J. Microbiol. Biotechnol. (2015), 25(10), 1606–1613
http://dx.doi.org/10.4014/jmb.1504.04007

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Identification of *Actinobacillus pleuropneumoniae* Genes Preferentially Expressed During Infection Using *In Vivo*-Induced Antigen Technology (IVIAT)

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Received: April 3, 2015
Revised: June 3, 2015
Accepted: June 3, 2015

First published online
June 9, 2015

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pISSN 1017-7825, eISSN 1738-8872

Porcine pleuropneumonia is an infectious disease caused by *Actinobacillus pleuropneumoniae*. The identification of *A. pleuropneumoniae* genes, specially expressed *in vivo*, is a useful tool to reveal the mechanism of infection. IVIAT was used in this work to identify antigens expressed *in vivo* during *A. pleuropneumoniae* infection, using sera from individuals with chronic porcine pleuropneumonia. Sequencing of DNA inserts from positive clones showed 11 open reading frames with high homology to *A. pleuropneumoniae* genes. Based on sequence analysis, proteins encoded by these genes were involved in metabolism, replication, transcription regulation, and signal transduction. Moreover, three function-unknown proteins were also indentified in this work. Expression analysis using quantitative real-time PCR showed that most of the genes tested were up-regulated *in vivo* relative to their expression levels *in vitro*. IVI (*in vivo*-induced) genes that were amplified by PCR in different *A. pleuropneumoniae* strains showed that these genes could be detected in almost all of the strains. It is demonstrated that the identified IVI antigen may have important roles in the infection of *A. pleuropneumoniae*.

Keywords: *Actinobacillus pleuropneumoniae*, *in vivo*-induced genes, differential expression

Introduction

Actinobacillus pleuropneumoniae has a large effect on the pig-breeding industry. After being infected with this pathogen, severe hemorrhagic necrotizing pleuropneumonia is caused [29]. In addition, clinical symptoms, including lethargy, vomiting, fever, and abdominal breathing, can also be observed in infected animals. Typically, severe respiratory symptom is caused in acute type disease, which can lead to death in a short time. Alternatively, chronic infection may occur, where the bacterium causes a subclinical pleuropneumonia without obvious symptoms [23].

Historically, most of the research on this pathogen has focused on the study of it cultured *in vitro*; however, this at best gives understanding of only part of the virulence-associated characteristics. Generally, genes of a pathogen specifically expressed *in vivo* may play a significant part in infection. The putative anaerobic-associated protein *Dms*A is one of the identified *in vivo*-induced (IVI) gene that was up-regulated under anaerobic surrounding. The *Dms*A deletion mutant was proved to be attenuated in acute infection [3]. Several genes involved in survival in infected host tissue were identified by different methods such as selective capture of transcribed sequences (SCOTS) and signature-tagged mutagenesis (STM) [26]. Global anaerobic regulator *HlyX* was identified as an IVI gene by SCOTS [1]. It was also reported that survival of wild strain without *HlyX* in tissue was impaired [4]. Subtilisin-like autotransporter serine protease, *Aas*P, was also screened as an IVI gene by SCOTS. Follow-up studies revealed that *Aas*P transcript cannot be detected in aerobic *in vitro* environment, but in anaerobic tissue [1].

With the purpose of analyzing *A. pleuropneumoniae* pathogenic mechanisms, antigenic proteins potentially expressed within the host during infection were identified

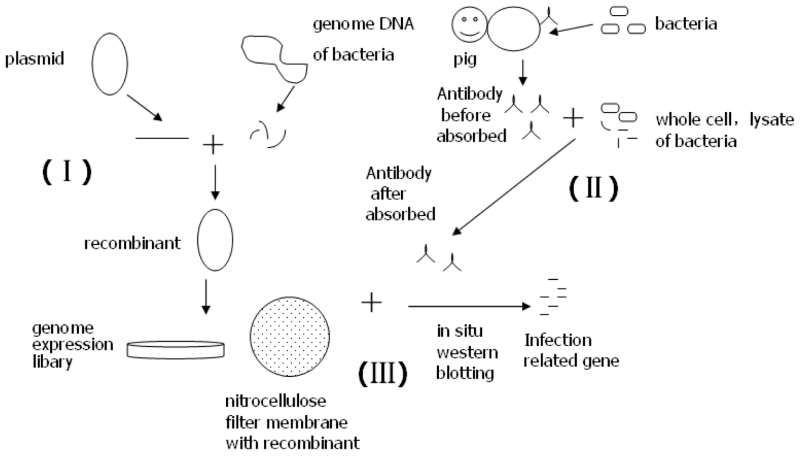

Fig. 1. Schematic representation of IVIAT: (I) construction of genome expression library, (II) preparation of screening serum, and (III) screening of *in vivo*-induced antigen.

