African Journal of Biotechnology Vol. 11(24), pp. 6550-6565, 22 March, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.4079
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Construction and analysis of subtractive hybridization library of differentially expressed genes in spleen of C57BL/6 and A/J mice with *Streptococcus suis* serotype 2 infection

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Accepted 14 February, 2012

Streptococcus suis **is an important swine and human pathogen, and it has presented a considerable challenge to the global public health community. Domínguez-Punaro et al. (2007) showed that two strains of mice were differentially susceptible to *S. suis*. The attainment of information on the differential expression of host response genes following *S. suis* infection is relevant to the understanding of the molecular mechanism of this disease. The intent of this study was to select and isolate differentially expressed genes from A/J and C57BL/6 mice that are associated with the host response to *S. suis* infection. These baseline data will allow for the screening and cloning of specific resistance genes, and further our understanding of the molecular mechanism of *S. suis* infection. Eight-week-old C57BL/6 and A/J mouse were infected with *S. suis* serotype 2, a 200-µL volume of a bacterial suspension (1×10^8 CFU/mL) was administrated by intraperitoneal injection to the mice, and cDNA subtraction libraries were constructed by suppression subtraction hybridization (SSH). Genes involved in immune function, such as lysozyme, interferon-active protein, macrophage activation 2-like protein, complement component-3 and Ly108 protein were up-regulated in the spleen of C57BL/6 mice to a greater extent than they were in the spleen of A/J mice, subsequent to infection with *S. suis* serotype 2. Interestingly, we observed that several splenic signaling factors associated with phospholipid metabolism were up-regulated to a greater extent as well in C57BL/6 mice than they were in A/J mice following infection with *S. suis* serotype 2. These data suggest that phospholipid metabolism may be involved in the host defense response to *S. suis* invasion, thereby contributing to the organism's overall immune response to this pathogen.**

Key words: *Streptococcus suis* serotype 2, suppression, subtractive hybridization, differentially expressed genes, mouse spleen.

INTRODUCTION

Streptococcus suis (*S. suis*) is an important zoonotic pathogen and is implicated in the etiology of several diseases, including arthritis, endocarditis, meningitis, pneumonia and septicemia (Gottschalk and Segura., 2000; Messier et al., 2008). The ability of *S. suis* to cause disease is not limited to swine. A severe outbreak of invasive infection in humans and pigs occurred in Sichuan and Jiangsu Provinces, resulting in high levels of mortality (Tang et al., 2006; Feng et al., 2009). *S. suis* infection has also caused sporadic human illness in China (Feng et al., 2009), Thailand (Wangsomboonsiri et

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al., 2008; Navacharoen et al., 2009), the United Kingdom (Watkins et al., 2001), Portugal (Taipa et al., 2008), the Netherlands (van de Beek et al., 2008), the United States (Smith et al., 2008; Fittipaldi et al.,2009), and in Australia (Tramontana et al., 2008), and it has been recognized as the most common cause of adult meningitis in Vietnam (Wertheim et al., 2009). Although 35 serotypes have been described to date, *S. suis* serotype 2 (SS2) is still the most virulent and frequently isolated form of the disease in animals (Higgins and Gottschalk.2005). Due to the serious public health risk imposed by SS2, focused research efforts on *S. suis* are warranted. Previous studies have been performed primarily on the pathogenic bacteria and on a several virulence-associated factors, including Rgg (Zheng et al., 2011), LuxS (Wang et al., 2011; Cao et al., 2011), enolase (Esgleas et al., 2008; Feng et al., 2009; Zhang et al., 2009), fibronectin-binding protein (de Greeff et al., 2002), glyceraldehyde-3-phosphate dehydrogenase (Brassard et al., 2004), and IgA1 protease (Zhang et al., 2010). However, the pathogenesis of *S. suis* infection is not fully understood. In studies, it has been suggested that the host response plays an essential roles in the development of the disease (Li et al., 2010). Domínguez-Punaro Mde et al. (2008) showed that A/J mice were more susceptible than C57BL/6 mice to *S. suis* infection. They also confirmed that IL-10 was responsible, at least in part, for the high survival rate, which suggests that an aberrant innate immune response contributes to the development of SS2 diseases (Domínguez-Punaro Mde et al., 2008). Therefore, in order to better understand the molecular mechanism of the disease process, it is necessary to screen and identify differentially expressed genes associated with the host response to *S. suis* infection. In this study, a subtractive hybridization library was constructed, and differentially expressed genes from the spleen of C57BL/6 and A/J mice infected with *S. suis* serotype 2 were analyzed. Differentially expressed gene functional analyses can provide baseline data that will be useful for the screening and cloning of specific resistant genes, and they may also contribute to a better understanding of the molecular mechanism of *S. suis* diseases.

MATERIALS AND METHODS

Reagent

The TRIZOL reagent kit was purchased from Invitrogen (CA, USA), the Oligotex® mRNA Kits from Qiagen (Germany), the PCR-Select™ cDNA subtraction kit, Advantage cDNA PCR kit and Polymerase Mix from Clontech (USA), and the 2 × Taq PCR Master Mix, Agarose Gel DNA purification kit, pMD®19-T Vector, DNA marker DL-2000, Φ×I74-Hae III digest from TaKaRa Bio Inc. (Shiga, Japan). The E.Z.N.A. cycle-pure kit (Omega, USA), Regular agarose G-10, tryptone and yeast extract were obtained from Tiangen Biotechnology Institution (Nanjing, China), and the Tris-phenol was purchased from Beijing Shuangxiang Biotechnology Institution (Beijing, China).

Bacterial strain and culture conditions

The HA9801 strain of SS2 was isolated in 1998 from diseased pigs in Jiangsu province, and it was confirmed virulent through animal experimentation (Zhang and Lu.,2007; Wu et al., 2008). Bacteria were grown overnight in Todd-Hewitt broth (THB) at 37°C, and isolated colonies were then inoculated in 2-mL of THB and incubated for 8 h at 37°C while being agitated. Working cultures were prepared by transferring 100 µL of 1:100 dilutions of the 8 h culture media into 10-mL of THB, which was further incubated and agitated for 12 h at 37°C. Stationary-phase bacteria were washed twice in phosphate-buffered saline (PBS) (pH 7.4). The bacterial pellet was re-suspended and adjusted to a concentration of 3.89×10^8 CFU/mL, and this solution was diluted in THB to a final concentration of 1×10^8 CFU/mL (Domínguez-Punaro Mde et al., 2008). The final suspension was plated onto blood agar to determine CFU/mL.

Animal and sample preparation

Eight-week-old C57BL/6 and A/J mice (obtained from the experiment center, Nanjing University, Jiangsu Province, China) were acclimatized to a standard 12-h-light/12-h-dark cycle, and they were allowed free access to rodent chow and water. All animal handling procedures were performed in strict accordance with guidelines established by the People's Republic of China. On the day of the experiment, 200 µL of the bacterial suspension (1×10^8 CFU/mL) were administrated to the animals by intraperitoneal injection. All mice were sacrificed at 72 h post-injection. Tissue samples obtained from the spleen were immediately frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

RNA extraction, cDNA synthesis and SSH

Total RNA was isolated from spleen tissue using the TRIZOL reagent kit, according to the manufacturer's instructions. The RNA concentration was quantified by measuring absorbance at 260 nm in a photometer (Eppendorf Biophotometer). Absorption ratios (260/280 nm) for all preparations were between 1.8 and 2.0. RNA sample aliquots were subjected to electrophoresis through a 1.0% agarose/formaldehyde gel to verify their integrity. The mRNA was extracted to determine total RNA, using the Oligotex mRNA kit, according to the manufacturer's instructions, and in each case, spleen samples from multiple individuals were pooled to extract mRNA for subtraction.

