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Differential Gene Expression in the Pathogenic Strains of *Actinobacillus pleuropneumoniae* Serotypes 1 and 3

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The limited information on differential gene expression in the different serotypes of *Actinobacillus pleuropneumoniae* has significantly hampered the research on the pathogenic mechanisms of this organism and the development of multivalent vaccines against *A. pleuropneumoniae* infection. To compare the gene expressions in the *A. pleuropneumoniae* strains CVCC259 (serotype 1) and CVCC261 (serotype 3), we screened the differentially expressed genes in the two strains by performing representational difference analysis (RDA). Northern blot analyses were used to confirm the results of RDA. We identified 22 differentially expressed genes in the CVCC259 strain and 20 differentially expressed genes in the CVCC261 strain, and these genes were classified into 11 groups: (1) genes encoding APX toxins; (2) genes encoding transferrin-binding protein; (3) genes involved in lipopolysaccharide (LPS) biosynthesis; (4) genes encoding autotransporter adhesin; (5) genes involved in metabolism; (6) genes involved in the ATP-binding cassette (ABC) transporter system; (7) genes encoding molecular chaperones; (8) genes involved in bacterial transcription and nucleic acid metabolism; (9) a gene encoding protease; (10) genes encoding lipoprotein/membrane protein; and (11) genes encoding various hypothetical proteins. This is the first report on the systematic application of RDA for the analysis of differential gene expression in *A. pleuropneumoniae* serotypes 1 and 3. The determination of these differentially expressed genes will serve as an indicator for future research on the pathogenic mechanisms of *A. pleuropneumoniae* and the development of a multivalent vaccine against *A. pleuropneumoniae* infection.

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Porcine contagious pleuropneumonia, caused by *Actinobacillus pleuropneumoniae*, is an extremely contagious and often fatal respiratory disease characterized by necrotic and hemorrhagic lung lesions, coughing, and severe respiratory distress [26]. This disease is found in the countries that have a swine industry, and it is responsible for enormous economic losses to the swine industry. To date, 15 serotypes of *A. pleuropneumoniae* have been described [3]. All serotypes are capable of causing disease, but they show significant differences in pathogenicity and immunogenicity [15, 25]. Therefore, vaccines raised against a specific serotype do not confer protection against infection by other serotypes [7, 25]. Owing to the limited information on the differential gene expression among the serotypes, studies on the pathogenic mechanisms of different serotypes and development of multivalent genetically engineered vaccines have been significantly hampered. Therefore, the differential gene expression among the principal serotypes should be identified and suitably exploited.

A. pleuropneumoniae serotypes 1 and 3 show the most significant variation in pathogenicity [15]. Serotype 1 is highly virulent, and infection of this serotype is associated with epidemic outbreak, high mortality, and severe lung lesions. However, serotype 3 is considered to be less virulent [4, 10]. Moreover, the two serotypes show significantly different immunogenicities, and the available vaccines for the 2 serotypes do not provide cross-protection [7, 35].

Representational difference analysis (RDA) is a polymerase chain reaction (PCR)-coupled subtractive hybridization technique that was originally designed to identify differences

between genomic DNA populations [24]. Subsequently, RDA has become a powerful tool that can be used to isolate both up- and downregulated genes in two different cDNA populations, thereby enabling identification of differentially expressed genes [30]. Therefore, in this study, we used cDNA-based RDA to identify the differentially expressed genes in the *A. pleuropneumoniae* pathogenic strains CVCC259 (serotype 1) and CVCC261 (serotype 3) and lay the foundation for further research on the pathogenic mechanisms of the different serotypes of *A. pleuropneumoniae* and the development of new multivalent vaccines.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

A. pleuropneumoniae strains CVCC259 (serotype 1) and CVCC261 (serotype 3) were purchased from China Institute of Veterinary Drug Control and cultured in brain heart infusion (BHI; Difco Laboratories, U.S.A.) containing nicotinamide adenine dinucleotide (NAD; 10 μg/ml; Sigma, U.S.A.) at 37°C for 5 h with shaking at 150 rpm. The RDA library was constructed in strain DH5α (TaKaRa, Japan) by using the pGEM T-Easy vector system (Promega, USA).

