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Identification and Sequencing of Two Expression Genes of Porcine *Pasteurella multocida in vivo*

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Abstract: According to the published sequence of 14 avian *Pasteurella multocida* genes required for survival and expression *in vivo*, the swine *P. multocida* infected models in septicemic mice were established and 14 pairs of primers were designed and synthesized to amplify the corresponding gene of swine *P. multocida* CVCC 432 strain. Successfully, two genes, pm0221 and pm1069 were identified as *in vivo*-expression gene using RT-PCR. Their nucleotide sequences were compared with those of avian *P. multocida* pm70 strain and the homology were 98.8 and 94.8%, respectively.

Key words: *Pasteurella multocida*, *in vivo*-expressed gene, identification, cloning, sequencing, RT-PCR

INTRODUCTION

Pasteurella multocida (*P. multocida*) is an important pathogen hazardous to aniaml and human beings with world wide distribution. Certain serotypes are the etiologic agents of several types of pasteurellosis, such as fowl cholera in avian species, hemorrhagic septicemia in cattle and buffalo and atrophic rhinitis in swine. Despite considerable research into the mechanisms of immunity, virulence and pathogenesis, safe and effective vaccines against pasteurellosis are still lacking and little is known of the molecular mechanisms of pathogenesis. Based on the difference of capsule, 5 capsule serogroups were recognized. Based on the difference of somatic antigens, 12 somatic types were recognized (1~12). Based on the difference of Heat Stable (HS) antigen, 16 HS serogroups were recognized (1~16) (Yang, 2007). As there are different *P. multocida* serotypes, although it has been demonstrated that natural infection with one serotype can lead to good immunological protection against multiple serotypes and that cross-protection can also be stimulated by using inactivated bacteria grown *in vivo*, but traditional killed whole cell bacterins normally provide only serotype-specific protection, so immune failure were common found in pig industry, causing severe economic failure.

Since 2001, *in vivo* Expression Technology (IVET) (Hunt *et al*., 2001), Signature-Tagged Mutagenesis (STM) (Fuller, 2000; Harper *et al*., 2003) and DNA microarray methods (Boyce *et al*., 2002) were developed to identify the *in vivo* expression gene of *P. multocida* of poultry and cattle origin and Numerous genes, such as those encoding outermembrane lipoproteins, metabolic and biosynthetic enzymes and a number of hypothetical proteins, were identied. Although, those method were prove to be useful targets for attenuating mutation and/or warrant further investigation for their roles in immunity and/or pathogenesis, but study on the *in vivo*-expressed gene of swine *P. multocida* have not reported now.

In order to investigate whether there are differences in *in vivo*-expression pattern between avian *P. multocida* and swine *P. multocida*, 14 published *in vivo*-expression genes of avian *P. multocida* Pm70 strain were selected as reference genes and RT-PCR technology were used to select the *in vivo*-expression gene of swine *P. multocida* strain using a mouse infection model. The result may form the basis for producing recombinant antigens to establish an indirect ELISA for discriminating infection and inactivated vaccine immunized pigs.

MATERIALS AND METHODS

Bacterial strains, vectors and grown conditions: *Escherichia coli* strains DH5a (invitrogen) were used for cloning. Swine *P. multocida* strain CVCC (432) was purchased from china institute of veterinary drug control.

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pMD18-T (Takara) is a high efficence T-cloning vector. *E. coli* was grown at 37°C in Luria-Bertani broth or on Luria-Bertani broth agar supplemented, when necessary with ampicillin (50 μg mL^{-1}) for selection and maintenance of recombinant plasmids and additionally with 125 μM X-Gal for blue-white selection with pMD18-T. The swine *P. multocida* was cultured at 37°C on blood nutrient agar or brain heart infusion broth (BHI; Oxoid).

Primers: According to the 14 published *in vivo*-expressed genes sequence of avian *P. multocida* Pm70 strain (genBank AE004439), computer software Primer Premier (Version 5.0, PREMIER Biosoft International, 3786 Cornia Way, Palo Alto, CA 94303-4504) was used to design 14 paires of primers (Table 1). The oligonucleotides were synthesized by shanghai shenggong bioengineering company.

