) (Figure 16).

Comparison of proteins of *S. suis* between serotype and each strains found that 7 proteins were up-regulated only in TSK 10.4 strain (keto-acid reductoisomerase, phosphate acyltransferase, chorismate synthase, 30S ribosomal protein S7, 30S ribosomal protein S8, UPF0348 protein SSU98_0368 and elongation factor 4) as shown in Table 17. For LPH 210/53 strain, 2 proteins including aspartate-tRNA ligase and ATP-dependent helicase/nuclease subunit A were specifically up-regulated as shown in Table 18. Three proteins that were specifically up-regulated in only MNCM 07 including tRNA threonylcarbamoyladenosine biosynthesis protein Gcp, Queuine tRNA-ribosyltransferase and Purine nucleoside phosphorylase DeoD-type as shown in Table 19. 50S ribosomal protein L2 was the only protein specifically expressed in TD 2.2 strain (Table 20). In addition, comparison of proteins similary expressed between in all 5 strains of serotype 2 and serotype 14 showed that 46 proteins were similary expressed (Table 16) including 6-phosphofructokinase, phosphopentomutase, DNA polymerase III PolC-type, dihydroxy-acid dehydratase, GTPase Era, chaperone protein DnaK, glycine-tRNA ligase beta subunit, argininosuccinate lyase, 50S ribosomal protein L18, peptidyl-tRNA hydrolase and protein translocase subunit SecA.

Moreover, comparison of the protein profiles between serotype of *S. suis* showed that 3 proteins (Alanine-tRNA ligase, 50S ribosomal protein L1 and UDP-N-acetylglucosamine pyrophosphorylase) were up-regulated in all serotype 2 isolates (Table 21). Whereas, only 1 protein (30S ribosomal protein S5) was up-regulated in both isolates of serotype 14 (Table 22). Finally, comparison of the protein profiles between origin of isolates (clinical and animal) showed that 2 proteins (50S ribosomal protein L33 and enolase) were up-regulated in only the isolates from pigs (Table 23). Most of the proteins were associated with translation including metabolic enzymes and protein synthesis within the cell.

(a)

(b)