applying *in vivo*-induced antigen technology (IVIAT) (Fig. 1) [9]. For the reason that *in vitro* research cannot clarify the physiology, metabolism, and regulation of gene expression in the host, as well as pathogenic mechanisms of the pathogen, IVIAT was widely used for various pathogens, including bacteria (intracellular bacteria and extracellular bacteria) [16, 21] and parasites [28].

In the present study, we applied IVIAT to indentify antigens of *A. pleuropneumoniae* expressed preferentially during infection. After screening the library against the adsorbed antisera, 28 positives were obtained and 11 single genes were identified by sequencing and bioinformatic analysis. The up-regulation of IVI genes *in vivo* during infection were confirmed by qRT-PCR.

Materials and Methods

Bacterial Strains and Growth Conditions

Strains of *A. pleuropneumoniae* were cultured in Tryptic soy broth (TSB) (Difco; USA) and Tryptic soy agar (TSA) (Difco) at 37°C and were both supplemented with NAD (10 μg/ml). *E. coli* strains DH5α and BL21 (DE3) were cultured in lysogeny broth (LB). Kanamycin (Tiangen, China) was added at 50 mg/ml when required.

Construction of a Genomic Expression Library of *A. pleuropneumoniae* L20

The freeze-dried *A. pleuropneumoniae* strain L20, placed in a −70°C refrigerator, was recovered at 37°C, overnight. The cultured L20 cells were detected and about 5×10^9 were centrifuged for genomic DNA extraction. The supernatant was discarded and genomic DNA was extracted from the sediment by use of a Bacterial DNA Kit (Omega Inc. USA). The extracted DNA was diluted and the concentration and purity were detected with a spectrophotometer (SmartSpec Plus; Bio-Rad, USA). Then the genomic DNA was partially digested using the enzyme *Sau*3AI (Thermo Inc, USA). The gene segments (0.5 to 1.5 bp) were separated, on a 0.9% agarose gel using a gel extraction kit (Omega Inc. USA), from the incomplete restricted digestion DNA products of *A. pleuropneumoniae* genome DNA. The isolated fragments were ligated into a pool of *Bam*HI-digested pET28a, pET28b, and pET28c plasmids (Novagen, USA) and the recombinant vectors were electroporated into competent *E. coli* DH5a and spread on kana-LB plates. The quality of library was tested by PCR amplification and restriction enzyme analysis. A total of 40,000 colonies were collected and the recombinant plasmids were extracted for the next step using plasmid Mini preps (Omega Inc.). Purified plasmids were stored at −80°C.

Preparation of Antisera

Six *A. pleuropneumoniae*-negative healthy 6-week-old piglets were infected *via* the intranasal route. The animals were infected with *A. pleuropneumoniae* L20 at a total dose of 5×10^8 CFU. This pig model was used for generating anti-APP pig antibodies for screening the L20 genomic library. Serum was obtained by precaval vein puncture and stored at −70°C. All animal experiments were processed according to national guidelines and were approved by the Office of Laboratory Animal, Sichuan Agricultural University. To reduce animal-to-animal variability, all of the sera obtained were pooled and mixed thoroughly. The mixed sera were then filtered and absorbed with whole cells and lysates of *A. pleuropneumoniae* L20, as well as *E. coli* BL21(DE3) [17]. The pooled serum was absorbed six times with each antigen. Reactivity to bacteria was detected by ELISA to ensure removal of antibody to *in vitro* expressed antigens.

In Vivo-Induced Antigen Screening

The plasmid DNA library was diluted successively and

transformed into *E. coli* BL21(DE3) to get 200–300 colonies on one plate after being incubated at 37°C overnight. The plasmid library was diluted according to previous optimized concentration and transformed into *E. coli* BL21 to get a batch plate with 200–300 colonies. The colonies on the plates were transferred onto an NC nitrocellulose membrane (Millionpore, Kent, UK), and then the membrane was placed on LB agar containing 30 μg/ml kanamycin and 1 mM isopropyl-β-D-thiogalactoside (Tiangen, China) with the colonies facing upward. After incubation at 37°C for 4 h, the membrane was exposed to chloroform vapor to lyse the cells. The next steps to screen the positive clones using western blot were carried out according to a previous study [17].