RNA isolation (Boyce *et al.*, 2002): For the isolation of in vitro-grown *P. multocida*, *P. multocida* were harvested from duplicate BHI cultures at late log phase (5×10^9 CFU mL^{-1}), added to 0.1 volume of ice-cold killing buffer (0.05 M Tris-HCl (pH7.5), 15 mg of sodium azide mL^{-1}) and pelleted by centrifugation at 6000 g. RNA was isolated from *P. multocida* by using Trizol reagent (Tiangen Beijing co.limited) as described by the manufacturer. Purified RNA was treated with Dnase (15 U for 10 min at 37°C) and the RNA was further purified on Rneasy minicolumns (Qiagen). For the isolation of *in vivo*-grown bacteria, 5 mice (Kunming genus from experimental animal center of Sichuan university, chengdu) were infected with 5×10^4 CFU of *P. multocida* strain by injection into the leg muscle. About 0.5 g Liver was recovered from asphyxia death mouse during the final stages of disease (about 15 h after infection), Clinical signs at the time of sampling indicated that each infection was in the terminal phase. The Liver was levigated and 2 ml PBS was added into the sample. Then 0.1 volume of ice-cold killing buffer were added into 0.8 mL mixture and the bacteria were separated from the liver cells by sucrose density centrifugation for 1 h at 3,000 g in the presence of 25% (wt vol^{-1}) sucrose. Bacteria were removed from above the sucrose cushion and concentrated by centrifugation for 5 min at 6000 g. All manipulations were carried out on ice or at 4°C and took less than 1.5 h. RNA was purified as described above for *in vitro*-grown bacteria.

RT-PCR

RT reaction: A total reaction volume of 20 μL was used, including 10 μL of the extracted RNA, 2 μL (25pmol μL^{-1}) of the random primer, 4 μL of 5×RT buffer, 2 μL of dNTP (10 mM of each nucleotide), 0.5 μL of RNA inhibitor, 0.5 μL of RT intensifier and 1 μL of RTase, mix. The reaction conditions were 30°C for 10 min, 42°C for 40 min, 98°C for 5 min and 4°C for 5 min, following which the RT products (cDNA) were collected.

PCR reaction: Amplification of the cDNA was performed using 2×PCR mix 10 μL, Forward primer 0.5 μL, Reverse primer 0.5 μL, RT products 1 μL, 8 μL water, comprising a total volume of 20 μL. The optimum conditions for PCR were established according to the Tm value of primers and amplification length of target genes. Then 5 μL of each PCR product was analyzed in 0.9% agarose (BIO RAD) in TAE buffer gel containing 0.5 mg mL^{-1} ethidium bromide. The gels were electrophoresed at 80 V and the DNA bands were visualized and photographed using an imaging system (Versa Doc-manufactured by BIO RAD). The PCR product was sequenced by TaKaRa Dalian, China.

Cloning and seqencing of complete Open Reading Frame (ORF) of *in vivo*-expressed gene: According to the published in-vivo expressed genes of avian *P. multocida*, Primer 5.0 was used to design two paires of primers. The

Table 1: Primers used for RT-PCR of swine *P. multocida in vivo*-expressed gene

Gene (Locus tag)	Forward primer (5'→3')	Reverse primer (5'→3')	Predicted function or homologe	Amplification length (bp)
Dcd (PM0951)	GACTGGCTACCGCACTTT	GAGCATTCCGCACCTTAT	Deoxycytidine deaminase	430
ackA (Pm0704)	GGTTTAGCCGAAGCATTT	CCGAGTAAGCCTGATTTT	Fermation of acetyl-CoA	740
glpQ (PM1444)	TCGCATACACTGATTGGC	AAGGCAGGTAACGCATCT	Glycerol metabolism	265
Lpp (Pm0554)	AGTTTAGGCTTAGCAGGTT	GATTTCTTGTCCGTTGTC	Outer membrane-associated lipoprotein	336
deoC (PM1343)	ATTCCGTTAGCCAAACAA	CACCACGATTGAAACCAG	Deoxyribose synthesis	352
pm1294	CGAGATGTTTAGTAGGGTCA	GCAGGTCCTAATGAAAGTG	Heptosyl transferase	259
DcaA (PM0023)	AGTGCGAGCAATGTAGGGAC	TGTTGACAATCTGAAATCGG	Phosphoethanolamine transferase	815
Yebl (pm0272)	ATAACGAAGGCGAGATTA	ACCGATAGTGGTATAGGGT	Unkown	266
purN (PM0020)	CCATCGCATACCCTTGTG	CATCCTTCGCTTCTCCCT	De novo purine biosynthesis	310
nrfE (PM0027)	TCTGCTACTTGGGGTGGACAT	ACACTGACCGTGGCAATCGT	Formate-dependent nitrite reduction protein	839
ponC (PM0644)	TTTTTAGGTCCCGTCAGTGT	GCTTCTGCCGTAATCATCTG	Penicillin binding	1155
speF (PM0806)	ATTCTGCGTGGGTAGGTTATG	GCAAAGACGACGGATGGTA	Inducible ornithine decarboxylase	841
dsbD (PM0221)	CTATCCACCTGAAACCCGA	AGAACGCAAACGCTAAACTG	Thiol-disulfide interchange protein	858
pm1069	GGATTTCTTTGCCACCTGTC	TGCTGCCGCTTTCCAACT	Homology with *H. influenzae* P1 surface protein	743