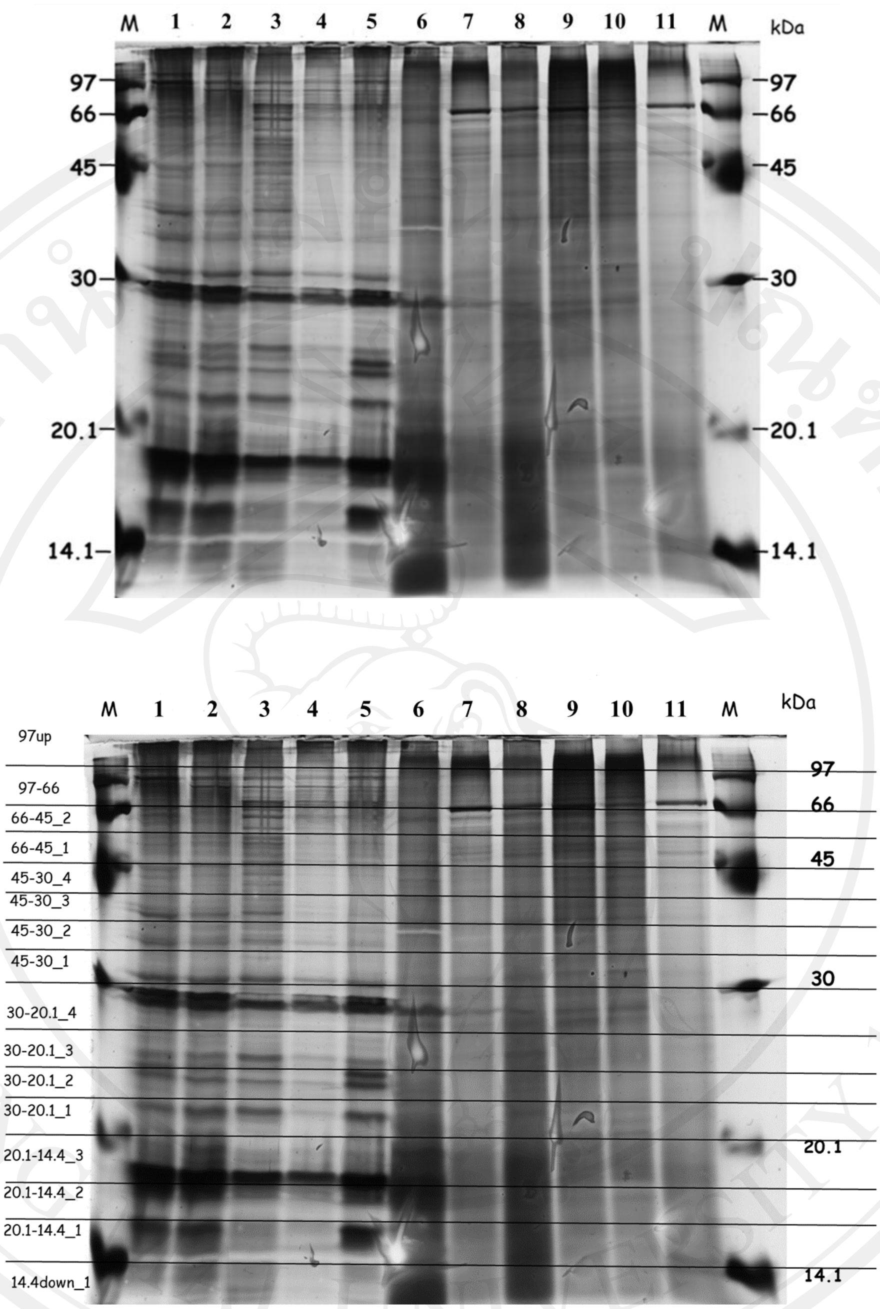

Figure 15 SDS-PAGE of proteins from *S. suis* samples. (on 12.5% polyacrylamide gel stained with silver) (a) Uncut, (b) Cut protein 15 bands. Lanes: M, the standard marker (Amersham Biosciences, UK); 1 to 5, control of *S. suis* proteins including lane 1, P1/7 (diseased pig); 2, LPH 210/53 (patient); 3, TSK 10.4 (healthy pig); 4, MNCM 07 (patient); 5, TD 2.2 (healthy pig); lane 6, control of human macrophage cell lines (U 937) and lane 7 to 11, intracellular bacteria (bacteria with in macrophage cells) including 7, P1/7 (diseased pig); 8, LPH 210/53 (patient); 9, TSK 10.4 (healthy pig); 10, MNCM 07 (patient); 11, TD 2.2 (healthy pig).

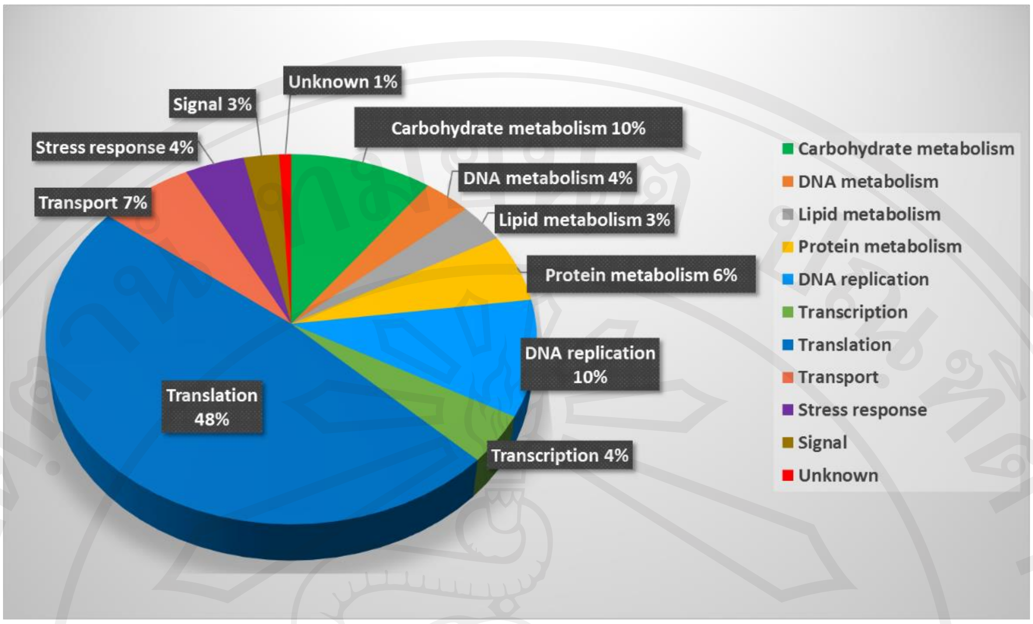

Figure 16 Functional categorization of significantly differential protein expression of *S. suis* after exposure to the human macrophage (U 937)