Subtractive hybridization libraries, including SSH-TA (a forward library tester cDNA using A/J mouse spleen, and a driver cDNA using C57BL/6 mouse spleen) and SSH-TB (a reverse library tester cDNA using C57BL/6 mouse spleen, and a driver cDNA, using A/J mouse spleen) were constructed using a PCR-Select™ cDNA subtraction kit according to the manufacturer's instructions, as schematically described in Figure 1. Briefly, cDNA was generated from 2 µg of poly (A+) mRNA from each of the tester and driver populations, and it was converted by reverse transcription and digestion with Rsa I (1.5 h at 37°C) to produce short, blunt-ended fragments. The tester cDNA was subdivided into two portions and ligated with a different adaptor that was provided in the cDNA subtraction kit (Clontech). Ligation efficiency was evaluated by PCR, using primers specific to human skeletal muscle G3PDH gene sequences, and then two hybridization steps were performed. For the first hybridization, an excess of the driver cDNA was added to each of the adaptor-ligated tester samples and then heat-denatured and allowed to anneal. In the second hybridization, the two primary hybridization samples were mixed together without denaturizing them, and nested PCR amplification was then performed. After amplifying the tester-specific sequences of hybridization samples by

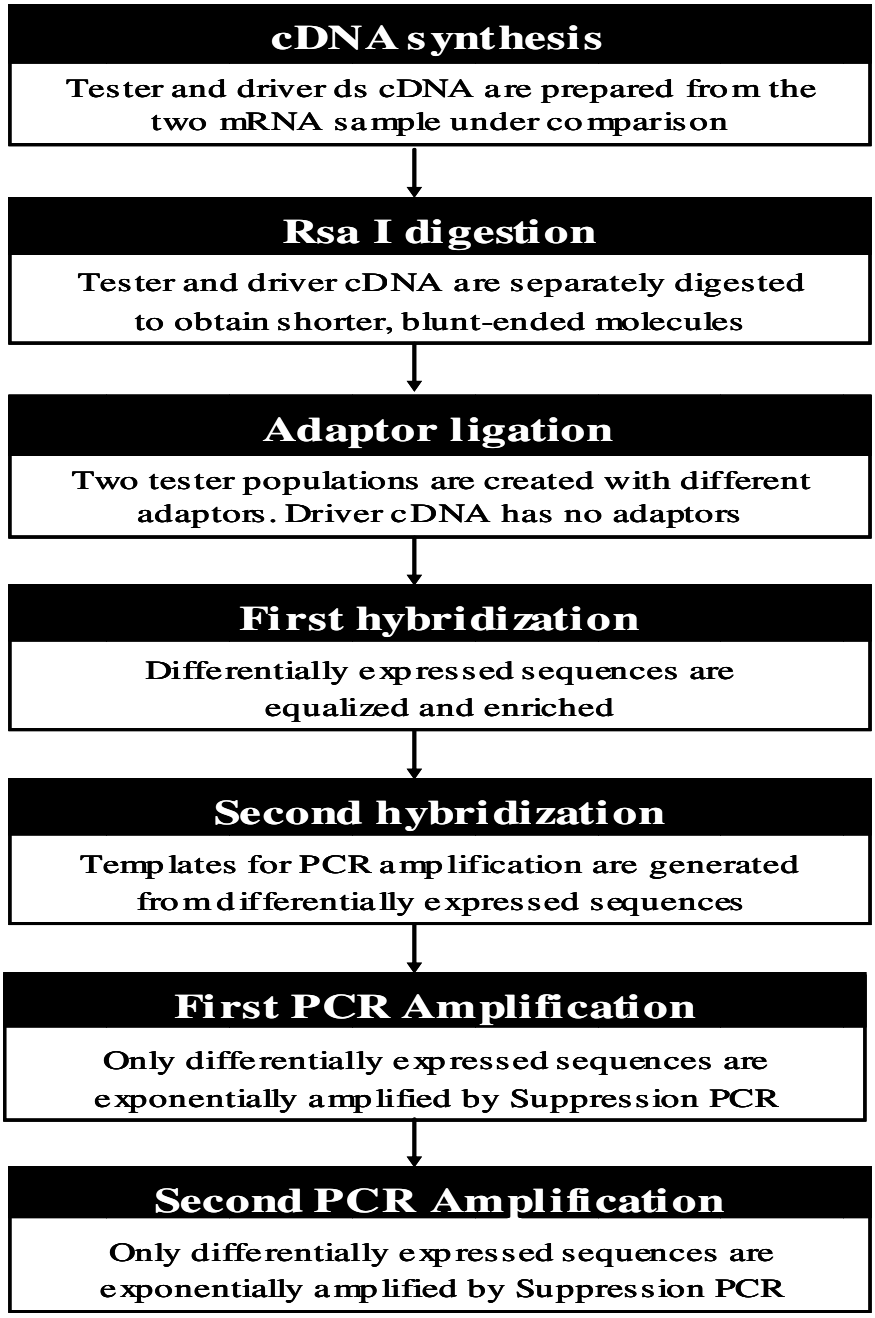

Figure 1. Overview of all steps of the subtractive hybridization procedure.

PCR, the specific fragments obtained were ligated to pMD®19-T vectors. The ligation mixture was then transformed into *Escherichia coli* DH-5α cells and cultured on Luria Bertani (LB) agarose plates containing ampicillin and X-Gal/IPTG. The white clones were selected to construct the forward and reverse subtracted cDNA library. A total of 421 recombinant clones were selected randomly and verified by PCR to check the quality of the library.

Detection of positive clones

All white clones were selected from LB plates and added to 200 µL of liquid LB medium containing 100 µg/mL of ampicillin and shaken for 6 h at 37°C. The cDNA inserted fragments were amplified using the nested PCR primers 1 (5'-TGCAGCGGCCGCCCGGGCAGGT-3') and 2R (5'-AGCGTGGTCGCGGCCGAGGT-3') that were provided in the PCR-selected cDNA subtraction kit, and insert fragments were detected by 2.0% agarose gel electrophoresis.

Sequencing, expressed sequence tags (ESTs) and bioinformatics analysis

All selected clones were sequenced by Shanghai Majorbio Bio-Pharm Technology Co. (Shanghai, China). After removing low quality bases, uninformative sequences, adaptor sequences and

Table 1. Mortality of HA9801 strain on A/J mice and C57BL/6 mice.

Strain	Infection dose	Mice strain	Amount of mice	Death/Total	Mortality (%)
HA9801	10^8	A/J	16	11/16	68.75
HA9801	10^8	C57BL/6	16	4/16	25

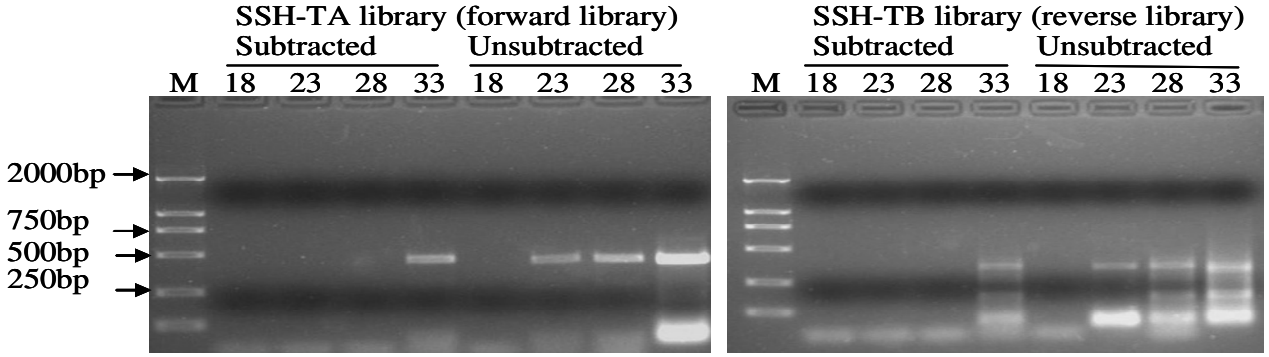

Figure 2. Reduction in G3PDH gene abundance by suppression subtractive hybridization. PCR analysis was performed on subtracted and unsubtracted secondary PCR products for different cycles in forward library (SSH-TA) and reverse library (SSH-TB). Lane 18: 18 cycle; Lane 23: 23 cycle; Lane 28: 28 cycle; Lane 33: 33 cycle. M: molecular size marker DL2000 (Takara).

vector sequences, each unidentified sequence was subjected to BlastX and BlastN alignment against the non-redundant (nr) protein database and GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Database searches were limited to ESTs > 100 bp in length, and matches with a bit score higher than 40 were considered significant. To further study the function and characteristics of differentially expressed genes, sequence analysis using online GO (Gene ontology category, http://www.geneontology.org/) software was employed to obtain information regarding functional annotation.