RNA Isolation and cDNA Synthesis

Total RNA from *A. pleuropneumoniae* strains CVCC259 (serotype 1) and CVCC261 (serotype 3) was extracted by using the RNeasy minikit (Qiagen, U.S.A.), and cDNA was prepared using the M-MLV RTase cDNA Synthesis kit (TaKaRa, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from 800 ng of RNA by using reverse transcriptase (M-MLV; TaKaRa, Japan). A 20-μl aliquot of first-strand cDNA was used as the template for second-strand synthesis. The double-stranded cDNA was subsequently purified using the Qiaquick PCR purification kit (Qiagen, U.S.A.).

Representational Difference Analysis

Representational difference analysis was performed according to a previously described procedure [22, 24]. We used the following adapters and primers for RDA (listed in Table 1): R-Bgl 12/R-Bgl 24, J-Bgl 12/J-Bgl 24, and N-Bgl 12/N-Bgl 24. Briefly, the cDNA fragments were digested with *Sau*3AI restriction enzyme (TaKaRa, Japan), and the resultant products were purified using the Qiaquick PCR purification kit (Qiagen, U.S.A.). The R-Bgl 12/R-Bgl 24 adapters were ligated to the digested DNA to be used as the tester. The first differential product (DP1) was obtained by performing hybridization (20 h at 67°C) with a driver:tester ratio of 100:1, which was followed by PCR amplification with the R-Bgl 24 primer. The second (DP2) and third (DP3) differential products were obtained by ligating the N-Bgl and J-Bgl adapters to the tester in the second and third rounds of subtractive hybridization; the driver:tester ratios for these two rounds of subtractive hybridization were 400:1 and 8,000:1, respectively. To obtain a subtracted cDNA library for the *A. pleuropneumoniae* strain CVCC259, cDNAs from CVCC259 were used as the testers and cDNAs from CVCC261 were used as the drivers; this combination of differentially expressed genes was designated as "c." To obtain a subtracted cDNA library of the *A. pleuropneumoniae* strain CVCC261, cDNAs from CVCC261 were used as the testers and cDNAs from CVCC259 were used as the drivers; this combination of differentially expressed genes was designated as "d."

Table 1. Primers used for RDA.

Primers	Sequence	Source
R-Bgl 24	5'-AGCACTCTCCAGCCTCTCACCGCA-3'	[24]
R-Bgl 12	3'-AGTGGCGTCTAG-5'	
	Primer pair to generate RDA adaptor	
J-Bgl 24	5'-ACCGACGTCGACTATCCATGAACA-3'	[24]
J-Bgl 12	3'-GTACTTGTCTAG-5'	
	Primer pair to generate RDA adaptor	
N-Bgl 24	5'-AGGCAACTGTGCTATCCGAGGGAA-3'	[24]
N-Bgl 12	3'-GCTCCCTTCTAG-5'	
	Primer pair to generate RDA adaptor	
recFF	5'-TTTCCGAAATCTCCAATCTCTC-3'	This study
recFP	5'-TTCGCTTTCTTGCCATTGC-3'	
	Primer pair for amplification of *recF* gene fragment	

Cloning and Sequencing of the Differentially Expressed Genes

The final RDA PCR products were column purified with the Qiaquick PCR purification kit (Qiagen, U.S.A.) and ligated into the pGEM-T vector (Promega, U.S.A.) to produce pGEM-TDP3 plasmids. *Escherichia coli* DH5α cells were transformed with the plasmids using the pGEM T-Easy Vector System, and the procedure was performed according to the protocol provided by the manufacturer. Plasmid isolation was performed by growing the *E. coli* DH5α cells containing the pGEM-TDP3 vector in 5 ml of Luria–Bertani medium supplemented with ampicillin (100 μg/ml); this culture was incubated overnight at 37°C with constant agitation. The inserts were sequenced using the T7 or M13 reverse primers supplied by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (http://www.sangon.com/). The detected sequences were compared with those in the GenBank database by using the BLASTN program.