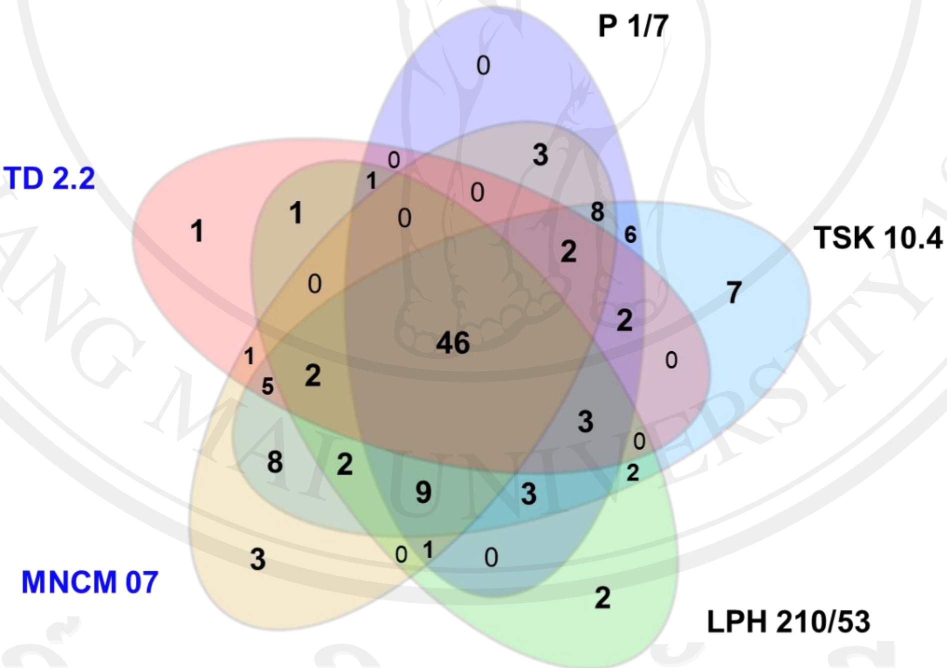

Figure 17 The number of differentially expressed proteins of *S. suis* serotypes 2 and 14 during exposure to human macrophages

(○ P1/7, ○ TSK 10.4, ○ LPH 210/53 and ○ MNCM07, ○ TD 2.2).

Table 16 Proteins of *S. suis* serotype 2 and serotype 14 that were similarly up-regulated during exposure to human macrophages

Protein identification	Peptide sequences	ID score	Function
Galactokinase	RAECEK	9.38	Carbohydrate metabolism
6-phosphofructokinase	TFVVEVMGR	16.01	Carbohydrate metabolism
N-acetylmannosamine-6-phosphate 2-epimerase	EEGGIMPLLVK	4.77	Carbohydrate metabolism
Phosphoenolpyruvate carboxylase	RFIDK	8.04	Carbohydrate metabolism
Phosphopentomutase	IGLGNIPR	12.05	Carbohydrate metabolism
Dtdp-D-Glucose 4,6-Dehydratase	QITNILAGIKPK	0.71	Carbohydrate metabolism
Adenine phosphoribosyltransferase	VLMHY	7.66	DNA metabolism
DNA polymerase III PolC-type	LAAGAAGVKR	15.59	DNA replication
DNA-directed RNA polymerase subunit beta	EIAALKEELK	16.16	DNA replication
Holliday junction ATP-dependent DNA helicase RuvB	IFIEAAK	11.01	DNA replication
DNA-directed RNA polymerase subunit omega	MMLKPSIDTLLDK	2.06	DNA replication
Holliday junction ATP-dependent DNA helicase RuvA	GILTKITAK	4.02	DNA replication

Table 16 (Continued)

Protein identification	Peptide sequences	ID score	Function
Dihydroxy-acid dehydratase	KADIEEAGR	12.94	Protein metabolism
Gamma-glutamyl phosphate reductase	IDLLVPR	5.76	Protein metabolism
GTPase Era	GIIIGK	12.87	Signal transduction
Acetate kinase	EVERLK	7.63	Stress response
Chaperone protein DnaK	AQALAVK	11.64	Stress response
competence-damage inducible protein	VRTPNNER	7.95	Stress response
Transcriptional repressor NrdR	GSKVKPGKK	14.1	Transcription
Polyribonucleotide nucleotidyltransferase	QANGAVVVR	6.9	Transcription
Glycine-tRNA ligase beta subunit	VGAVLALADK	10.56	Translation
tRNA dimethylallyl transferase	QVLYDR	9.53	Translation
Ribosome-recycling factor	MTADKEKELLEV	8.35	Translation
Lysine--tRNA ligase	MTALAEQGIDPFG K	8.15	Translation
UPF0298 protein SSU05_1549	ATILKLKK	3.03	Translation
Argininosuccinate lyase	KNPDMAELIR	11.86	Translation
50S ribosomal protein L18	LNIFR	23.31	Translation

Table 16 (Continued)

Protein identification	Peptide sequences	ID score	Function
Proline--tRNA ligase	AADGIVEVK	11.37	Translation
30S ribosomal protein S2	MEEDGTFEVLPK	1.81	Translation
Glutamate--tRNA ligase	SIQHIDNMLKSL	4.44	Translation
UPF0374 protein SSU05_0445	TWRDTMVLK	1.29	Translation
Methylenetetrahydrofolate--tRNA-(uracil-5-)-methyltransferase TrmFO	RLDSIIMR	7.47	Translation
Ribosome maturation factor RimP	KEVTIPYQTVAK	2.05	Translation
50S ribosomal protein L35	RTGSGGLKR	12.74	Translation
50S ribosomal protein L24	GVEAVVVTALPK	4.36	Translation
50S ribosomal protein L5	LVTVSLPR	11.39	Translation
MutS2 protein	ARLDLR	21.77	Translation
tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG	GLENAQMMR	7.54	Translation
50S ribosomal protein L22	AKGSASPINK	7.34	Translation
30S ribosomal protein S15	NLLAYLRR	7.2	Translation
Peptidyl-tRNA hydrolase	LIIGLGNPGDR	5.66	Translation
30S ribosomal protein S12	SPALNVGYNSRKK	4.2	Translation
UDP-N-acetylmuramoyl-L-alanyl-D-glutamate-L-lysine ligase	LLAFTGTKGK	2.16	Transport
Protein translocase subunit SecA	DEAIDGIIK	5.73	Transport