RESULTS

Mortality

The mortality rates for the A/J and C57BL/6 mice following injection with the HA9801 suspension are shown in Table 1. Mortality for the A/J and C57BL/6 mice were 68.75 and 25.0%, respectively. This indicates that the A/J mice were more susceptible to HA9801 than were the C57BL/6 mice.

Library construction

Subtraction efficiency was evaluated by PCR amplification of the G3PDH housekeeping gene. If subtraction is efficient, a reduction in the housekeeping gene transcripts should be observed. A comparison of abundance before and after subtraction suggests that the quantity of G3PDH gene was significantly reduced following subtraction (Figure 2). The PCR products of the G3PDH gene can be observed after 23 and 33 cycles for unsubtracted and subtracted cDNAs, which indicates that G3PDH abundance was theoretically reduced by ~2(33-23) = 210. The results indicate that specific genes in the spleen of A/J and C57BL/6 mice infected with SS2 strain HA9801 were enriched by ~210 times by SSH.

PCR product examination of differentially expressed cDNAs

All differentially expressed cDNA clones (total 441) were screened by PCR amplification from the two subtraction libraries. As some of the clones were found several times, 174 clones from the SSH-TA library and 183 clones from the SSH-TB library were successfully amplified. The maximum efficiency was 84.8% in the two subtraction libraries. Agarose gel electrophoresis results show that the cDNA insert size ranged from 150 to 720 bp following removal of the primer and adapter sequences (Figure 3).

EST sequence analysis

Production of ESTs from SSH library

Three hundred and fifty-seven ESTs were obtained from the SSH library after low-quality and repeated sequences were eliminated . BLAST was used to analyze ESTs with database nucleotide collection (nr/nt), as some ESTs shared overlapping regions, or represented different

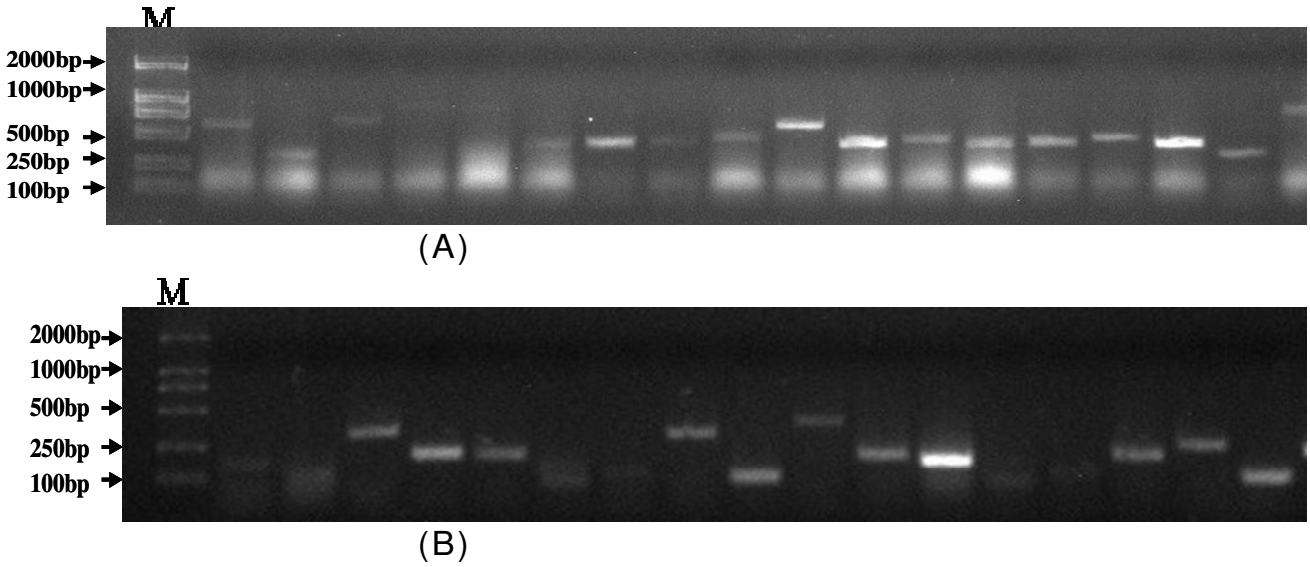

Figure 3. Electrophoresis patterns of PCR products amplified from the inserted fragments. M: DL2000; A: SSH-TA library; B: SSH-TB library.

fragments of the same gene. Finally, the results revealed that 116 EST fragments from the SSH-TA library and 138 EST fragments from the SSH-TB library were highly homologous (> 95%), with known sequences deposited in GenBank. All EST sequences listed in Table 2 (from the SSH-TA library) and in Table 3 (from the SSH-TB library) were deposited in GenBank. Also, three EST fragments from the SSH-TA library and two EST fragments from the SSH-TB library were novel genes with no homology within the GenBank database (data not shown).

Basic function annotation

To assess the role of the ESTs, gene-ontology was used for functional annotation of the differentially expressed genes. The results indicate that binding sequences (25%) contribute to the majority of the total unique transcripts obtained in the SSH-TA library, followed by genes involved in physiological pathways (15%), transcription and translation (14%), signal transduction (11%), regulatory enzyme activity (10%), catalytic activity (6%), cytes pathways (5%), development (4%), transport (4%) and unknown functions (6%) (Figure 4A). The results indicate that genes involved in binding sequences (21%) are also the largest contributor to the total obtained in the SSH-TB library, followed by physiological pathways (19%), transcription and translation (14%), signal transduction (12%), catalytic activity (7%), regulatory enzyme activity (6%), regulatory apoptosis (5%), cytes pathways (4%), transport (4%), development (3%) and unknown functions (5%) (Figure 4B). Interestingly, the regulatory signal transduction genes increased from 11% in the SSH-TA library to 12% in the SSH-TB library, and regulatory cell apoptosis genes appeared only in the SSH-TB library. The data indicate that C57BL/6 mice cells may be more susceptible to apoptosis following infection with the *S. suis* pathogen than are cells from A/J mice.

Putative differentially expressed proteins

To determine the primary cell functions of the 254 unique transcripts identified through significant matches, we subjected them all to Blast homology searches, and a summary of these searches is contained in Table 4. Some proteins that were expressed in C57BL/6 mice have been implicated in general and specific disease resistance mechanisms, and in the body's immune response. Others are involved in cell signal transduction and as membrane proteins, transcriptional factors and biological activity regulators. However, relatively few binding proteins were differentially expressed in A/J mice. As well, some proteins were differentially expressed both in A/J and C57BL/6 mice (data not shown). Interestingly, as many as eight differentially expressed proteins, as identified by BLAST software analysis, may have impacted upon anti-disease activity in C57BL/6 mice, including interferon activity protein, lysozyme, Ly108 protein, macrophage activation 2 like protein, complement component-3, phosphatase, phosphatidylserine decarboxylase, serine/threonine kinase and CDC-like kinase-1.