Reverse Northern Hybridization Analysis

The differentially expressed genes were confirmed using a novel reverse Northern hybridization approach, which was performed according to a previously described protocol [20, 22]. The differentially expressed genes from each of the sequenced clones were amplified using the J-Bgl 24 primer, successively spotted onto a nylon membrane, and cross-linked to the membrane by baking the membrane for 30 min at 120°C. The probes were prepared using 6 μg of the *Sau*3AI-digested genomic DNA and cDNA obtained from the CVCC259 and CVCC261 strains. The probes were separately labeled using digoxigenin (DIG)-High Prime (Roche, Germany). Nonradioactive labeling, hybridization, and detection were performed using the DIG-High Prime DNA Labeling and Detection Starter Kit (Roche, Germany) according to the manufacturer's instructions. Since all the amplified differentially expressed genes contained the J-Bgl 24 primer, the J-Bgl 24 primer was considered as the negative control. The DIG-labeled control DNA supplied in the kit was considered as the positive control in the Southern blot analysis. The housekeeping gene *recF* was considered as the positive control in Northern blot analysis [28]. To

determine the relative expression level of each differentially expressed gene, the density ratios of the corresponding dots were measured using the spot densitometry analysis tool of an Alpha Imager HP imaging system (Alpha Innotech Corp., U.S.A.) after normalization using the spot intensity for the housekeeping gene.

GenBank

The nucleotide sequences of the 42 differentially expressed genes reported in this study have been deposited in the GenBank database under accession numbers GR930350–GR930391.

RESULTS

Construction of *A. pleuropneumoniae* cDNA RDA Libraries

To identify the genes that are differentially expressed in the *A. pleuropneumoniae* strains CVCC259 (serotype 1) and CVCC261 (serotype 3), we adapted the RDA subtractive technology that has been successfully used for such studies in other systems [1]. The CVCC259 and CVCC261 strains were cultured in BHI medium for 6 h; RNA was extracted, and double-stranded cDNA was prepared using the RNA. The sizes of the cDNA fragments obtained after digestion with *Sau*3AI were approximately 100–800 bp, and the digested products were ligated with an R-adaptor for RDA (data not shown). The differentially expressed genes in the CVCC259 and CVCC261 strains were identified using RDA. After the first, second, and third rounds of subtractive hybridization, the differential expressions of these genes became increasingly apparent. The products of the third round of subtractive hybridization were analyzed by neutral polyacrylamide gel electrophoresis. We detected sequences with sizes of approximately 150–700 bp and obtained clear bands of gene fragments (Fig. 1).

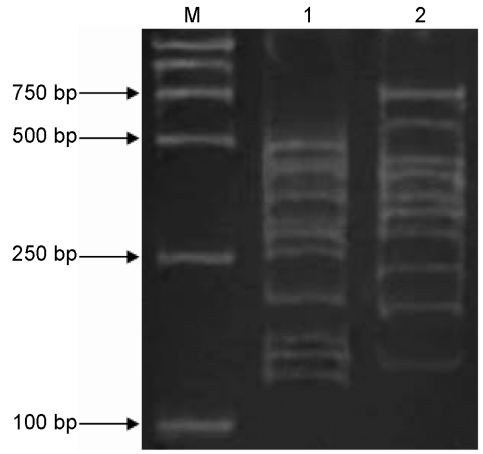

Fig. 1. The differential products of cDNA RDA.
Lane M indicates the DL2000 DNA Marker; lane 1 indicates the third differential products (DP3) of CVCC259 cDNA; and lane 2 indicates the third differential products (DP3) of CVCC261 cDNA.

Cloning and Sequence Analysis of the Differentially Expressed Genes

The RDA products were ligated onto the pGEM-T vector, and *E. coli* DH5α cells were transformed using this vector. The cells containing an RDA product insert were selected by PCR and sequenced. The sequences were searched against the GenBank database, European Molecular Biology Laboratory (EMBL) database, DNA databank of Japan (DDBJ), and Protein database (PDB) for identification. A total of 22 differentially expressed genes were screened from the CVCC259 strain (Table 2), and 20 differentially expressed genes were screened from the CVCC261 strain (Table 3).

Reverse Northern Hybridization Analysis

To corroborate the RDA results, we initially performed reverse Southern hybridization to determine whether the differentially expressed genes were derived from the *A. pleuropneumoniae* chromosome. All the 22 gene fragments screened from the CVCC259 strain were able to hybridize with the genomic DNA probes from strain CVCC259 and produced 22 blots with strongly positive hybridization (Fig. 2A). Furthermore, all the 20 gene fragments screened from the CVCC261 strain were able to hybridize with the genomic DNA probes from strain CVCC261 and produced 20 blots with strongly positive hybridization (Fig. 2B).