Table 16 (Continued)

Protein identification	Peptide sequences	ID score	Function
Pyrrolidone-carboxylate peptidase	AMVEAIHR	3.23	Transport
UDP-N-acetylenol pyruvoylglucosamine reductase	MKDKIR	1.19	Transport

Table 17 Proteins up-regulated in *S. suis* strain TSK 10.4 during exposure to human macrophages

Protein identification	Peptide sequences	ID score	Function
Ketol-acid reductoisomerase	VGAELRKAMPFV GRNDDDAFK	3.01	Carbohydrate metabolism
Phosphate acyltransferase	KKEASMVLATK	0.81	Lipid metabolism
Chorismate synthase	VAVGAVAKR	4.85	Protein metabolism
30S ribosomal protein S7	EIMDAANNTGAA VKK	8.31	Translation
UPF0348 protein SSU98_0368	GADQLDLVR	5.84	Translation
30S ribosomal protein S8	RVSKPGLR	3.7	Translation
Elongation factor 4	AGKKR	0.6	Translation

Table 18 Proteins up-regulated in *S. suis* strain LPH 210/53 during exposure to human macrophages

Protein identification	Peptide sequences	ID score	Function
Aspartate--tRNA ligase	AIVVKGAADSYSRK	4.38	Translation
ATP-dependent helicase/nuclease subunit A	ILDKLKR	11.16	DNA replication

Table 19 Proteins up-regulated in *S. suis* strain MNCM 07 during exposure to human macrophages

Protein identification	Peptide sequences	ID score	Function
tRNA threonylcarbamoyladenosine biosynthesis protein Gcp	VGRVMGLPYPAGR	5.56	Translation
Queuine tRNA-ribosyltransferase	LTSYHNLYFLINLMK	2.3	Translation
Purine nucleoside phosphorylase DeoD-type	ILLPGDPLR	3.13	DNA metabolism

Table 20 Proteins up-regulated in *S. suis* strain TD 2.2 during exposure to human macrophages

Protein identification	Peptide sequences	ID score	Function
50S ribosomal protein L2	NKDGVEAIVK	1.27	Translation

Table 21 Proteins up-regulated in all strains of *S. suis* serotype 2 during exposure to human macrophages

Protein identification	Peptide sequences	ID score	Function
Alanine--tRNA ligase	AIVVKGAADSYSRK	12.36	Translation
50S ribosomal protein L1	SKNLLAALEK	6.30	Translation
UDP-N-acetylglucosamine pyrophosphorylase	ILDKLKR	1.00	DNA replication

Table 22 Proteins up-regulated in both strains of *S. suis* serotype 14 during exposure to human macrophages

Protein identification	Peptide sequences	ID score	Function
30S ribosomal protein S5	AEEVAALR	6.93	Translation

Table 23 Proteins up-regulated in *S. suis* isolates from pigs both serotype 2 and serotype 14

Protein identification	Peptide sequences	ID score	Function
50S ribosomal protein L33	RNTPDRLQLK	9.35	Translation
Enolase	GLVTAVGDEGGFAPK	57.28	Unknown

Protein expression pattern of *S. suis* during exposure to human macrophages

The expression pattern of 118 proteins expressed in *S. suis* serotype 2 and serotype 14 during exposure to human macrophages were analysed using Multi Experiment Viewer (MeV). The biggest group was proteins-related to translation (57 proteins) and the second most expressed were protein involved in carbohydrate metabolism (12 proteins) and DNA replication (12 proteins). It was found that 16 of 57 proteins of translation group were similary expressed in all strains of *S. suis* such as 30S ribosomal protein S15, 50S ribosomal protein L5, MutS2 protein, UPF0374 protein SSU05_0445, glutamate-tRNA ligase, lysine-tRNA ligase and ribosomal-recycling factor. Two proteins were specifically expressed only in MNCM 07 (such as tRNA threonylcarbamoyl adenosine biosynthesis protein Gcp and queuine tRNA-ribosyltransferase), 1 protein expressed only in LPH 210/53 strain (as aspartate-tRNA ligase) and 1 protein expressed only in TSK 10.4 and TD 2.2 (as 30S ribosomal protein S7 and 50S ribosomal protein L2, respectively) (Figure 18). In carbohydrate metabolism group, 5 proteins were similary expressed in all strains of *S. suis* such as phosphopentomutase, galactokinase, 6-phosphofructokinase and phospho enolpyruvate carboxylase, 1 protein was specifically expressed only in TSK 10.4 strain (as keto-acid reductoisomerase) (Figure 19). Besides, 4 proteins within DNA replication group (DNA polymerase III PolC-type, DNA-directed RNA polymerase subunit β, DNA-directed RNA polymerase subunit omega and holliday junction ATP-dependent DNA helicase RuvA) were similary expressed in all strains of *S. suis* and 1 protein was specifically expressed only in LPH 210/53 strain (as ATP-dependent helicase/nuclease subunit A) (Figure 20).