DISCUSSION

A severe *S. suis* outbreak in humans and pigs has seriously challenged public health officials in

Table 2. List of identified genes from SSH-TA library.

Classification	Identified gene	GenBank accession number of EST	Length (bp)	Identities	E-value
Binding	*M. musculus* DNA for desmin-binding fragment DesA7	AJ403263.1	409	96	3E-168
	M. musculus DNA for desmin-binding fragment DesA17	AJ403273.1	512	91	2E-145
	M. musculus DNA for desmin-binding fragment DesA25	AJ403281.1	392	94	2E-86
	M. musculus DNA for desmin-binding fragment DesB4	AJ403301.1	402	95	3E-89
	M.musculus DNA for desmin-binding fragment DesB15	AJ403312.1	508	91	9E-134
	M.musculus DNA for desmin-binding fragment DesB16	AJ403313.1	569	90	3E-153
	M. musculus DNA for desmin-binding fragment DesB20	AJ403317.1	519	91	3E-143
	M. musculus DNA for desmin-binding fragment DesB29	AJ403326.1	545	93	4E-157
	M. musculus DNA for desmin-binding fragment DesB38	AJ403335.1	431	92	1E-157
	M. musculus DNA for vimentin-binding fragment VimC35	AJ403170.1	460	94	2E-109
	M. musculus DNA for vimentin-binding fragment VimC44	AJ403179.1	498	92	1E-137
	M. musculus DNA for vimentin-binding fragment VimE5	AJ403237.1	627	89	5E-176
	M. musculus DNA for vimentin-binding fragment VimE9	AJ403241.1	455	97	3E-168
	M. musculus DNA for vimentin-binding fragment VimE22	AJ403254.1	488	93	3E-83
	M. musculus DNA for GFAP-binding fragment GFAPE3	AJ403645.1	309	95	8E-164
	M. musculus DNA for GFAP-binding fragment GFAPE11	AJ403653.1	354	93	2E-81
	Mus musculus guanylate binding protein 4 (Gbp4), mRNA	NM_008620.3	221	99	9E-109
	Mus musculus guanylate binding protein 6 (Gbp6), mRNA	NM_194336.2	232	93	5E-91
	poly(A) binding protein, cytoplasmic 1 (Pabpc1 protein)	AAH04587.1	503	100	7E-16
	poly(A) binding protein, cytoplasmic 2	AAB70164.1	338	75	5E-08
	poly(A) binding protein, cytoplasmic 4	CAM46095.1	398	100	1E-11
	poly(A) binding protein, cytoplasmic 6	EDL02091.1	362	80	3E-10
	Mus musculus RNA binding motif protein 39 (Rbm39), mRNA	NM_133242.2	498	99	0
	Murine gene 37 for pot. membrane bound protein	Y00629.1	307	96	7E-55
	Rattus norvegicus RNA-binding region containing protein 2-like, mRNA	NM_001177904.1	528	95	5E-146
	Mus musculus poly(A)binding protein, cytoplasmic-1, mRNA, complete cds	BC003870.1	343	99	5E-146
	M. musculus mRNA for poly(A) binding protein	X65553.1	347	100	3E-128
Physiology pathway	*Mus musculus* mRNA for mKIAA4245 protein	AK220567.1	303	99	1E-111
	Mus musculus mRNA for estrogen responsive finger protein, complete cds	D63902.1	365	99	1E-142
	Mus musculus premature mRNA for mFLJ00102 protein	AK220304.1	408	98	8E-174
	Mus musculus glucocorticoid-attenuated response gene 49(GARG-49/IRG2) mRNA, complete cds	U43086.1|MMU43086	604	99	7E-135
	HUMRNPB1A Human hnRNP B1 protein mRNA	M29064.1	465	99	0
	Homo sapiens mRNA for BM-010 variant protein	AB209021.1	190	95	3E-79
	Mus musculus tripartite motif-containing 25 (Trim25), mRNA	NM_009546.2	372	98	8E-159

Table 2 Contd

	Samd9l protein (sterile alpha motif domain containing 9-like)	AAH31151.2	781	100	4E-84
	Pb-fam-2 protein	XP_675977.1	407	77	E-133
Catalytic activity	*Mus musculus GTF2IRD1* and *CYLN2* genes complete cds	AF289667.1|AF289667	273	94	9E-79
	AF081957 Synthetic construct aminoglycoside 3'-phosphotrans ferase mutant (mNeo) gene	AF081957.1	258	100	2E-11
	Mus musculus GTPase, IMAP family member 6 (Gimap6), mRNA	BC028779.1	408	98	2E-170
	Rattus norvegicus NCK associated protein 1 like (Nckap1l), mRNA	NM_001108119.1	459	92	1E-151
	PREDICTED: *Mus musculus* heterogeneous nuclear ribonucleoproteins A2/B1-like (LOC100045191)	XM_001473825.2	470	100	4E-47
Regulatory transcription and translation	*Mus musculus* eukaryotic translation initiation factor 4A2 (Eif4a2), mRNA	BC094422.1	223	98	1E-107
	Mus musculus eukaryotic translation initiation factor 4A2 (Eif4a2), transcript variant 3, mRNA	NM_001123038.1	199	100	9E-99
	Homo sapiens replication factor C (activator 1) 4 (37kD) (RFC4) gene, complete cds	AF538718.1	201	95	3E-79
	Mus musculus eukaryotic translation elongation factor 1 alpha 1, mRNA, complete cds	BC108391.1	308	100	2E-135
	MUSMHCQA1A *Mus musculus* MHC class I Qa-1a antigen mRNA, complete cds	L00606.1	319	99	4E-132
	Rattus norvegicus similar to chromosome 1 open reading frame 63, mRNA, complete cds	BC085888.1	359	87	2E-124
	MUSSATA Mouse satellite DNA	M17407.1	389	94	1E-152
	Human DNA sequence from clone RP11-449I17 on chromosome 10, A transcriptional regulator(yeast) (SIN3A) and a CpG island, complete sequence	AL161651.13	580	80	5E-116
	Mus musculus PRP38 pre-mRNA processing factor 38 (yeast) domain containing B, mRNA	NM_025845.2	388	100	7E-80
	PRP38 pre-mRNA processing factor 38 (yeast) domain containing B	BAB30042.1	298	100	1E-25
Signal transduction	*Mus musculus* KH domain containing, RNA binding, signal transduction associated 1, mRNA	NM_011317.3	433	99	2E-180
	Homo sapiens mRNA for proto-oncogene protein, complete cds	D14497.1|HUMPOPSTK	528	79	3E-108
	Mus sp. JAK1 protein tyrosine kinase mRNA, complete cds	S63728.1|S63728	284	99	2E-110
	Homo sapiens mitogen-activated protein kinase kinase kinase 8 (MAP3K8), mRNA	NM_005204.2	563	80	5E-116
	Clk4 protein	AAH02220.1	355	83	6E-62
	Homo sapiens cDNA FLJ56064 complete cds, highly similar to Dual specificity protein kinase CLK1	AK294295.1	426	86	2E-145
	Human protein kinase mRNA	M59287.1|HUMKINCDC	542	86	8E-144
	Homo sapiens CDC-like kinase 4, mRNA, complete cds	BC136261.1	537	78	2E-61
	CDC-like kinase 1	EDL00074.1	507	100	1E-75
Regulatory enzyme activity	Putative protein kinase C regulatory protein	S55223.1	435	82	3E-79
	Mus musculus tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide,mRNA	BC049070.1	471	98	2E-154
	Homo sapiens mRNA similar to protein kinase, cAMP dependent regulatory seroserotype I beta	BC002470.1	518	90	3E-163
Transport	*Mus musculus* ATPase, Na^+/K^+ transporting, beta 3 polypeptide (Atp1b3), mRNA	NM_007502.4	441	99	0
	Atp1b3 protein (ATPase, Na^+/K^+ transporting, beta 3 polypeptide)	AAI14531.1	441	97	9E-39