Then, we performed reverse Northern hybridization to determine whether the differentially expressed genes were derived from the *A. pleuropneumoniae* cDNA, and the levels of differential expression were quantified after normalization using the levels of the housekeeping gene. The 22 gene fragments screened from the CVCC259 strain were able to hybridize with the cDNA probes from the CVCC259 strain and produced 22 blots with strongly positive hybridization (Fig. 3A). However, we did not observe any strongly positive hybridization blots in the hybridization experiment performed using the 22 gene fragments screened from the CVCC259 strain and cDNA probes from the CVCC261 strain, which produced 22 blots with weakly positive hybridization (Fig. 3B). The expression levels of these 22 gene fragments in the CVCC259 strain were upregulated in comparison with their expression levels in the CVCC261 strain (Fig. 3C). All the 20 gene fragments screened from the CVCC261 strain were able to hybridize with the cDNA probes from the CVCC261 strain and produced strongly positive hybridization blots (Fig. 4A). However, in the hybridization experiment conducted using the 20 gene fragments screened from the CVCC261 strain and cDNA probes from the CVCC259 strain, we observed negative hybridization or weakly positive hybridization blots (Fig. 4B). The expression levels of these 20 gene fragments in the CVCC261 strain were upregulated in comparison with their expression levels in the CVCC259 strain (Fig. 4C).

Table 2. Homology sequence search results for differentially expressed genes from *A. pleuropneumoniae* strain CVCC259.

Clone no.	Fragment size	Homologous genes and protein function	Homologous fragment size	Identities	Accession no.
APX toxins					
c12	284	*apxIA* for Apx I toxin determinant A	284/285	99%	GR930354
c28	256	*apxIA* for Apx I toxin determinant A	256/256	100%	GR930357
c49	145	*apxID* for Apx I toxin secretion component	145/145	100%	GR930362
c79	403	*apxIB* for Apx I toxin translocation ATP-binding protein	403/404	99%	GR930366
Involved in lipopolysaccharide (LPS) biosynthesis					
c30	227	Putative O-antigen biosynthesis gene	227/227	100%	GR930358
c34	195	Putative O-antigen biosynthesis gene	195/196	99%	GR930359
Transferrin-binding protein					
c9	289	*tbpB* for transferrin-binding protein B	289/289	100%	GR930353
c86	244	*tbpB* for transferrin-binding protein B	244/244	100%	GR930368
Adhesin					
c67	332	Autotransporter adhesin	310/335	92%	GR930371
c85	415	Autotransporter adhesin	384/415	92%	GR930367
c100	373	Autotransporter adhesin	269/336	80%	GR930369
Involved in ATP-binding cassette (ABC) transporter system					
c42	323	Hypothetical ABC transporter ATP-binding protein	322/323	99%	GR930361
c55	303	Putative ABC transporter permease protein	302/303	99%	GR930363
Involved in metabolism					
c37	152	Carbonic anhydrase	152/152	100%	GR930360
c58	286	*maeB* for NADP-dependent malic enzyme	283/286	98%	GR930364
c76	201	*lysC* for lysine-sensitive aspartokinase 3	201/201	100%	GR930365
Molecular chaperone					
c7	149	*slyD* for FKBP-type peptidyl-prolyl *cis–trans* isomerase SlyD	132/144	91%	GR930352
c17	146	*hslR* for heat-shock-protein-15-like protein	146/146	100%	GR930356
Involved in bacterial transcription and nucleic acid metabolism					
c3	216	*udp* for uridine phosphorylase	213/216	98%	GR930351
c14	148	*trmD* for tRNA (guanine-*N*1)-methyltransferase	148/148	100%	GR930355
Protease					
c33	220	*clpP* for ATP-dependent Clp protease proteolytic subunit	204/204	100%	GR930370
Hypothetical protein					
c2	350	Hypothetical protein	350/350	100%	GR930350

Analysis of the Differentially Expressed Genes from the CVCC259 and CVCC261 Strains

The homologies of these differentially expressed genes were determined by searches in the GenBank, EMBL, DDBJ, and PDB databases. Although genome sequencing of *A. pleuropneumoniae* serotype 1 has not been completed, the 22 differentially expressed genes of the CVCC259 strain were found to show high homologies with the corresponding sequences of the *A. pleuropneumoniae* (Table 2). Furthermore, the 20 differentially expressed genes of the CVCC261 strain were also found to show high homologies with the corresponding sequences of the *A. pleuropneumoniae* strain JL03 (serotype 3), except d36, for which genome sequencing has been completed (Table 3).