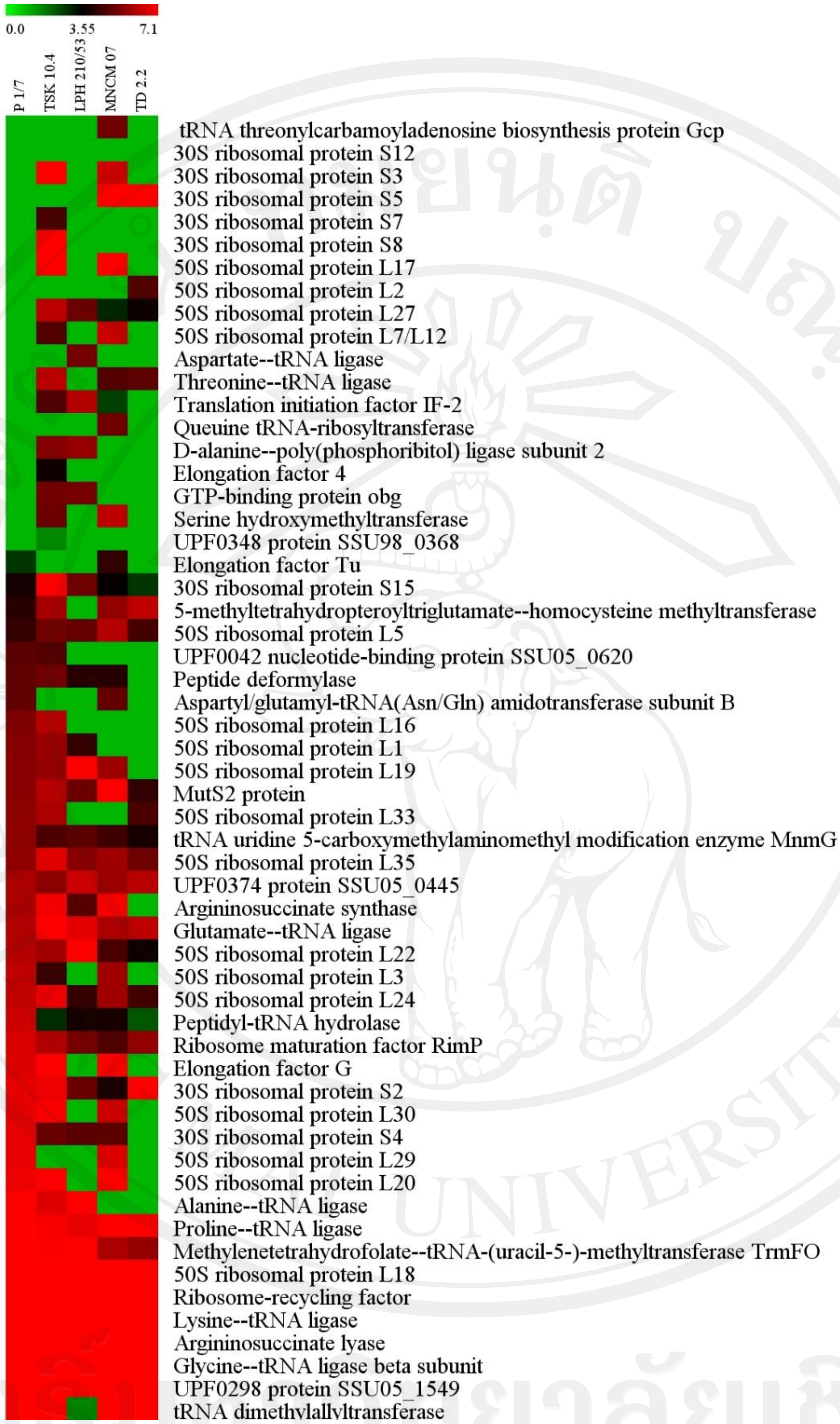

Figure 18 Heat map representation of the translation-related proteins of *S. suis 5* strains; P1/7, TSK 10.4, LPH 210/53, MNCM 07 and TD 2.2. Level of protein expression is marked using a color scale ranging from green (no change), dark (a little) and red (the highest) up-regulation ratio.