Isolation of RNA and RT-PCR

Four-week-old BALB/c mice (Dashuo, China) were used in this study. The experimental infection mice model was produced by intraperitoneal injection below lethal doses of *A. pleuropneumoniae*. Total RNA was extracted from the exponentially growing *A. pleuropneumoniae* (OD_{600} = 0.6) and *A. pleuropneumoniae*-infected lungs of mice using the Total RNA Extractor SK1312 (Sangon Biotech, China) according to the manufacturer protocol. In the process of RNA isolation, besides the reagent in the Total RNA Extractor kit, chloroform, isopropanol, and absolute ethyl alcohol were also needed. The integrality and contamination of RNA were detected by agarose gel electrophoresis (1.5% agarose, 1× TAE electrophoretic buffer solution). The absorption values of RNA at 260 and 280 nm were detected to assess the concentration and purification. An OD260/280 of RNA ranging from 1.8 to 2.0 was considered pure enough to use for the next operation. The RT-PCR was carried out according to the kit's manufacturer protocol (Sangon Biotech, China).

Relative Expression Level Analysis

qRT-PCR was processed on a ABI Stepone plus real-time PCR instrument using SYBR green I detection. The 16S rRNA gene was used as a control to normalized the results of the tested genes for relative quantification [15]. To make conclusions of trials more convincing, the *Gapdh* (housekeeping gene) gene was also used as another control. ApxIV is a known *in vivo*-induced protein described in a former study, which is widely used as an object to detect the infection of *A. pleuropneumoniae*. As a control, the expression of the ApxIV gene *in vitro* and *in vivo* was also tested. The specificity of reactions of all the sample was also tested by melting analysis. cDNA of each sample was diluted 8-fold for detection. In brief, 10 μl of 2× SYBR Green qPCR Master Mix (Sangon Biotech), 1 μl of each strand primer, 2 μl of template (cDNA), and 6 μl of ddH2O were added to the reaction tube and mixed as reaction mixture in 20 μl total volume. The primers of

Table 1. Primers used in this study.

Gene	Primer sequence (5′--3′)	Annealing temp. (°C)	Length of result	Source of primer
16srRNA-F	5′ GAACCTTACCTACTCTTGACATCCA 3′	59.0		This article
16srRNA-R	5′ TTCACAACACGAGCTGACGAC 3′	59.2	106 bp	This article
gapA-F	5′ TCAACTCACGGTCGTTTCAATG 3′	60.9		This article
gapA-R	5′ TTACGCGAATCGCTTTACCA 3′	59.7	79 bp	This article
ftsA-F	5′ TTTTGTGCTTACGGGTGGTG 3′	59.3		This article
ftsA-R	5′ TTGCCCTTTTCATCCGAGTT 3′	59.7	192 bp	This article
rnhB-F	5′ CTATCTCCTTTTATGACCGCTTGT 3′	59.3		This article
rnhB-R	5′ CACGCAACAATGCTCGCTAT 3′	59.8	128 bp	This article
ftsK-F	5′ TTCAGCGGAGGAGTATGTTGC 3′	60.3		This article
ftsK-R	5′ CGGATCTTGTAATTCGGTTGTG 3′	59.9	160 bp	This article
galU-F	5′ ATGCTTCTACCGTTTCTTGCTC 3′	58.4		This article
galU-R	5′ TCCGCAGGTATTTGGGATTTA 3′	59.9	109 bp	This article
galT-F	5′ TAAACGAAGCGGAACGAGC 3′	59.0		This article
galT-R	5′ AACGGCGGATAGAAATGGG 3′	59.9	169 bp	This article
malT-F	5′ TGTGCCATCAAATCACCTTCA 3′	59.5		This article
malT-R	5′ TCGCCTGTTACAAACTTCTGC 3′	58.7	192 bp	This article
HP-F	5′ AAGGCAGAAACCTCAACTCGT 3′	58.2		This article
HP-R	5′ AAACTGACTCGGCTCACAACAT 3′	58.9	80 bp	This article
hflX-Fn	5′ TAGTCCGTCACAAACCCGTAA 3′	58.2		This article
hflX-Rn	5′ GTAATTTTCCTTCGTGCGATCT 3′	58.3	116 bp	This article
Apx-F	5′ TCCGAACCGCTAACAGACA 3′	57		This article
Apx-R	5′ GCAACGACAGTAAGATTGAAGG 3′	57.1	132 bp	This article