Table 2 Contd

Cytes pathway	AF248636 *Mus musculus* lymphocyte antigen 108 isoform s mRNA, complete cds	AF248636.1	544	100	1E-176
Development	*Mus musculus* histocompatibility 2, T region locus 23, mRNA, complete cds	BC005648.1	319	99	2E-139
	myeloid nuclear differentiation antigen like	D0QMC3.1	427	99	4E-85
	Mouse DNA sequence from clone RP23-353G2 on chromosome 2, complete sequence	AL837506.3	421	91	1E-171
	Mnda protein (myeloid cell nuclear differentiation antigen)	AAI45398.1	812	92	7E-76
Others	*Mus musculus* activated spleen cDNA, RIKEN full-length enriched library, clone: F830006H06 product: unclassifiable, full insert	AK171721.1	369	94	2E-155
	mCG10725, isoform CRA_b	EDL25591.1	485	100	4E-38
	mCG10725, isoform CRA_a	EDL20953.1	485	97	9E-39
	mCG1027072	EDL10687.1	369	98	6E-35
	mCG1031612	EDK97144.1	298	83	2E-26
	mCG1029410	EDL13997.1	422	100	1E-81
	rCG41011	EDL84379.1	408	96	2E-78

Table 3. List of identified genes from SSH-TB library.

Classification	Identified gene	GenBank accession number of EST	Length (bp)	Identities	E-value
Binding	*M. musculus* DNA for desmin-binding fragment DesA7	AJ403263.1	409	96	3E-168
	M. musculus DNA for desmin-binding fragment DesA17	AJ403273.1	512	91	2E-145
	M. musculus DNA for desmin-binding fragment DesA25	AJ403281.1	392	94	2E-86
	M. musculus DNA for desmin-binding fragment DesB15	AJ403312.1	508	91	9E-134
	M. musculus DNA for desmin-binding fragment DesB16	AJ403313.1	569	90	3E-153
	M. musculus DNA for desmin-binding fragment DesB20	AJ403317.1	519	91	3E-143
	M. musculus DNA for desmin-binding fragment DesB29	AJ403326.1	545	93	4E-157
	M. musculus DNA for desmin-binding fragment DesB38	AJ403335.1	431	92	1E-157
	M. musculus DNA for desmin-binding fragment DesD19	AJ403398.1	445	93	1E-82
	M. musculus DNA for desmin-binding fragment DesD23	AJ403402.1	515	94	2E-134
	M. musculus DNA for desmin-binding fragment DesD25	AJ403404.1	477	91	3E-163
	M. musculus DNA for vimentin-binding fragment VimC2	AJ403137.1	293	94	7E-85
	M. musculus DNA for vimentin-binding fragment VimE5	AJ403237.1	627	89	5E-176
	M. musculus DNA for vimentin-binding fragment VimE9	AJ403241.1	455	97	3E-168
	M. musculus DNA for vimentin-binding fragment VimE22	AJ403254.1	488	93	3E-83
	M. musculus DNA for vimentin-binding fragment VimG17	AJ403224.1	384	94	8E-15

Table 3 Contd

	M. musculus DNA for GFAP-binding fragment GFAPE3	AJ403645.1	309	95	8E-164
	M. musculus DNA for GFAP-binding fragment GFAPE5	AJ403647.1	349	94	3E-168
	M. musculus DNA for GFAP-binding fragment GFAPE20	AJ403402.1	272	93	3E-83
	Mus musculus guanylate binding protein 4 (Gbp4), mRNA	NM_008620.3	221	99	9E-109
	Mus musculus guanylate binding protein 6 (Gbp6), mRNA	NM_194336.2	232	93	5E-91
	M. musculus mRNA for poly(A) binding protein	X65553.1	347	100	3E-128
	Murine gene 37 for pot. membrane bound protein	Y00629.1	307	96	7E-55
	poly(A) binding protein, cytoplasmic 1 (Pabpc1 protein)	AAH04587.1	503	100	7E-16
	poly(A) binding protein, cytoplasmic 2	AAB70164.1	338	75	5E-08
	poly(A) binding protein, cytoplasmic 4	CAM46095.1	398	100	1E-11
	poly(A) binding protein, cytoplasmic 6	EDL02091.1	362	80	3E-10
	Rattus norvegicus RNA-binding region containing protein 2-like, mRNA	NM_001177904.1	528	95	5E-146
Physiology pathway	*Mus musculus* tripartite motif-containing 25 (Trim25), mRNA	NM_009546.2	372	98	8E-159
	Mus musculus lysozyme 2, mRNA (cDNA clone MGC:62600, IMAGE:6514977), complete cds	BC054463.1	218	100	2E-109
	Mus musculus lysozyme 1 (Lyz1), mRNA	NM_013590.4	210	96	2E-95
	Mus musculus mRNA for mKIAA4245 protein	AK220567.1	303	99	1E-111
	Mus musculus macrophage activation 2 like protein, mRNA , complete cds	BC007143.1	258	93	2E-95
	MUSEFP *Mus musculus* mRNA for estrogen responsive finger protein, complete cds	D63902.1	375	99	1E-142
	Mus musculus interferon-induced protein with Tetratricopeptide repeats 3, mRNA, complete cds	BC003804.1	604	99	4E-137
	Mus musculus interferon activated gene 204 (Ifi204), mRNA	NM_008329.2	530	98	0
	Mus musculus interferon activated gene 205 (Ifi205), mRNA	NM_172648.3	510	93	0
	Mus musculus interferon activated gene 202B (Ifi202b), transcript variant 1, mRNA	NM_008327.2	502	82	3E-78
	PREDICTED: *Mus musculus* interferon-activable protein 202-like, mRNA	XM_001473873.2	502	82	2E-76
	Mus musculus glucocorticoid-attenuated response gene 49 (GARG-49/IRG2) mRNA, complete cds	U43086.1|MMU43086	604	99	7E-135
	HUMRNPB1A Human hnRNP B1 protein mRNA	M29064.1	465	99	0
	interferon-activatable protein	AAA39313.1	658	95	1E-77
	Mus musculus strain NZB lupus susceptibility protein p202 (Ifi202a) mRNA	DQ222946.1	604	82	7E-75
	phosphatidylserine decarboxylase, isoform CRA-d(EC:4.1.1.65)	EAW59990.1	188	79	1E-18
	phosphatidylserine decarboxylase, isoform CRA-b (EC:4.1.1.65)	EAW59985.1	188	83	5E-18
	Mus musculus premature mRNA for mFLJ00102 protein	AK220304.1	408	98	8E-174
	Homo sapiens mRNA for BM-010 variant protein	AB209021.1	190	95	3E-79
	Samd9l protein (sterile alpha motif domain containing 9-like)	AAH31151.2	781	100	4E-84
	Pb-fam-2 protein	XP_675977.1	407	77	E-133
	Mus musculus phosphatase and tensin homolog (Pten), mRNA (EC:3.1.3.36)	NM_008960.2	262	99	3E-50
	Mus musculus phosphatidylserine decarboxylase (Pisd), mRNA (EC:4.1.1.65)	NM_177298.3	721	96	5E-63