DISCUSSION

Although genome sequencing of *A. pleuropneumoniae* strain JL03 (serotype 3) has been completed, the differences between gene expression in serotypes 1 and 3 have not been identified. In the current experiment, we used the

Table 3. Homology sequence search results for differentially expressed genes from *A. pleuropneumoniae* strain CVCC261.

Clone no.	Fragment size	Homologous genes and protein function	Homologous fragment size	Identities	Accession No.
APX toxins					
d8	345	*apxIIIA* for ApxIII toxin determinant A	344/345	99%	GR930373
d94	629	*apxIIIA* for ApxIII toxin determinant A	629/630	99%	GR930383
Involved in lipopolysaccharide (LPS) biosynthesis					
d104	316	*glf* for UDP-galactopyranose mutase	316/317	99%	GR930384
d106	467	*wzz* for Wzz protein	467/467	100%	GR930385
d119	494	*rafG* for lipopolysaccharide biosynthesis glycosyltransferase	494/495	99%	GR930389
d40	540	Glycosyltransferase	539/540	99%	GR930379
d16	342	Putative sugar transferase	342/342	100%	GR930377
Involved in metabolism					
d86	298	*dhal* for PTS-dependent dihydroxyacetone kinase, ADP-binding subunit DhaL	248/248	100%	GR930382
d1	327	*cysD* for sulfate adenylate transferase subunit 2	326/327	99%	GR930372
d9	275	*cysG* for uroporphyrinogen-III methylase	274/275	99%	GR930374
d108	295	*cysH* for phosphoadenosine phosphosulfate reductase	295/295	100%	GR930386
Lipoprotein/membrane protein					
d10	127	*hlpB* for lipoprotein HlpB	65/65	100%	GR930375
d57	391	*tolA2* for colicin import membrane protein	391/391	100%	GR930381
Hypothetical protein					
d36	96	*Mycoplasma hyorhinis* isolate N1 virulence specific noncoding region genomic sequence	29/29	100%	GR930378
d11	152	Hypothetical protein	151/153	98%	GR930376
d43	474	Hypothetical protein	474/474	100%	GR930380
d112	177	Hypothetical protein	177/177	100%	GR930387
d118	465	Hypothetical protein	465/466	99%	GR930388
d141	422	Hypothetical protein	422/422	100%	GR930390
d162	171	Hypothetical protein	171/171	100%	GR930391

RDA method to screen differentially expressed genes of the CVCC259 (serotype 1) and CVCC261 (serotype 3) strains. The differentially expressed genes of the two pathogenic *A. pleuropneumoniae* strains can be classified into the following groups: (1) genes encoding APX toxins, including *apxIA*, *apxIB*, *apxID*, and *apxIIIA*; (2) genes encoding the transferrin-binding protein (*i.e.*, *tbpB*); (3) genes involved in lipopolysaccharide (LPS) biosynthesis, including a putative O-antigen biosynthesis gene, *glf*, *wzz*, *rafG*, a gene encoding glycosyltransferase, and a putative sugar tranferase-