genes were designed using the genes in the genomic DNA sequences of L20 (serovar 5b), which are available on the NCBI database. The eight gene forward and reverse primer sequences used are listed in Table 1. The reaction procedure of qRT-PCR was as follows: 95°C for 3 min; and 40 cycles of 95°C for 15 sec, 60°C for 40 sec, and 15 sec at 68°C. The data represent the fold changes in mRNA expression relative to the control (*in vitro*). All experiments were performed in triplicate. Blank controls were also included. The $2^{-\Delta\Delta CT}$ method was applied to evaluate the relative expression level.

Detection of 8 Selected Genes in 17 *A. pleuropneumoniae* Strains

Seventeen *A. pleuropneumoniae* strains were detected for the eight selected genes by PCR in this work. The primers used in this section were the same as that for qRT-PCR (Table 1). A 50 µl bacterium solution of each strain was transferred into 200 µl eppendorf tubes respectively and boiled for 10 min to lyse the cells. The PCR procedure consisted of 2 min at 95°C, followed by 34 cycles of 30 sec at 95°C, 30 sec at 54°C, 45 sec at 72°C, 10 min at 72°C, and 16°C thereafter. The results of PCR amplification were detected by agarose gel electrophoresis (1.5% agarose, 1× TAE electrophoretic buffer solution).

Results

Construction of the Genomic Expression Library of *A. pleuropneumoniae* L20

As is known, the necessary number of recombinants of the genomic expression library the study needed can be calculated as described in a previous study [17]. According to the formula, it is estimated that approximately 1.0×10^4 recombinants are required theoretically. In fact, approximately 3.6×10^4 recombinants were obtained. The results of PCR and restriction enzyme analysis demonstrated that more than 80% clones contained DNA inserts with the theoretical size. This indicated that this genomic library was qualified to be applied to the downstream experiments. Results of sequencing showed that there was a 100% similarity between the DNA inserts and the strain L20 gene fragment.

Sera Adsorption and Identification of Genes Identified by IVIAT

As shown in Fig. 2, a dramatic decline of the absorbance of the serum was observed after the reactivities of the pooled sera with each antigen by ELISA. Additionally, the biggest decline was detected after adsorption with the whole cells of L20 and BL21(DE3). Adsorbed thoroughly with all kinds of antigens, the absorbance was down to about 0.2 by ELISA. In the present study, approximately 20,000 clones were screened against adsorbed sera and 11 genes were identified after initial screening and twice

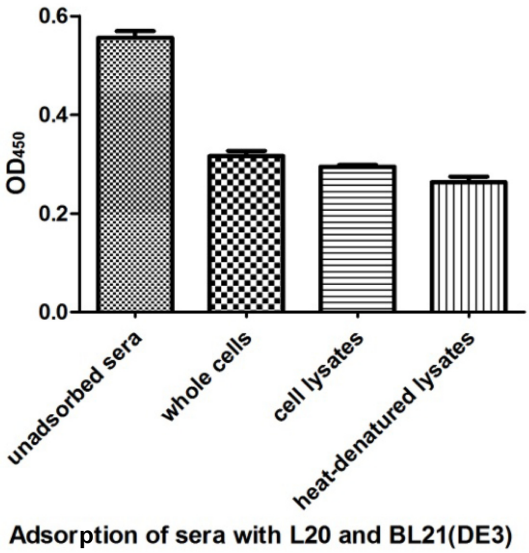

Fig. 2. ELISA results after each step in adsorption of pooled patients sera.
Pooled convalescent sera were sequentially adsorbed against whole cells, native cell lysates, and heat-denatured cell lysates of *in-vitro*-grown *A. pleuropneumoniae* L20 and *E. coli* BL21(DE3) containing native pET28a/b/c plasmid. In bar graphs, data are expressed as OD_{450} readings (means plus standard deviations) and corrected for background during adsorption. The standard deviations are presented from three independent experiments.

repeat screening by western blotting. The positive clones identified by IVIAT were detected by PCR using the pET-28 universal primers to determine whether the DNA fragments were inserted into the plasmid. The determined BL21(DE3) cells with positive plasmid were stored in glycerin at 4°C and sent to be sequenced (Sangon Biotech, China). The results of sequencing were analyzed by sequence alignment using the free online software BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). At last, 11 different single genes were obtained and all of them existed in the three whole-genome sequence, including L20, JL03, and AP76. Four genes indentified(APL_1166, *galU*, *galT*, *rnhB*), were predicted to be related to metabolism. The genes related to replication, transcription regulation, and signal transduction were screened as *in vivo*-induced genes. Moreover, some function-unknown genes were also obtained as in the previous study. The list of the screened genes are presented in Table 2. The localization of IVI antigen was processed by PSORTb ver. 3.0.2 (http://www.psort.org/psortb/).

