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Genomic Differences Between *Actinobacillus pleuropneumoniae* Serotypes 5b and 3 and their Distribution and Transcription Among 15 Serotypes

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Abstract The development of serotyping-based diagnostic methods and multivalent vaccines has been significantly hampered due to the limited information available on the genetic differences among the 15 currently known serotypes of *Actinobacillus pleuropneumoniae*. In this study, using the GenomeComp informatics software, differential genes were screened and identified between the complete genome sequences of the serotypes 5b (L20 strain, highly virulent) and 3 (JL03 strain, weakly virulent), 84 presented uniquely in strain L20, while 57 were only found in JL03 strain. Of these, 75 encode putative proteins and 66 encode hypothetical proteins, including phage-related proteins, Apx toxin, capsular polysaccharide biosynthesis proteins, ATP-binding cassette (ABC) transporters, Clp-like proteases, fimbrial protein (Flp), various glycosyltransferases, methylases, integrases, and other proteins related to virulence. To confirm and further characterize the differential genes, we carefully selected 34 proven or putative virulence genes which were extremely useful on researching into detection and vaccine of *A. pleuropneumoniae,* and investigated the distribution and transcription of these genes among the 15 serotypes through polymerase chain reaction, reverse transcriptase-polymerase chain reaction and sequencing, and different distribution and transcription patterns of the differential genes in each serotype were first found and described. These information of these differential genes among the 15 serotypes of *A. pleuropneumoniae* may greatly serve as an indicator for future research on the pathogenic mechanisms of different serotypes, serotyping-based diagnostic methods, and multivalent vaccines.

Feng Yang and Fang Xie are the co-first authors. Xin Feng and Liancheng Lei contributed equally to this work.

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Introduction

Porcine contagious pleuropneumonia is caused by *Actinobacillus pleuropneumoniae* [7]. This disease, which has been described worldwide, affects swine of all the ages and has a major impact on economy, ecology, and animal welfare in the pig-rearing industry. On the basis of the antigenic properties of capsular polysaccharides and the cell wall lipopolysaccharides, *A. pleuropneumoniae* has been divided into 15 serotypes [1]. Compared with the low virulence serotypes 3 and 6, serotypes 1, 5, 9, and 11 are the most virulent, causing high mortality and severe pulmonary pathologic changes [12]. Current *A. pleuropneumoniae* vaccines targeting a specific serotype do not confer protection from infection by the other serotypes [9]. Owing to the limited information on the genetic differences among the serotypes, studies on the immunity mechanisms of different serotypes, typing-based diagnosis, and multivalent genetically engineered vaccines have been significantly hampered. Therefore, the genomic differences among the principal serotypes should be identified and suitably exploited.

To date, the complete genome sequences of *A. pleuropneumoniae* strains L20 (serotype 5b; accession no. CP000569), *A. pleuropneumoniae* JL03 (serotype 3; accession no. CP000687), and *A. pleuropneumoniae* AP76 (an isolate of serotype 7; CP001091) have been determined. In this study, we first used GenomeComp informatics software (genome sequence alignment software) to compare the complete genome sequences of *A. pleuropneumoniae* serotypes 5b (highly virulent) and 3 (weakly virulent) in order to screen all the differential genes from each strain. And then, to confirm and further characterize the differential genes, we also selected primary proven or putative virulence genes [2] and investigated the distribution and transcription of these genes among the 15 serotypes of *A. pleuropneumoniae* through PCR, RT-PCR, and sequencing.

Materials and Methods

Bacterial Strains and Growth Conditions

The 15 serotypes of the reference strains of *A. pleuropneumoniae* used in this study are listed in Table 1. *Actinobacillus pleuropneumoniae* reference strains of serotype 1–10 were purchased from the China Institute of Veterinary Drug Control (Beijing, China) and cultured in brain heart infusion (BHI; Difco Laboratories, Detroit, MI) supplemented with nicotinamide adenine dinucleotide (NAD) (10 μg/ml; Sigma) at 37°C for 6 h, while shaking at 150 rpm. The chromosomal DNA of the reference strains of serotype 11–15 were kindly provided by Dr. Shuqing Li (Shanghai Entry-Exit Inspection and Quarantine Bureau, Shanghai, P.R. China). The *A. pleuropneumoniae* strain JL03 (serotype 3) was provided by Dr. Bin Wu (Huazhong Agricultural University, Wuhan, P.R. China).