Table 3 Contd

Catalytic activity	*Rattus norvegicus* NCK (choline kinase) associated protein 1 like (Nckap1l), mRNA	NM_001108119.1	459	92	1E-151
	Cytochrome oxidase subunit 1 (EC 1.9.3.1) (Cytochrome c oxidase polypeptide I) (Cytochrome c oxidase subunit I) homolog	AK136262.1	344	100	1E-157
	Mus musculus GTF2IRD1 (glucosyltransferase 1, insulin resistance diabetes1) and CYLN2 genes complete cds	AF289667.1|AF289667	568	94	9E-79
	cytochrome c oxidase subunit I	BAA95618.1	344	87	2E-27
	cytochrome c oxidase subunit III	ABK79255.1	438	90	1E-50
	Mus musculus GTPase, IMAP family member 6 (Gimap6), mRNA	BC028779.1	408	98	2E-170
	PREDICTED: *Mus musculus* heterogeneous nuclear ribonucleoproteins A2/B1-like (LOC100045191)	XM_001473825.2	470	100	4E-47
Regulatory transcription and translation	*Mus musculus* lysozyme 2, mRNA (cDNA clone MGC:62600 IMAGE:6514977), complete cds	BC094422.1	223	98	1E-107
	Mus musculus eukaryotic translation initiation factor 4A2 (Eif4a2), transcript variant 3, mRNA	NM_001123038.1	199	100	9E-99
	Homo sapiens replication factor C (activator 1) 4 (37kD) (RFC4) gene, complete cds	AF538718.1	201	95	3E-79
	Mus musculus eukaryotic translation elongation factor 1 alpha 1, mRNA, complete cds	BC108391.1	308	100	2E-135
	MUSEFTU *Mus musculus* protein synthesis elongation factor Tu (eEF-Tu, eEf-1-alpha) mRNA, complete cds	M22432.1	302	98	5E-111
	MUSMHCQA1A *Mus musculus* MHC class I Qa-1a antigen mRNA, complete cds	L00606.1	319	99	4E-132
	Rattus norvegicus similar to chromosome 1 open reading frame 63, mRNA, complete cds	BC085888.1	359	87	2E-124
	MUSSATA Mouse satellite DNA	M17407.1	389	94	1E-152
	Mus musculus PRP38 pre-mRNA processing factor 38 (yeast) domain containing B, mRNA	NM_025845.2	388	100	7E-80
	Human DNA sequence from clone RP11-449I17 on chromosome 10, A transcriptional regulator (yeast) (SIN3A) and a CpG island, complete sequence	AL161651.13	580	80	5E-116
Signal transduction	*Mus musculus* KH domain containing, RNA binding, signal transduction associated 1 (Khdrbs1), mRNA	NM_011317.3	433	99	2E-180
	Mus sp. JAK1 protein tyrosine kinase mRNA,complete cds	S63728.1|S63728	284	99	2E-110
	Homo sapiens mRNA similar to protein kinase, cAMP dependent Regulatory seroserotype I beta	BC002470.1	518	90	3E-163
	CDC-like kinase 1	EDL00074.1	507	100	1E-75
	serine threonine tyrosine kinase	AAA40151.1	589	99	1E-74
	Clk4 protein	AAH02220.1	355	83	6E-62
	Homo sapiens cDNA FLJ56064 complete cds, highly similar to Dual specificity protein kinase CLK1 (EC 2.7.12.1)	AK294295.1	426	86	2E-145
	Homo sapiens mitogen-activated protein kinase kinase kinase 8 (MAP3K8), mRNA	NM_005204.2	563	80	5E-116
	Human protein kinase mRNA	M59287.1|HUMKINCDC	542	86	8E-144
	Homo sapiens CDC-like kinase 4, mRNA (cDNA clone MGC:167871 IMAGE:9020248), complete cds	BC136261.1	536	78	2E-61
	Homo sapiens mRNA for proto-oncogene protein, complete cds	D14497.1|HUMPOPSTK	528	79	3E-108
Regulatory enzyme activity	AF081957 Synthetic construct aminoglycoside 3'-phosphotransferase,mutant (mNeo) gene, complete cds	AF081957.1	258	100	2E-11
	Mus musculus tyrosine 3-monooxygenase/tryptophan 5-monooxygenase, activation protein, beta polypeptide,mRNA	BC049070.1	471	98	2E-154
	14-3-3 protein beta subseroserotype=putative protein kinase C, regulatory protein	S55223.1	435	82	3E-79

Table 3 Contd

Regulatory apoptosis	*Mus musculus* ribosomal protein S28, mRNA ,complete cds	BC010987.1	340	98	1E-106
	Mus musculus ribosomal protein S28, mRNA ,complete cds	BC090982.1	361	96	1E-96
	Mus musculus C57BL/6J ribosomal protein S28 mRNA, complete cds	U11248.1|MMU11248	298	99	3E-94
	Rattus norvegicus ribosomal protein S15a, mRNA, complete cds	BC058452.1	353	94	3E-163
	PREDICTED: *Rattus norvegicus* similar to 40S ribosomal protein, S28 (LOC689805), mRNA	XM_002725838.1	210	92	5E-34
	ribosomal protein S25	P62852.1	366	100	4E-36
	Ly108 (SLAM family member 6)	ACF05482.1	544	100	4E-07
Transport	*Mus musculus* ATPase, Na^+/K^+ transporting, beta 3,polypeptide (Atp1b3), mRNA	NM_007502.4	441	99	0
	Atp1b3 protein (ATPase, Na^+/K^+ transporting, beta 3 polypeptide)	AAI14531.1	441	97	9E-39
Cytes pathway	AF248636 *Mus musculus* lymphocyte antigen 108 isoforms mRNA, complete cds	AF248636.1	544	100	1E-176
Development	*Mus musculus* histocompatibility 2, T region locus 23, mRNA, complete cds	BC005648.1	319	99	2E-139
	Mouse DNA sequence from clone RP23-353G2 on chromosome 2, complete sequence	AL837506.3	421	91	1E-171
	myeloid nuclear differentiation antigen like	D0QMC3.1	427	99	4E-85
	Mnda protein (myeloid cell nuclear differentiation antigen)	AAI45398.1	812	92	7E-76
Others	*Mus musculus* activated spleen cDNA, RIKEN full-length enriched library, clone: F830006H06 product: unclassifiable, full insert	AK171721.1	369	94	2E-155
	mCG10725, isoform CRA_b	EDL25591.1	485	100	4E-38
	mCG21656, isoform CRA_a	EDL20953.1	485	97	9E-39
	mCG1027072	EDL10687.1	369	98	6E-35
	mCG1031612	EDK97144.1	298	83	2E-26
	mCG1029410	EDL13997.1	422	100	1E-81
	rCG41011	EDL84379.1	408	96	2E-78

China. SS2 is the prevalent *S. suis* serotype in Chinese diseased pigs, and although several proteins have been identified as candidate vaccines to control *S. suis* (Baums and Valentin-Weigand, 2009; Feng et al., 2010), the mechanism of SS2 pathogenesis is still not well understood. Several pathogenic factors were successfully identified, and these have helped to improve our understanding of the virulence of this bacterium. As infectious disease results from interplay between the pathogen and the host defense mechanism, characterization of the host immune response is essential to a full understanding of the disease process (de Greeff et al., 2010). In the present study, the mortality rates for A/J and C57BL/6 mice infected by HA9801 were 68.75 and 25.0%, respectively. This infers that A/J mice are more susceptible to HA9801 infection than are C57BL/6 mice, which is consistent with the results of Domínguez-Punaro Mde et al. (2008). This latter group reported that A/J mice are more susceptible to *S. suis* infection than are C57BL/6 mice, especially during the acute septic phase of the infection, and that a balance between pro- and anti-inflammatory mediators is crucial for survival during the septic phase.