Assessment of IVI Gene Expression *In Vivo* by qRT-PCR

The relative expression levels of IVI genes expressed *in*

Table 2. Results of screening of IVI genes.

Functional category	Gene locus tag	Gene name	Predicted gene product	Prediction of protein localization	Reference supports
Metabolism	APL_1166	_	Putative ubiquinone/menaquinone biosynthesis methyltransferase	Bacterial cytoplasm	
	APL_0651	*galU*	UTP--glucose-1-phosphate uridylyltransferase	Outer membrane or periplasmic protein	
	APL_0994	*galT*	Galactose-1-phosphate uridylyltransferase	Bacterial cytoplasm	[5, 14]
	APL_0129	*rnhB*	Ribonuclease HII	Bacterial inner membrane	[11, 12]
Replication, recombination, and repair	APL_0022	*ftsA*	Cell division protein FtsA	Bacterial inner membrane	[10, 25]
	APL_0618	*ftsK*	DNA translocase FtsK	Bacterial inner membrane	[20, 27]
Transcription regulation	APL_1233	*malT*	HTH-type transcriptional regulator	Bacterial cytoplasm	
Signal transduction	APL_1962	*hflX*	GTP-binding protein hflX	Bacterial cytoplasm	[21]
Function unknown	APL_1709	_	Hypothetical protein	Bacterial inner membrane	
	APL_1061	_	Hypothetical protein	Outer membrane or periplasmic protein	
	APL_1060	_	Hypothetical protein	Outer membrane or periplasmic protein	

vivo relative to *in vitro* were tested by qRT-PCR. Of the eight genes tested except *hflX*, the relative expression levels were up-regulated in mice lungs relative to cultured *in vitro* (Fig. 3). More than 3,000-fold up-regulation was obtained for *gal*T. More than 100-fold up-regulation were obtained in three other genes containing HP1709, *rnh*B, and *fts*K. The other three genes *mal*T, *gal*U, and *fts*A were not up-regulated as sharply as the four genes already mentioned, although a more than 10 fold up-regulation could be tested in each of them. Nevertheless, the expression of *hfl*X was not detected in all of the *A. pleuropneumoniae* infected lung samples. The relative expression trends of all of the genes tested were similar to one another, after normalized to the 16S rRNA gene and *Gapdh* gene. Moreover, the expression of the control gene *Apx*IV was up-regulated more than 100-fold. The results were further proved by the control gene.

Detection of 8 Selected Genes in 17 *A. pleuropneumoniae* Strains

In this section, we examined the distribution of eight selected genes in 17 *A. pleuropneumoniae* strains, including

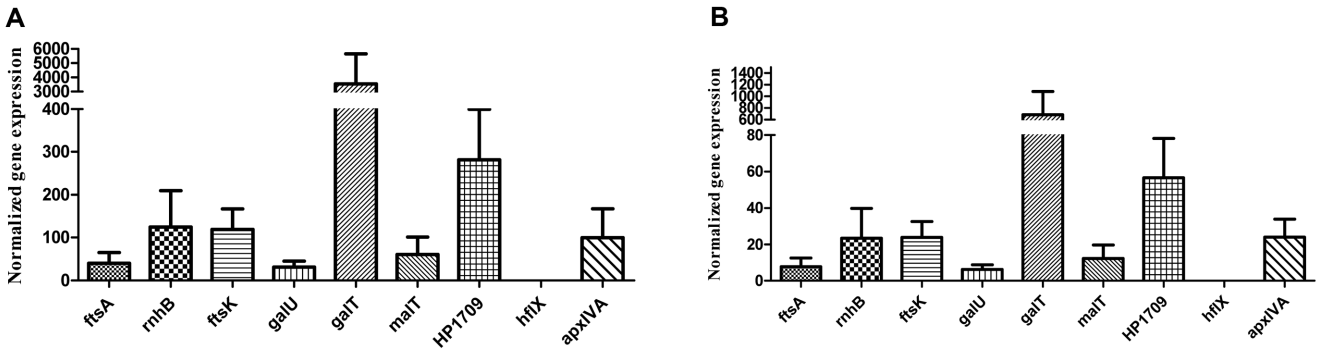

Fig. 3. qRT-PCR was used to measure the up-regulation of genes *in vivo* (mouse model) as compared with *in vitro* control (cultured in TSB).