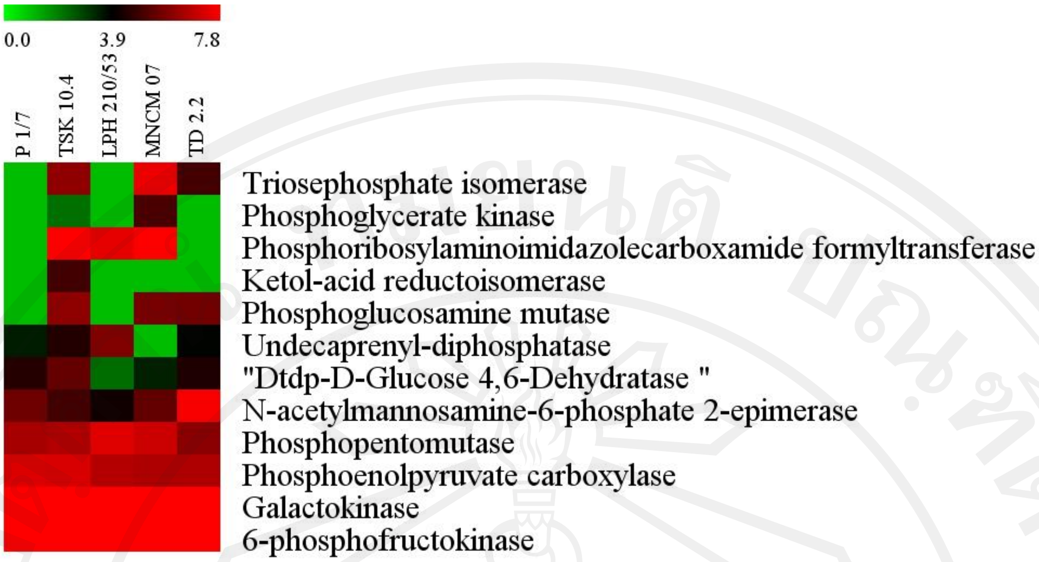

Figure 19 Heat map representation of the carbohydrate metabolism-related proteins of *S. suis 5* strains; P1/7, TSK 10.4, LPH 210/53, MNCM 07 and TD 2.2. Level of protein expression is marked using a color scale ranging from green (no change), dark (a little) and red (the highest) up-regulation ratio.

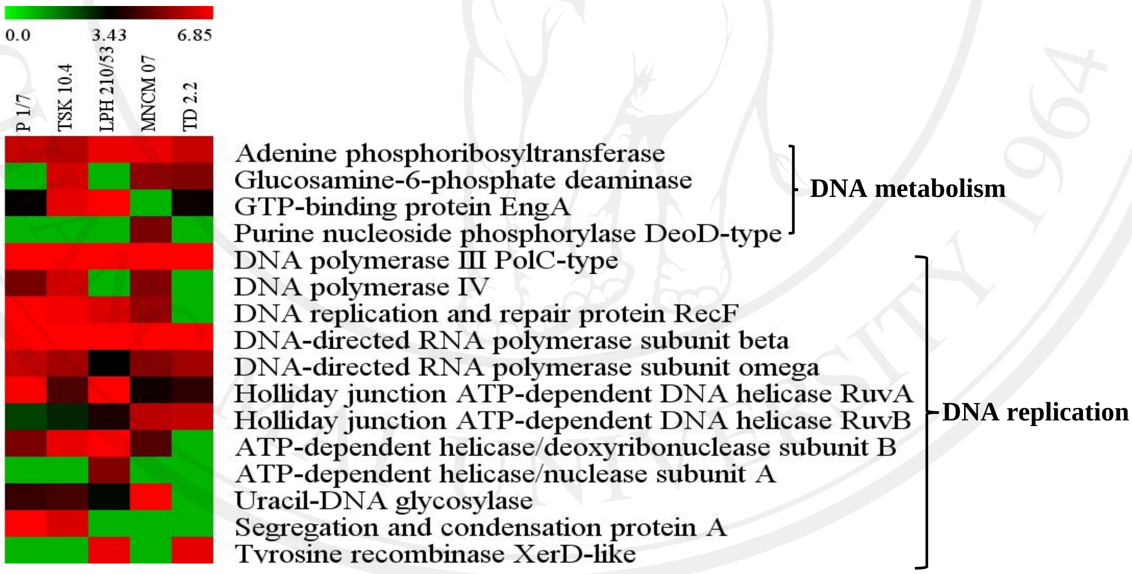

Figure 20 Heat map representation of the DNA metabolism and DNA replication-related proteins of *S. suis 5* strains; P1/7, TSK 10.4, LPH 210/53, MNCM 07 and TD 2.2. Level of protein expression is marked using a color scale ranging from green (no change), dark (a little) and red (the highest) up-regulation ratio.

5.5 Distribution of virulence-associated genes of *S. suis*

Virulence-associated genes of *S. suis* were detected by multiplex polymerase chain reaction (M-PCR) analysis. Those genes were suilysin (*sly*) with the amplicon size of 248 bp, extracellar factor protein (*epf*) with the amplicon size of 744 bp and muramidase released protein (*mrp*) with the amplicon size of 188 bp (Silva *et al*., 2006). In this study, 50 isolates of *S. suis* selected were isolated from patients (25 isolates) and healthy pigs (25 strains). M-PCR analysis of patient isolates showed that, of 25 isolates, 5 isolates carried *sly*, *epf* and *mrp* (20%), 7 isolates carried only *sly* (28%), 10 isolates carried only *mrp* (40%) and 2 isolates carried *sly* and *mrp* (8%). Whereas, 1 isolate did not carry any of the virulence genes detected (4%) as shown in Table 24.

Moreover, The distribution of genes in 25 healthy pigs isolates were found that 8% of the isolates (2 isolates) carried *sly*, *epf* and *mrp*, 4 isolates carried only *sly* (16%), 11 isolates carried only *mrp* (44%) and 1 isolate carried both *sly* and *mrp* (4%) and 7 isolates did not carry any of the 3 virulence genes detected (28%) as shown in Table 25.