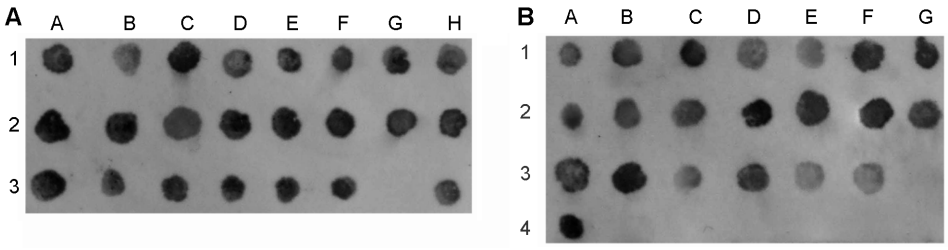

Fig. 2. Southern blot of the differentially expressed genes.
Panel A indicates the spots corresponding to the hybridization of the 22 differentially expressed genes of CVCC259 with the genomic DNA probes from CVCC259: A1: c2; B1: c3; C1: c7; D1: c9; E1: c12; F1: c14; G1: c17; H1: 28; A2: c30; B2: c33; C2: c34; D2: c37; E2: c42; F2: c49; G2: c55; H2: c58; A3: c67; B3: c76; C3: c79; D3: c85; E3: c86; F3: c100; G3: Negative control (J- Bgl 24); H3: positive control (control DNA from kit). Panel B indicates the spots corresponding to the hybridization of the 20 differentially expressed genes of CVCC261 with the genomic DNA probes from CVCC261: A1: d1; B1: d8; C1: d9; D1: d10; E1: d11; F1: d16; G1: d36; A2: d40; B2: d43; C2: d57; D2: d86; E2: d94; F2: d104; G2: d106; A3: d108; B3: d112; C3: d118; D3: d119; E3: d141; F3: d162; G3: Negative control (J- Bgl 24); A4: positive control (control DNA from kit).

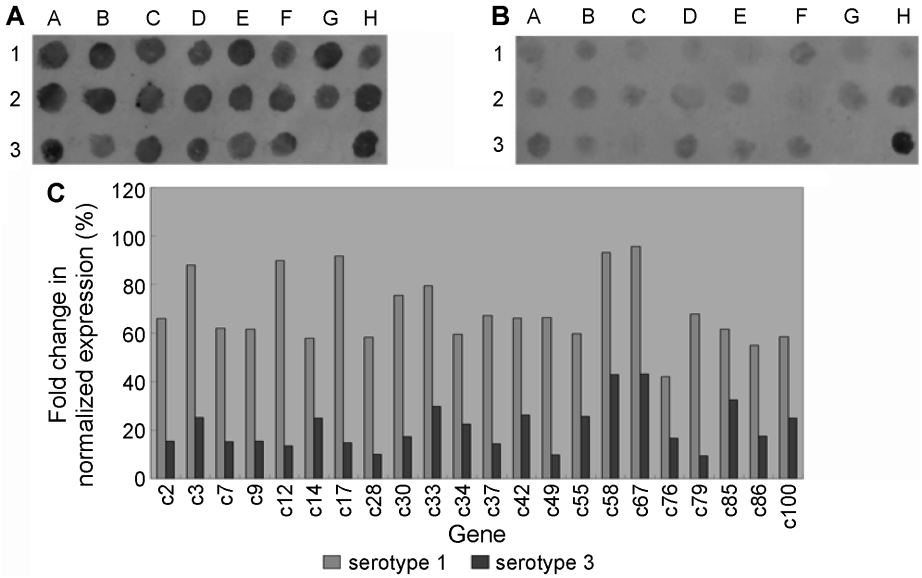

Fig. 3. Northern blot of the differentially expressed genes from CVCC259.
Panel A indicates the spots corresponding to the hybridization of the 22 differentially expressed genes of CVCC259 with the cDNA probes from CVCC259; panel B indicates the spots corresponding to the hybridization of the 22 differentially expressed genes of CVCC259 with the cDNA probes from CVCC261; panel C indicates the relative expression levels of the 22 differentially expressed genes in CVCC259 and CVCC261 after normalization using the spot intensity for the *recF* gene. A1: c2; B1: c3; C1: c7; D1: c9; E1: c12; F1: c14; G1: c17; H1: 28; A2: c30; B2: c33; C2: c34; D2: c37; E2: c42; F2: c49; G2: c55; H2: c58; A3: c67; B3: c76; C3: c79; D3: c85; E3: c86; F3: c100; G3: Negative control (J- Bgl 24); H3: positive control (*recF* gene).

encoding gene; (4) genes encoding adhesin (*i.e.*, autotransporter adhesin); (5) genes involved in metabolism, including *maeB*, *lysC*, *dhal*, *cysD*, *cysG*, *cysH*, and a gene encoding carbonic anhydrases; (6) genes involved in the ATP-binding cassette (ABC) transporter system; (7) genes encoding molecular chaperones, including *hslR* and *slyD*; (8) genes involved in bacterial transcription and nucleic acid metabolism, including *trmD* and *udp*; (9) a gene