The relative expression level of eight selected *in vivo*-induced genes were normalized to the 16S rRNA gene (**A**) and *Gapdh* gene (**B**). ApxIVA is a known *in vivo*-induced gene, which was also tested as a control gene in this study. The relative expression level *in vitro* of the nine genes are all designated one and the data presented in the figure represents relative expression level *in vivo* of the nine tested genes.

Table 3. Distribution of the 8 IVI genes in 17 *A. pleuropneumoniae* strains.

Strain	Serotype	Gene names							
		(*fts*A)	(*rnh*B)	(*fts*K)	(*gal*U)	(*gal*T)	(*mal*T)	APL_1709	(*hfl*X)
4074(ATCC27088)	1	+	+	+	+	+	+	+	+
S1536(ATCC27089)	2	+	+	+	+	+	+	+	+
S1421(ATCC27090)	3	+	+	+	+	+	+	+	+
M62(ATCC33378)	4	+	+	+	+	+	+	+	+
L20	5	+	+	+	+	+	+	+	+
WF83	7	+	+	+	+	+	+	+	+
D13039	10	_	+	+	_	+	+	+	+
MS71	1	+	+	+	+	+	+	+	+
MS31	1	+	+	+	+	+	+	+	+
MS32	1	+	+	+	+	+	+	+	+
MS33	1	+	+	+	+	+	+	+	+
MS34	1	+	+	+	+	+	+	+	+
MS51	1	+	+	+	+	+	+	+	+
MS52	1	+	+	+	+	+	+	+	+
MS53	1	+	+	+	+	+	+	+	+
MS54	1	+	+	+	+	+	+	+	+
GA16	7	+	+	+	+	+	+	+	+

some reference strains and those isolated recently from farms in Sichuan Province, China. PCR was employed to analyze the distribution of eight IVI genes in *A. pleuropneumoniae* strains. Seventeen APP strains were analyzed, and PCR results showed that the distribution ratio of these IVI genes were as follows (Table 3). Except genes *ftsA* and *galU*, which were not detected in reference strain D13039 (serotype 10), the rest of the selected genes were detected in all 17 strains.

Discussion

A. pleuropneumoniae remains a threat to the pig industry worldwide, causing significant economic losses. Survival of *A. pleuropneumoniae* depends on its ability to sense and adapt to a variety of environmental signals in host lungs. Therefore, the identification and characterization of genes expressed differentially in hosts might give insight of the colonization and survival of *A. pleuropneumoniae*. To identify *in vivo*-induced genes, different methods like STM [7, 26], *in vivo* expression technology (IVET) [8], and SCOTS [1, 2] were carried out and a lot of *in vivo* critical genes were identified.

IVIAT is a method that combines genome expression library construction and immunology, and widely applied to a lot of different microbes. *In vivo*-induced genes identified by IVIAT were proved to be useful for analyzing the pathogenic mechanism of microbes. Compared with the methods to identify *in vivo*-induced genes mentioned before, IVIAT is uncomplicated and with no need for animal models. This technology may be a huge supplement to the other methods and accelerate research processes of *A. pleuropneumoniae*.

Eleven genes were identified in this study by IVIAT. Sequencing and bioinformatic analysis results showed these genes belong to different functions such as metabolism, transcription regulation, replication, signal transduction, and functions-unknown (Table 2), and most of these genes have not been studied in *A. pleuropneumoniae* previously.