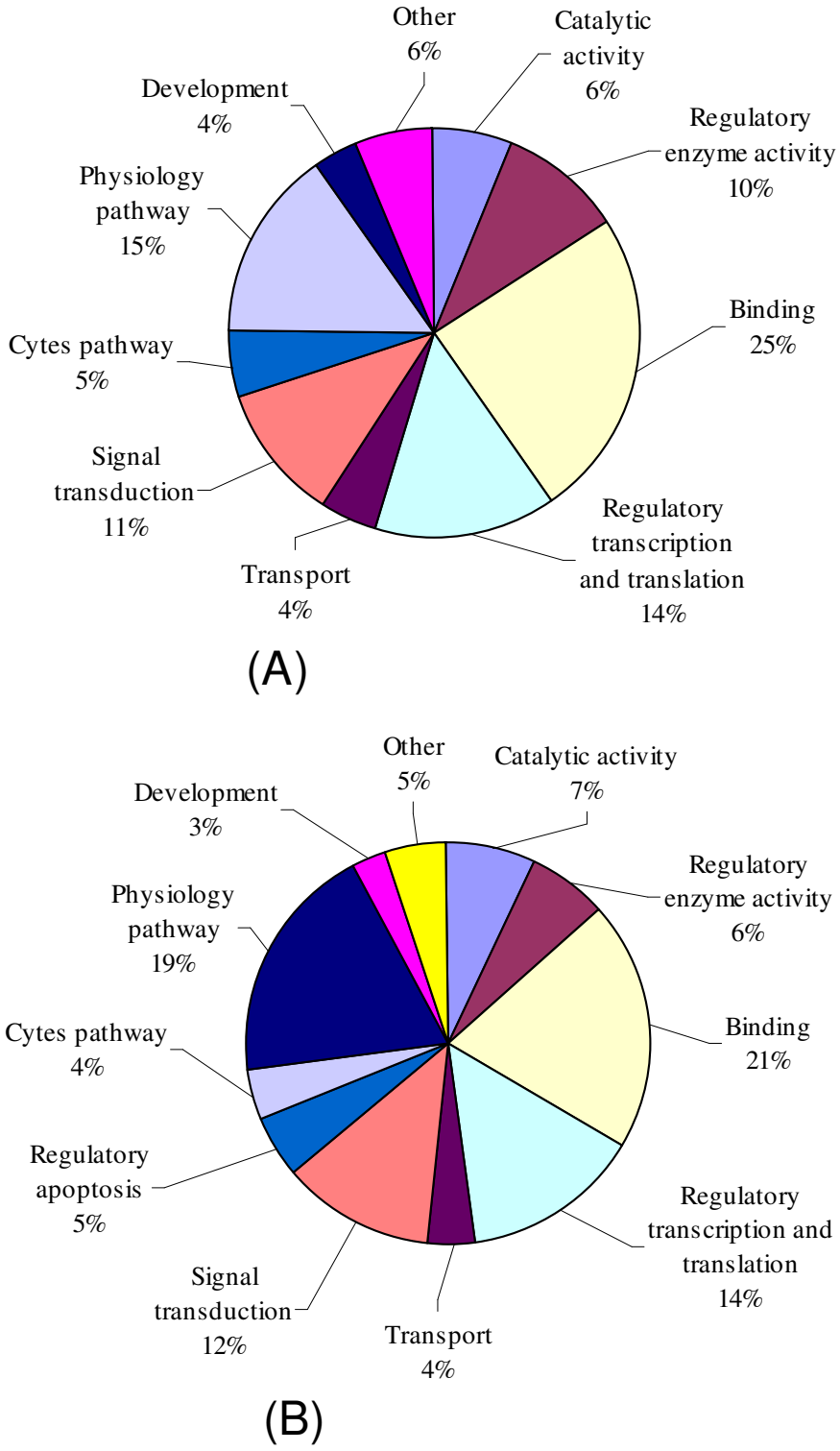

Figure 4. Functional categories analysis of differentially expressed tags functional annotation in mouse spleen infected with *S. suis* serotype 2. A: SSH-TA library; B: SSH-TB library.

The suppression subtractive hybridization (SSH) technique is an efficient and widely used PCR-based method that is used to isolate differentially expressed genes (Hayashi and Spencer., 2005; Tang et al., 2009). In

Table 4. List of possible differentially expression proteins in mouse spleen infected with *S. suis* serotype 2.

Strain	Putative differentially expressed protein
A/J mouse strain	desmin-binding fragment Des B4
	vimentin-binding fragment Vim (C35、C44)
	GFAP-binding fragment GFAPE11
	RNA binding motif protein 39 (Rbm39)
C57BL/6 mouse strain	desmin-binding fragment Des(B20、D19、D23、D25)
	vimentin-binding fragment Vim(C2、G17)
	GFAP-binding fragment (GFAPE5、GFAPE20)
	lysozyme-2
	lysozyme-1
	ribosomal protein-S28
	ribosomal protein-S25
	ribosomal protein-S15a
	macrophage activation 2 like protein
	protein synthesis elongation factor Tu (eEF-Tu, eEf-1-alpha)
	interferon-induced protein with Tetratricopeptide repeats 3
	interferon-activable protein 202-like
	NZB lupus susceptibility protein
	Cytochrome c oxidase subunit 1
	Cytochrome c oxidase subunit III
	Ly108 protein (SLAM family member 6)
	phosphatase
	Phosphatidylserine decarboxylase
	Phosphatidylserine decarboxylase -CRA-b
	Phosphatidylserine decarboxylase -CRA-d
	CDC-like kinase 1
	Serine/threonine kinase
	Clk4 protein

the current study, subtractive hybridization libraries were constructed by SSH, and differentially expressed genes (116 ESTs fragments from the SSH-TA library and 138 ESTs fragments from the SSH-TB library) were found to be highly homologous (above 95%), with known sequences deposited in GenBank. Further analysis indicated that the ESTs from the SSH-TA and SSH-TB library genes are involved in numerous and diverse physiological functions. Li et al. (2010) studied the gene expression profiles from spleen tissue obtained from pigs that had suffered from highly pathogenic *S. suis*, and the results showed that the majority of down-regulated genes were involved in transcription, transport, energy metabolism and immune function (the majority of the up-regulated genes). Interestingly, the regulatory signal transduction genes increased from 11% in the SSH-TA library to 12% in the SSH-TB library, and regulatory cell apoptosis genes appeared only in the SSH-TB library. The data indicate that C57BL/6 mice cells may be more susceptible to apoptosis following infection with the *S. suis* pathogen than are cells from A/J mice. Also, the results showed that some genes involved in immune function (lysozyme, interferon-actively protein, macrophage activation 2 like protein, complement component-3 and Ly108 protein), phospholipid metabolism (phosphatase), protein synthesis (transcription and elongation factors), signal transduction (CDC-like kinase 1, serine/threonine kinase, Clk4 protein) and ribosomal protein structure were selected from the spleen of C57BL/6 mice following HA9801 infection.

Lysozyme is a well-known anti-microbial protein that has been detected in the body fluids and tissues from many bivalve mollusks, and it is believed to play a role in host defense and digestion (Allam et al., 2000; Cronin et al., 2001). Ordás et al. (2000) reported that lysozyme concentrations changed in clams (*Tapes decussatus*) infected by *Perkinsus atlanticus*, and Chu and La Peyre. (1993) reported the same observation in oysters

(*Crassostrea virginica*) infected by *Perkinsus marinus*. Results reported herein describe up-regulation of lysozyme gene expression in the spleen of C57BL/6 mice infected by HA9801, which suggests that lysozyme may protect the body against damage by exogenous pathogenic bacteria.

Interferon is a broad-spectrum antiviral agent that does not directly inhibit or kill viruses. Instead, it induces anti-viral protein synthesis following interaction with cell surface receptors, thereby inhibiting viral replication. Data presented here show that interferon-activated protein and interferon-induced protein genes are highly expressed in C57BL/6 mice infected with strain HA9801. Li et al. (2010) reported that interferon-induced transmembrane protein levels increased 2-fold in spleens three days subsequent to *S. suis* infection, while Wu et al. (2010) found that the level of interferon regulatory factor 11 was elevated in zebrafish infected with SS2 strain HA9801. Interferon-activated protein is a cytokine that is produced by mononuclear cells and lymphocytes. It enhances the vitality of natural killer cells, macrophages and T lymphocytes, and it is involved in immunoregulation (Demmers et al., 2001). Given these results, we hypothesize that interferon protein gene expression in C57BL/6 mice infected with strain HA9801 is up-regulated to improve exogenous pathogen resistance by enhancing natural killer cell, macrophage and T lymphocyte vitality.