The results showed that genotypes of the isolates from the northern part of Thailand (50 isolates) comprised of 5 genotypes including *sly*-/*epf*-/*mrp*+ (42%), *sly*+/*epf*-/*mrp*- (22%), *sly*+/*epf*+/*mrp*+ (14%), *sly*+/*epf*-/*mrp*+ (6%), *sly*-/*epf*-/*mrp*- (16%) as shown in Table 26. The genotype *sly*-/*epf*-/*mrp*+ is the most prevalent genotype of the isolates. Interestingly, 40 % of the patient isolates (10 isolates) investigated were genotyped *sly*-/*epf*-/*mrp*+ in accordance with the majority of genes detected in healthy pig isolates (44%, 11 isolates). Thus, the distribution of 3 virulence-associated genes amongst the isolates in this study.

Table 24 Distribution of virulence-associated genes (*sly*, *epf*, *mrp*) amongst *S. suis* strains isolated from patients

Isolate no.	Site of isolation	Virulence-associated genes [a]	Diseases and symptoms	ST [b] (ST complex)
MNCM 01	Blood	*sly*+/*epf*+/*mrp*+	Endocarditis	1 (1)
MNCM 06	Blood, CSF	*sly*+/*epf*+/*mrp*+	Neck stiffness, deafness (meningitis)	1 (1)
MNCM 16	CSF	*sly*+/*epf*+/*mrp*+	Neck stiffness (meningitis)	1 (1)
MNCM 21	CSF	*sly*+/*epf*-/*mrp*-	Meningitis	101 (27)#
MNCM 25	Blood	*sly*-/*epf*-/*mrp*+	Neck stiffness (meningitis), diarrhea, death	102 (27)#
MNCM 26	Blood	*sly*-/*epf*-/*mrp*+	Endocarditis, deafness (meningitis)	25 (27)#
MNCM 43	Blood	*sly*-/*epf*-/*mrp*+	Endocarditis	28 (27)
MNCM 50	Blood	*sly*+/*epf*-/*mrp*-	Pulmonary edema, death	104 (27)#
MNCM 51	Blood	*sly*-/*epf*-/*mrp*+	Septicemia, diarrhea, death	25 (27)#
MNCM 54	Blood	*sly*-/*epf*-/*mrp*+	Neck stiffness (meningitis), diarrhea	102 (27)#
MNCM 55	Blood	*sly*-/*epf*-/*mrp*+	Septic shock, death	25 (27)#
LPH 03	Blood	*sly*-/*epf*-/*mrp*+	Meningitis	103 (27)#
LPH 04	Blood	*sly*-/*epf*-/*mrp*+	Septicemia, diarrhea	25 (27)#
LPH 05	Blood	*sly*-/*epf*-/*mrp*+	Septicemia	103 (27)#

Table 24 (Continued)

Isolate no.	Site of isolation	Virulence-associated genes [a]	Diseases and symptoms	ST [b] (ST complex)
LPH 12	Blood	*sly*-/*epf*-/*mrp*+	Septic shock, death	25 (27)#
H 131/53	Blood	*sly*+/*epf*-/*mrp*-	Septicemia	ND
H 132/53	Blood	*sly*+/*epf*-/*mrp*-	Septicemia	ND
H 148/53	Blood	*sly*+/*epf*-/*mrp*-	Septicemia	ND
H 153/53	Blood	*sly*+/*epf*-/*mrp*+	Septicemia	ND
H 187/53	Blood	*sly*+/*epf*+/*mrp*+	Septicemia	ND
H 219/53	Blood	*sly*+/*epf*+/*mrp*+	Septicemia	ND
H 240/53	Blood	*sly*-/*epf*-/*mrp*-	Septicemia	ND
H 244/54	Blood	*sly*+/*epf*-/*mrp*-	Septicemia	ND
H 286/54	Blood	*sly*+/*epf*-/*mrp*-	Septicemia	ND
H 290/54	Blood	*sly*+/*epf*-/*mrp*+	Septicemia	ND

MNCM and LPH were isolated from patients at Maharaj Nakorn Chiang Mai Hospital and Lamphun Hospital, Thailand, respectively.

[a] Virulence-associated gene profiling was done as described previously (Silva *et al*., 2006); *sly*, suilysin gene; *epf*, extracellular protein factor gene; *mrp*, muraminidase-released protein gene; +, positive; –, negative; [b] ST, sequence type; CSF, cerebrospinal fluid.

#ST25, ST101, ST102, ST103, and ST104 belong to the ST27 complex, only with a less-stringent approach that defines an ST complex by sharing of alleles at >5 of the 7 loci.

ND; Not determined

Table 25 Distribution of virulence-associated genes (*sly*, *epf*, *mrp*) amongst *S. suis* strains isolated from healthy pigs