primers for the amplification of Pm0221 gene were Pm0221F (AACGAATGGGGCTATCCTACA) and Pm0221D (GGCGA ATAGGACGATTAGGTTG), The primers for the amplification of Pm1069 gene were Pm1069F (TATCTGACTGGTCTGAACTCTTG) and Pm1069D (CATAAAGGTTCGC ACTGG CT). The designed amplification length of Pm0221 and Pm1069 were 2104 and 1395 bp, respectively. The oligonucleotides were synthesized by shanghai shenggong bioengineering company. Amplification of ORF of swine *P. multocida in vivo*-expressed gene was performed using 2×PCR mix 25 μL, Forward primer 1 μL, Reverse primer 1 μL, RT products 2 μL, 21 μL water, comprising a total volume of 50 μL. The optimum conditions for PCR were as follows: 94°C for 4 min, 14 cycles at 94°C for 30 sec, 61°C for 30 s (a decrease of 0.5°C for every cycle), 72°C for 50 sec, 25 cycles at 94°C for 30 sec, 54°C for 30 sec, 72°C for 50 sec and final elongation at 72°C for 10 min.

Products of PCR reactions, corresponding to the predicted size of the target gene, were isolated from agarose gels and cloned into the pMD18-T plasmid to yield recombinant plasmid. Confirmation of clones containing recombinant plasmid were achieved by PCR. The recombinant plasmid was sequenced by Sangon (China).

Bioinformatics analysis of identified swine *P. multocida in vivo*-expressed gene: The secondary structure, hydrophobicity and surface antigen of target protein were analyzed by utilizing the protean program in the Lasergene package (DNASTAR Inc., Madison, WI 53715, USA), the prediction of protein transmembrane domain was completed online htpp://www.ch.embnet.org/software/TMPRED.form.html, the prediction of antigenic determinant were analyzied online http://immunax.dfci.harvard.edu/Tools/antigenic.pl.

RESULTS

Analysis of in vivo-expressed gene by RT-PCR: To analyze the *in vivo*-expressed gene of swine *P. multocida* during growth within a natural host, we used mouse as the infection model. Fourteen genes, which were *in vivo*-expressed genes in avian *P. multocida* were analyzed to see where they were *in vivo*-expressed gene in swine *P. multocida*. The results of RT-PCR shows that pm0221 and pm1069 were in vivo expressed genes of swine *P. multocida* (Table 2 and Fig. 1).

Cloning and sequencing of ORF of pm0221 and pm1069: Fragments of 2104 and 1395 bp length long were successflly amplified, which conformed to the designed length (Fig. 2). The ORF of pm0221 gene of swine *P. multocida* is 1761 bp long, the same size with that of avian *P. multocida*, the percentige of identidy of swine swine *P. multocida* pm0221 gene with that of avian *P. multocida* is 98.8%. The OFR of pm1069 gene of swine *P. multocida* is 1332 bp long, 12 bp shorter than that of avian *P. multocida*, the percentige of identidy of swine swine *P. multocida* pm1069 gene with that of avian *P. multocida* is 94.8%. The sequence data have been submitted to the GenBank databases under accession No. GQ339604 (pm1069) and GQ339605 (pm0221), respectively.

Table 2: The identification results of swine *P. multocida in vivo*-expressed gene by RT-PCR

Genes	Length of target gene (bp)	RT-PCR results	
		In vitro	*In vivo*
Dcd	430	+	+
acka	740	+	+
glpQ	265	+	+
Lpp	336	+	+
deoC	352	-	-
pm1294	259	-	-
DcaA	815	-	-
yebL	266	-	-
purN	310	+	+
nrfE	839	-	-
ponC	1155	-	-
dsbD	858	-	+
speF	841	+	+
pm1069	743	-	+

"+"represent positive results of RT-PCR; "-" represent negative results of RT-PCR