Macrophage activation 2 like protein is initiated by lymphokines, and it can alter the morphology and functional activity of macrophages. Stimulated macrophages can produce interleukin (IL)-1β, tumour necrosis factor (TNF), IL-18 and antiviral interferon (IFN) (Pirhonen et al., 1999). In A/J mice, increased IL-12 expression may have been associated with the induction of high levels of IFN-γ, a cytokine known for its potent ability to activate macrophages and enhance TNF-γ synthesis, thereby exacerbating mortality (Heinzel et al., 1994). In this study, macrophage activation 2 like protein was highly expressed in HA9801 infected C57BL/6 mice. This is consistent with the work of Wu et al. (2010), who reported elevated levels of macrophage stimulating 1 in zebrafish infected with SS2 strain HA9801. Human and murine monocytes/macrophages recognize whole *S. suis* or its purified cell wall components primarily through a toll-like receptor 2 (TLR2)- dependent pathway (Graveline et al., 2007). Monocyte-derived macrophages also demonstrate a capacity to kill *S. suis* in the absence or presence of specific antibodies, depending on the bacterial strain they are exposed to (Andresen and Tegtmwier., 2001). High levels of cytokines and chemokines could be released by macrophages (Segura et al., 1999) and monocytes (Segura et al., 2002) by *S. suis* stimulation, and these could be important in the initiation and development of inflammation and meningitis (Domínguez-Punaro et al., 2007). This suggests that macrophage activation is sequelae to and a cellular source of anti-inflammatory cytokines in the spleen of mice following strain HA9801 infection.

The complement system is an important part of the immune system and induction of those genes associated with complement activation and other immune system components represents an essential step in the organism's overall defense against pathogen invasion. KEGG pathway analysis revealed that genes involved in complement and coagulation cascades were up-regulated in zebrafish following *S. suis* infection (Wu et al., 2010), and Chabot-Roy et al. (2006) demonstrated the importance of complement and specific antibodies in the bactericidal activity of leukocytes against *S. suis*. In this study, the complement component 3 (C3) gene was induced in C57BL/6 mice after infection with strain HA9801. Complement activation leads to cleavage of C3, and the resultant products initiate a cascade of events, producing several physiologically active molecules (Walport et al., 2001). C3 and its products migrate to sites of infection where they may activate pro-inflammatory cells. These cells secrete inflammatory mediators and cytokines, which assist in the overall immune response to the *S. suis* infected in C57BL/6 mice. Additional study of C3 and the pathogenesis of SS2 strain HA9801 infections in C56BL/6 mice will further clarify this relationship.

Ly108 is a member of the mouse CD family of cell surface proteins (Peck and Ruley., 2000). These proteins contain similar immunoglobulin domains, which activate natural killer (NK) cells and lymphocytes. Through the combined effects of homologous Src-2 protein domains, phosphatase SH2 domains and tyrosine phosphorylation, these proteins resist the invasion of foreign bacteria (Gray et al., 2000; Bottino et al., 2001; Valdez et al., 2004). Our results show that Ly108 protein gene expression was enriched in C57BL/6 mice after strain HA9801 infection. Although the exact mechanisms underlying the inflammatory response induced by *S. suis* is unknown, *in vitro* experiments have shown that cytokine and chemokine production by *S. suis*-activated phagocytes is mediated through CD14-dependent and independent pathways (Segura et al., 2002). Segura et al. (2002) also demonstrated that *S. suis* is recognized primarily by TLR2, which is associated with CD14, leading to the release of pro-inflammatory mediators (Graveline et al., 2007). Activation of TLR2 and CD14 was also observed in murine brain parenchyma after exposure to *S. suis* (Domínguez-Punaro et al., 2007). Li et al. (2010) found that TLR2 and CD14 were elevated 2-fold and 3.4-fold, respectively, at the transcript level in spleen tissue following infection with highly pathogenic *S. suis*. Thus, Ly108 protein could play an important role in the host response to infection by *S. suis* in C57BL/6 mice.

Genes involved in phospholipid metabolism were up-regulated in splenic tissue obtained from C57BL/6 mice infected with strain HA9801. Phospholipids are the main constituents of biological membranes, and they have

important regulatory as well as structural functions in membranes. Phosphatase gene expression was up-regulated in C57BL/6 mouse spleen after strain HA9801 infection. Phosphatase activity may generate phosphatidylinositols (PIs) which have been found to play an important role in cellular signaling and intracellular trafficking (Krauss and Haucke.,2007). Phosphotidylinositols can be phosphorylated by phosphatidylinositol kinase, producing a variety of second messengers, such as inositol-1,4,5-triphosphate (IP3) and diglyceride (DG) (Manning and Cantley, 2007). Those second messengers can activate protein kinase thus activating many downstream signaling proteins that regulate cell survival and cell cycle progress. Consistent with this result, serine/threonine kinase and CDC-like kinase-1 gene expression were also elevated in the spleen of C57BL/6 mice after strain HA9801 infection. Protein kinase activates MAP-kinases and the NF-κB signaling pathway, which are closely related to tumor cell apoptosis, regulation and development (Liu and Xia.,2006). de Greeff et al. (2010) reported that NF-kB and MAP-kinase signaling pathways were induced upon interaction with SS2. In a study by Domínguez-Punaro et al. (2007), robust and rapid expression of TLR2, IkBa and CCL_2 (MCP-1) was evident in the choroid plexus of mice soon after they were infected with *S. suis*. Recent work by Wichgers Schreur et al. (2010) revealed that components released during *S. suis* infection, as well as in penicillin-treated whole bacteria, could induce NF-kB activation through TLR2/6. Thus, we speculate that signaling pathways associated with phospholipid metabolism could play an important role in defending against *S. suis* infection in C57BL/6 mice. It was also observed that the phosphatidylserine decarboxylase gene was highly expressed in the spleen of C57BL/6 mice following strain HA9801 infection. Phos-phatidylserine decarboxylase catalyzes phos-phatidylserine decarboxylation, generating phos-phatidylethanolamine in prokaryotes (Kanfer and Kennedy, 1964). So, the precise mechanism by which the phosphatidylserine decarboxylase gene was induced in the spleen of C57BL/6 HA9801 infected mice is open to further investigation.

The data indicate that genes involved in the immune response and in phospholipid metabolism are up-regulated to a greater extent in C57BL/6 mice splenic tissue than are similar genes in A/J mice splenic tissue following infection with *S. suis* strain HA9801. Although further investigation of the role that phospholipid metabolism signaling pathways play in protecting against *S. suis* infection may be needed, this study provides evidence that, besides the host immune response, phospholipid metabolism represents another important host defense mechanism against *S. suis* infection.

ACKNOWLEDGMENTS

This work were supported by the national major special project on new varieties cultivation for transgenic organisms (No. 2009ZX08009-154B) and Priority Academic Program Development of Jiangsu Higher Education Institutions. We are also grateful to Dr. William W. Riley for his critical reading of the manuscript

Abbreviations:

***S. suis*,** *Streptococcus suis*; **SS2,** *Streptococcus suis* serotype 2; **SSH,** suppression subtractive hybridization; **THB,** Todd-Hewitt broth; **PBS,** phosphate-buffered saline; **i.p.,** intraperitoneal; **TNF,** tumor necrosis factor; **IFN,** interferon; **TLR,** Toll-like receptor; **C3**, complement component 3; **SH2,** Src homology 2; **cDNA,** complementary deoxyribonucleic acid; **PCR,** polymerase chain reaction.

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