Isolate no.	Site of isolation	Virulence-associated genes [a]	Diseases and symptoms	ST [b] (ST complex)
LDK 19.2	Tonsil	*sly-/epf-/mrp-*	None	ND
LSK 15.2	Tonsil	*sly-/epf-/mrp+*	None	ND
T 11.3 N	Tonsil	*sly-/epf-/mrp-*	None	ND
T 13.1 N	Tonsil	*sly+/epf-/mrp-*	None	ND
T 16.2 N	Tonsil	*sly-/epf-/mrp+*	None	ND
T 6.1 S	Tonsil	*sly-/epf-/mrp+*	None	ND
T 12.1 S	Tonsil	*sly-/epf-/mrp+*	None	ND
T 13.1 S	Tonsil	*sly+/epf-/mrp+*	None	ND
T 13.2 S	Tonsil	*sly-/epf-/mrp+*	None	ND
T 13.3 S	Tonsil	*sly-/epf-/mrp+*	None	ND
TCP 40.2S	Tonsil	*sly-/epf-/mrp-*	None	ND
TD 2.3	Tonsil	*sly+/epf+/mrp+*	None	ND
TDK 5.4	Tonsil	*sly+/epf+/mrp+*	None	ND
THD 10.2	Tonsil	*sly+/epf-/mrp-*	None	ND
THD 10.4	Tonsil	*sly-/epf-/mrp-*	None	ND
THD 10.6	Tonsil	*sly-/epf-/mrp-*	None	ND
THD 16.4	Tonsil	*sly-/epf-/mrp+*	None	ND
TJ 21.2 S	Tonsil	*sly-/epf-/mrp-*	None	ND
TP 12.2	Tonsil	*sly+/epf-/mrp-*	None	ND
TP 12.3	Tonsil	*sly+/epf-/mrp-*	None	ND
TSK 11.2	Tonsil	*sly-/epf-/mrp+*	None	ND
TSK 16.2	Tonsil	*sly-/epf-/mrp+*	None	ND
TSK 16.3	Tonsil	*sly-/epf-/mrp+*	None	ND
TSK 16.4	Tonsil	*sly-/epf-/mrp+*	None	ND
TSP 19.2	Tonsil	*sly-/epf-/mrp-*	None	ND

[a] Virulence-associated gene profiling was done as described previously (Silva *et al*., 2006); *sly*, suilysin gene; *epf*, extracellular protein factor gene; *mrp*, muraminidase-released protein gene; +, positive; –, negative. [b] ST, sequence type; ND, Not determined

Table 26 Percentage of genotypes amongst *S. suis* strains isolated from the northern part of Thailand

Genotype	Total isolates (%)	Number of isolates (%)	
		Healthy pig	Patient
sly+/epf+/mrp+	7 (14)	2 (8)	5 (20)
sly+/epf-/mrp-	11 (22)	4 (16)	7 (28)
sly-/epf-/mrp+	21 (42)	11 (44)	10 (40)
sly+/epf-/mrp+	3 (6)	1 (4)	2 (8)
sly-/epf-/mrp-	8 (16)	7 (28)	1 (4)

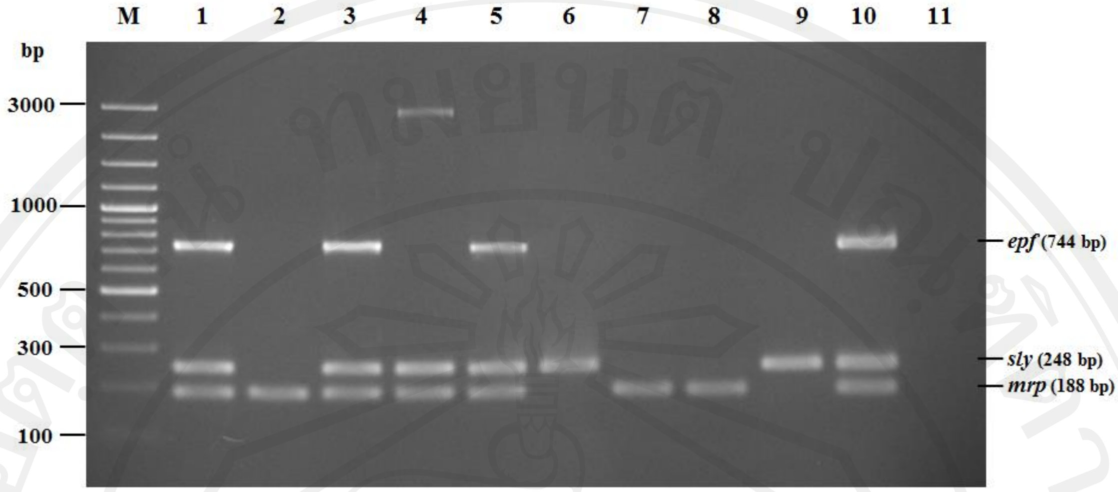

Figure 21 Detection of virulence-associated genes of *S. suis*: suilysin (*sly*), extracellular factor protein (*epf*) and muramidase released protein (*mrp*) by Multiplex-PCR. The amplicon size of those genes were 248 bp, 744 bp and 188 bp, respectively. Lanes: M, molecular mass marker (100 bp Plus DNA Ladder; Thermo scientific); 1, P1/7 (reference strain: diseased pig); 2, TSK 10.4 (healthy pig); LPH 210/53 (patient); MNCM 07 (patient); TD 2.2 (healthy pig); H 148/53 (patient); LSK 15.2 (healthy pig); MNCM 55 (positive control of gene *mrp*+); MNCM 21 (positive control of gene *sly*+); MNCM 06 (positive control of genes *sly*+, *epf*+, *mrp*+) and 11, negative control of the PCR (deionize water).