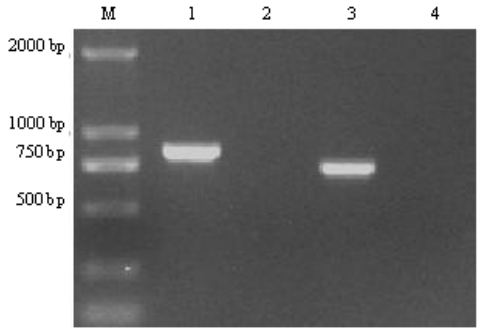

Fig. 1: The partial identification result of swine *P. multocida in vivo*-expressed gene by RT-PCR. Lane 1 and 2 corresponds to RT-PCR result of PM0221 gene *in vivo* and *in vitro*, respectively, while Lane 3 and 4 corresponds to RT-PCR result of PM1069 gene *in vivo* and *in vitro*, respectively. M refers to DNA Marker DL2000

Bioinformatics analysis: The result of deduced amino acid sequence analysis by utilyzing DNASTAR shows that the hydrophobic region and surface antigen of pm0221 protein were mainly located at 31-227 and

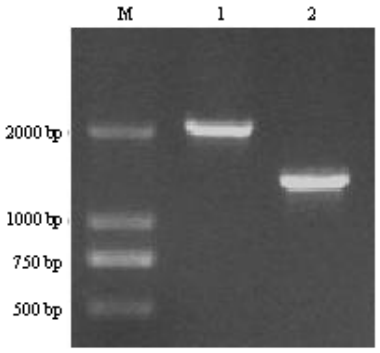

Fig. 2: The RT-PCR result of sequences containing the complete Open Reading Frame (ORF) of pm0221 and pm1069 genes of swine *P. multocida*. Lane 1 and 2 correspond to RT-PCR result of pm0221 and pm1069 genes, respectively. M refers to DNA Marker DL2000

426-582 aa. While those of pm1069 protein were mainly located at 142-431 aa. The prediction resultes of protein transmembrane domain completed online htpp://www.ch.embnet.org/software/TMPRED.form.html shows that pm0221 protein have 9 transmembrane domains, mainly located at 188-446 aa, while Pm1069 protein has only one transmembrane domain, mainly located at 26-44 aa.

The prediction resultes of antigenic determinant completed online http://immunax.dfci.harvard. edu/Tools/antigenic.pl results showed that pm0221 have 19 antigenic determinants, located at 4-574 aa, while pm1069 have 15 antigenic determinants, located at 22-434 aa.

DISCUSSION

A number of screening methods have been used previously to identify potential *in vivo*-expressed genes in *P. multocida*, including STM (Fuller, 2000; Harper *et al*., 2003), IVET (Hunt *et al*., 2001) and microarray (Boyce *et al*., 2002). Signature-Tagged Mutagenesis (STM) was first used to identify the *P. multocida* virulence genes in mouse or chicken model. *In vivo*-Expression Technology (IVET) was utilized to identify *in vivo*-expressed genes of *P. multocida*. Numerous genes, such as those encoding outer membrane lipoproteins, metabolic and biosynthetic. enzymes and a number of hypothetical proteins, were identified. DNA microarray Method was also used to make a genomic scale analysis of *Pasteurella multocida* gene expression during growth within the natural chicken host. The results of different method were different, many genes were identified in one study but not by other methods, indicating that neither of these methods produces a saturation coverage of the genome. One of the clear benefits of whole-genome expression profiling with microarrays is the ability to analyze the gene expression profiles of every gene under the conditions being tested. However, some genes were identified by STM and IVET methods that were not identified by DNA microarray Methods. This is not surprising, as STM and IVET analyses were carried out on different *P. multocida* strains and both used mouse infection models. Thus, infection model is significantly important.

Based on the published *in vivo*-expression gene identified by other method, RT-PCR were used to identify the *in vivo*-expressed gene of swine *P. multocida* in mice model. Only two genes pm0221 and pm1069 were identified as *in vivo*-expressed gene, while others were not *in vivo*-expressed gene, this results maybe related to the different strain of *P. multocida* and use of mouse infection model and identification method used in this study.