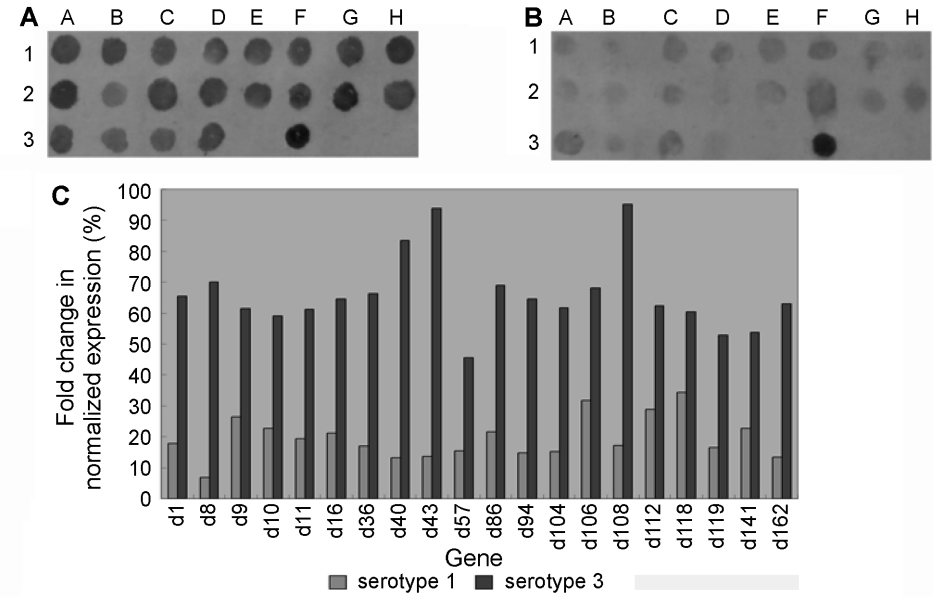

Fig. 4. Northern blot of the differentially expressed genes from CVCC261.
Panel A indicates the spots corresponding to the hybridization of the 20 differentially expressed genes of CVCC261 with the cDNA probes from CVCC261; panel B indicates the spots corresponding to the hybridization of the 20 differentially expressed genes of CVCC261 with the cDNA probes from CVCC259; panel C indicates the relative expression levels of the 20 differentially expressed genes in CVCC259 and CVCC261 after normalization using the spot intensity for the *recF* gene. A1: d1; B1: d8; C1: d9; D1: d10; E1: d11; F1: d16; G1: d36; H1: d40; A2: d43; B2: d57; C2: d86; D2: d94; E2: d104; F2: d106; G2: d108; H2: d112; A3: d118; B3: d119; C3: d141; D3: d162; E3: Negative control (J- Bgl 24); F3: positive control (*recF* gene).

encoding protease (*i.e.*, *clpP*); (10) genes encoding lipoprotein/membrane proteins, including *hlpB* and *tolA2*; and (11) genes encoding various hypothetical proteins.

Apx toxins and transferring-binding protein (TbpB) are the major pathogenic virulence factors in *A. pleuropneumoniae* [12, 18]. In our study, we found that the *apxIA*, *apxIB*, and *apxID* genes were specifically expressed in the CVCC259 strain, whereas the *apxIIIA* gene was specifically expressed in the CVCC261 strain. Our results were in agreement with those of a previous report [18]. The ApxI toxin has strong hemolytic activity and cytotoxicity, which may be the primary factors responsible for the stronger virulence of serotype 1. However, the ApxIII toxin has no hemolytic activity, and the absence of the *apxI* operon in serotype 3 is probably the most important factor responsible for the lower virulence of this serotype. Additionally, we screened two gene fragments of the *tbpB* gene from the *A. pleuropneumoniae* strain CVCC261. These results were in agreement with those of a previous study that proposed that TbpB is antigenically distinct among different *A. pleuropneumoniae* serotypes [12].