Proteins involved in biosynthesis and metabolism are essential for bacterial survival and growth *in vivo*. Using IVIAT, we identified four IVI genes that encode proteins involved in biosynthesis and metabolism (*galU*, *galT*, APL1166, *rnhB*). Of these genes, *galU*, *galT*, *rnhB* were selected to be analyzed for mRNA transcription level by qRT-PCR. Relative expression analysis demonstrated that all of the three genes were up-regulated *in vivo*. The relative expression levels of *galU*, *galT*, *rnhB* were up-regulated 30-fold, 3,541-fold, and 124-fold, respectively. Proteins encoded by these genes may play an important part for surviving *in vivo* surroundings. *galU* encodes the protein involved in LPS core biosynthesis, and is associated

with the adherence and the virulence of *A. pleuropneumoniae* [24]. GalU was proved to be virulence-associated in several animal-pathogenic bacteria since its product, UDP-glucose, is indispensable for the biosynthesis of virulence factors such as lipopolysaccharide and exopolysaccharide [18]. The *gal*U mutant was also proved to be associated with biofilm formation and autoagglutination [30]. In *E. coli*, *Klebsiella*, and *Salmonella* spp., galactose-1-phosphate uridyltransferase (*gal*T) is arranged in an operon induced by galactose [5]. Accumulation of galactose-1-phosphate in *gal*E or *gal*T mutants is lethal in *E. coli* [14]. However, study on *Erwinia amylovora* showed that a mutation in the *gal*T gene disabled galactose catabolism but did not affect amylovoran synthesis or virulence symptoms of mutant strains [22]. Multiple RNase H genes per genome is a general feature of both eukaryotic and prokaryotic organisms. In many pathogens, such as *E. coli* and *B. subtilis*, more than one RNase H were reported[11, 12].

In this study, genes encoding protein involved in cell division were identified, including cell division *ftsA* and DNA translocase *ftsK*. Differences of mRNA expression of the two identified genes were analyzed by qRT-PCR and the results confirmed the two genes were up-regulated in infected tissue. Expression levels of *ftsA* and *ftsK* were up-regulated 39-fold and 119-fold, respectively. In a previous study, the *ftsA* gene was identified as an *in vivo* expression gene in *Borrelia burgdorferi* during an active murine infection by IVET [6]. Cell division protein encoded by *fts*A, predicted to belong to the actin family, is one of the core proteins involved in cell division [10]. Previous study on *fts*A mutants demonstrated that inactivation of *fts*A causes extensive damage to division [25]. It is reported that FtsK is a septum-located DNA translocase, involved in cell division and chromosome segregation with cell division [20, 27].

Gene *mal*T is the only transcriptional regulator identified in the present study. In this study, the relative expression analysis indicated that the expression of *mal*T gene in infected mice lungs was about 60-fold higher than *in vitro*. This suggests *mal*T may play an important part in the process of infection. Mutant of *mal*T (transcriptional regulator) gene in *A. pleuropneumoniae* is attenuated in its growth rate. Analysis of a stringent type transcript profile in the *mal*T mutant demonstrated that *mal*T may be associated directly or indirectly with stringent response [19].

Using IVIAT, *Hfl*X was already identified as an IVI gene in *E. coli* O157:H7 during human infection [13]. GTP-binding protein was also identified as an *in vivo*-expressed gene in the transcription analysis of *B. burgdorferi in vivo* [6]. However, gene *hflX* was not detected by qRT-PCR *in vivo* in the present study.

Three functions-unknown genes, numbered apl_1709, apl_1060, and apl_1061, were also identified as IVI genes. Transcription analysis of the gene expression of apl_1709 by real-time quantitative RT-PCR showed that the relative expression level of that *in vivo* was 1,000-fold higher compared with that *in vitro*. Compared with the other analyzed genes, the amplification of the relative expression level of apl_1709 was the highest one. Apl_1709 may be essential for the course of *A. pleuropneumoniae* infection and further study should be carried out to analyze the function of apl_1709.

PCR amplification results of the selected eight IVI genes among *A. pleuropneumoniae* reference stains and farm isolates showed that almost all the eight genes were detected in the tested strains, except *fts*A and *gal*U in strain D13039 (Table 3). These results showed that most of the IVI genes existed in almost all of the pathogens, consistent with previous reports.

In summary, we have identified 11 *A. pleuropneumoniae* antigens expressed *in vivo*. Our results confirmed eight already known virulence factors and suggested three new potential virulence factors. Additionally, we confirmed IVI genes using *A. pleuropneumoniae* infection in a mouse model. Nevertheless, further studies on the newly identified IVI genes are necessary to advance our knowledge of their role in infection.

Acknowledgments

This study was supported by a grant from the Special Fund for Agro-Scientific Research in the Public Interest (No. 201303034) and a grant from the Special Fund for Sichuan Science and Technology Support Program (No. 2013NZ0056).

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