The homologue of avian *P. multocida* pm1069 were the *E. coli* protein FadL and the P1 outer membrane protein (Munson and Hunt, 1989) from *H. influenzae*. In *E. coli*, FadL was identified as an outer membrane porin required for the uptake of long-chain fatty acids, mutants lacking FadL are unable to grow on medium containing long-chain fatty acids as a sole carbon source (Nunn and Simons, 1978). The *H. influenzae* homologue P1 has been considered a potential vaccine candidate for both type b and nontypeable strains of *H. influenzae*, as both polyclonal and monoclonal antibodies raised against P1 protect against bacteremia in the infant rat model (Hansen *et al*., 1982; Loeb, 1987). The pm1069 gene of swine *P. multocida* CVCC432 strain is 12 bp shorter than that of avian *P. multocida* Pm70 strain, but the nucleotide acid sequence identidy is still high (94.8%), this 12 bp deletion maybe related to the host specificity of *Pasteurella multocida* and phylogenetic analysis of pm1069 gene with more strains of swine and avian *P. multocida* should be undertaken.

The avian *P. multocida* pm0221 gene encoding DsbD protein (Hunt *et al*., 2001). In *E. coli*, DsbD is a integral membrane protein involved in disulfide bond formation in periplasmic proteins (Missiakas *et al*., 1995). As disulfide bonds are often essential for the proper folding, stability and activity of many extracellular proteins, the expression of such a gene *in vivo* may have implications in bacterial pathogenesis for the secretion of toxins or other virulence factors. Additionally, DsbD is required for c-type cytochrome biogenesis under anaerobic conditions and plays a role in copper (Cu^{2+}) tolerance in bacteria such as *E. coli*, *S. enterica* serovar Typhimurium and *P. aeruginosa* (Crooke and Cole, 1995; Gupta *et al*., 1997; Page *et al*., 1997).

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RERERENCES

Boyce, J.D., I. Wilkie, M. Harper, M.L. Paustian, V. Kapur and B. Adler, 2002. Genomic scale analysis of *Pasteurella multocida* gene expression during growth within the natural chicken host. Infection Immun., 70: 6871-6879.

Crooke, H. and J. Cole, 1995. The biogenesis of c-type cytochromes in *Escherichia coli* requires a membrane bound protein, DipZ, with a protein disulphide isomerase-like domain. Mol. Microbiol., 15: 1139-1150.

Fuller, T.E., M.J. Kennedy and D.E. Lowery, 2000. Identification of *Pasteurella multocida* virulence genes in a septicemic mouse model using signature tagged mutagenesis. Microbol. Pathog, 29: 25-38.

Gupta, S.D., H.C. Wu and P.D. Rick, 1997. A *Salmonella typhimurium* genetic locus which confers copper tolerance on copper-sensitive mutants of *Escherichia coli*. J. Bacteriol., 179: 4977-4984.

Hansen, E.J., S.M. Robertson, P.A. Gulig, C.F. Frisch and E.J. Haanes, 1982. Immunoprotection of rats against *Haemophilus influenzae* type B disease mediated by monoclonal antibody against a haemophilus outer membrane protein. Lancet, 1: 366-368.

Harper, M., J.D. Boyce, I.W. Wilkie and B. Adler, 2003. Signature-tagged mutagenesis of *Pasteurella multocida* identifies mutants displaying differential virulence characteristics in mice and chickens. Infection Immun., 71: 5440-5446.

Hunt, M.L., D.J. Boucher, J.D. Boyce and A. Ben, 2001. *In vivo*-expressed genes of *Pasteurella multocida*. Infection Immun., 5: 3004-3012.

Loeb, M.R., 1987. Protection of infant rats from *Haemophilus influenzae* type b infection by antiserum to purified outer membrane protein a. Infection Immun., 55: 2612-2618.

Missiakas, D., F. Schwager and S. Raina, 1995. Identification and characterization of a new disulfide isomerase-like protein (DsbD) in *Escherichia coli*. EMBO J., 14: 3415-3424.

Munson, R. and Jr. A. Hunt, 1989. Isolation and characterization of a mutant of *Haemophilus influenzae* type b deficient in outer membrane protein P1. Infection Immun., 57: 1002-1004.

Nunn, W.D. and R.W. Simons, 1978. Transport of long-chain fatty acids by *Escherichia coli*: Mapping and characterization of mutants in the fadL gene. Proc. Natl. Acad. Sci., 75: 3377-3381.

Page, M.D., N.F. Saunders and S.J. Ferguson, 1997. Disruption of the *Pseudomonas aeruginosa* dipZ gene, encoding a putative protein-disulfide reductase, leads to partial pleiotropic deficiency in c-type cytochrome biogenesis. Microbiology, 143: 3111-3122.

Yang, H.C., 2007. Pasteurellaceae and Correlated Genus. In: Veterinary Microbiology, Liu, C.P. (Ed.). 4th Edn., China Agriculture Press, Beijing, China, pp: 134-137.