In Gram-negative bacteria, LPS is an important structural component of the cell membrane and is considered to be a virulence determinant and an immunodominant molecule [17]. LPS is composed of three components: the lipid A, the core oligosaccharide, and the polysaccharide O-antigen [33]. In this study, we screened two gene fragments of a putative O-antigen biosynthesis gene that were specifically expressed in the CVCC259 strain. The O-antigen plays a critical role in the bacterium–host interaction that provides protection from the host defenses [14, 21]. Genomic comparison of the sequenced *A. pleuropneumoniae* strains also revealed serotype-based variability in the genes involved in O-antigen synthesis [38]. Furthermore, we also screened five genes that encoded enzymes involved in lipopolysaccharide metabolism and were specifically expressed in the CVCC261 strain: *glf*, *wzz*, *rafG*, a glycosyltransferase-encoding gene, and a putative sugar transferase-encoding gene. The *glf* gene encodes UDP-galactopyranose mutase, which is a key enzyme of galactofuranose metabolism, thereby controlling the biosynthesis of galactomannan and galactofuranose containing glycoconjugates [36]. Targeted gene deletion of the *glf* gene attenuates the virulence of the organism [19, 36]. The *wzz* gene encodes the Wzz protein, which regulates the length of the O-antigen chain attached to LPS and plays an important role in the virulence of several bacteria [21, 34]. Glycosyltransferase was also involved in the virulence of several bacteria [5, 9, 27]. Therefore, we speculate that these genes may be previously unidentified virulence factors of *A. pleuropneumoniae* and may provide new avenues for the development of an *A. pleuropneumoniae* vaccine.

Adhesion to host cells is a critical step in the pathogenesis of infectious microorganisms. We screened three gene sequences of autotransporter adhesion from strain CVCC259, and these sequences showed 80–92% homology with the annotated autotransporter-adhesin gene in the genome of *A. pleuropneumoniae* strain AP76 (serotype 7) (GenBank Accession No. CP001091.1). Members of the autotransporter-adhesin family are important virulence factors in several Gram-negative pathogens, and these adhesins mediate adherence to eukaryotic cells [13]. Furthermore, a gene with high homology to autotransporter adhesin was upregulated when the *A. pleuropneumoniae* was exposed to porcine lung epithelial cells. The autotransporter adhesin of *Bordetella pertussis*, pertactin (PRN), is used as a component of the commercial multivalent vaccine [16]. Therefore, the autotransporter adhesin of *A. pleuropneumoniae* strain CVCC259 may be a new virulence factor that is involved in adhesion and colonization of *A. pleuropneumoniae*, and it may serve as an effective component of a multivalent vaccine.

ATP-binding cassette transporters include membrane-transport proteins that couple the energy derived from ATP hydrolysis to the translocation of solutes across biological membranes [31]. We screened one fragment of a gene encoding a hypothetical ABC transporter protein and one fragment of a gene encoding a putative ABC transporter permease protein, both of which were specifically expressed in CVCC259. In bacteria, the ABC transporters are important virulence factors because they play roles in secretion of toxins [8] and may be suitable targets for the development of antibacterial vaccines, which can be achieved either through the development of live attenuated bacteria or through the development of protein- and DNA-based subunit vaccines [11]. Therefore, we speculated that these genes may serve as potential candidates for a multivalent vaccine.

Notably, in this study, the *clpP* gene, which encodes the ATP-dependent Clp protease proteolytic subunit, was upregulated in CVCC259. The bacterial Clp proteases are a class of ATP-dependent serine proteases that are involved in bacterial adaptation to stress factors (heat shock, starvation, oxidative stress, *etc*.) [32]. Deletion of these genes reduces the organism's ability to withstand exposure to low pH and oxidative agents [2]. In addition, the Clp proteases of several pathogenic bacteria are associated with virulence [39]. Clp proteases play key roles in the processing and maturation of several important proteins, including many virulence factors [6]. Interestingly, Clp proteases may affect biofilm formation in several bacteria [23, 29, 37]. Therefore, we speculated that Clp protease may be an important virulence factor involved in the pathogenicity of *A. pleuropneumoniae* serotype 1; however, this hypothesis should be confirmed in further studies.

In addition, we also screened genes encoding lipoprotein, membrane protein, and various hypothetical proteins. The functions of these specifically expressed genes are not

clear, and further analysis is required to elucidate these findings.

In this study, we have shown the feasibility of using the RDA technique to identify differentially expressed genes among *A. pleuropneumoniae* strains CVCC259 and CVCC261. The results of this study extend our understanding of the differences between the gene expression in *A. pleuropneumoniae* serotypes 1 and 3. The analysis of these differentially expressed genes will provide an indicator for future research on the pathogenic mechanisms of *A. pleuropneumoniae* and facilitate the development of a multivalent vaccine against *A. pleuropneumoniae* infection.

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