Table 1 *Actinobacillus. pleuropneumoniae* reference strains used in this study

Strain	Serotype
4074	1
1536	2
1421	3
JL03	3
M62	4
K17	5a
L20	5b
FemΦ	6
WF83	7
405	8
13261	9
13039	10
56153	11
8329	12
N-273	13
3606	14
HS143	15

DNA sequences

The complete genome sequences of both *A. pleuropneumoniae* strain L20 (serotype 5; accession no. CP000569) and *A. pleuropneumoniae* strain JL03 (serotype 3; accession no. CP000687) were obtained from the NCBI Genbank database (http://www.ncbi.nlm.nih.gov/).

Genomic differences analysis

GenomeComp, which provides a graphic user interface (GUI), is a software for DNA sequence comparisons [14]. First, we used this software to compare the two sequences (CP000569 and CP000687) and to identify their differential genes. Then, in order to confirm these results, we used the programs BlastN, BlastP, and BlastX (http://www.ncbi.nlm.nih.gov/) to locate the sequence similarities in the nucleotide and protein databases.

Identification of the differential genes by polymerase chain reaction

To confirm and further characterize the differential genes, we selected the 34 proven and putative virulence genes (18

differential genes of strain L20, 16 differential genes of JL03 strain) which were extremely helpful on researching into detection and vaccine of APP, and designed specific primers using the Primer 5.0 software (listed in Supplementary Table 1). The genomic DNA of reference strain L20 (serotype 5b) and strain JL03 (serotype 3) was extracted using an AxyPrep Bacterial Genomic DNA Miniprep kit (Axygen). The following temperature program was used for polymerase chain reaction (PCR): 95°C for 2 min, followed by 30 cycles of 94°C for 40 s, 52°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min.

Analysis of the Distribution of the 34 Selected Differential Genes in 15 Serotypes

To further clarify the distribution of the 34 selected differential genes among the 15 serotypes of *A. pleuropneumoniae*, we extracted the genomic DNA of 17 reference strains using the AxyPrep Bacterial Genomic DNA Miniprep kit (Axygen). The extracted DNA was then used for the PCR-based identification of the differential genes in 15 serotypes.

Analysis of Transcription of the 34 Selected Differential Genes by Reverse Transcriptase-Polymerase Chain Reaction

To further characterize the expression of the 34 selected differential genes among the 15 serotypes of *A. pleuropneumoniae*, we extracted and purified the total RNA of 17 reference strains using the E.Z.N.A.® Bacterial RNA Kit (OMEGA) for reverse transcriptase-polymerase chain reaction (RT-PCR) as manufacturer described. 1 μg of total RNA was employed to generate cDNA with a RevertAid™ First Strand cDNA Synthesis kit (Fermentas life sciences), according to the manufacturer's instructions. Then, 1 μl of synthesized cDNA was used for analysis of the expression of the 34 selected differential genes by PCR as previously described.

Results

Genetic Differences Analysis

The complete genome sequences of serotypes 3 (JL03 strain) and 5b (L20 strain) were compared using the GenomeComp software. We identified a total of 84 differential genes from strain L20, and 57 differential genes from JL03 strain.

The nucleotide similarities of these genes were determined using the GenBank database. Compared with the JL03 strain, the L20 strain has 84 differential genes, which encode 41 hypothetical proteins and 43 putative proteins, including phage-related proteins, various methylases, endolysins, Clp-like proteases, fimbrial protein (Flp), ABC transporters, UDP-galactopyranose mutases, various glycosyltransferases, capsular polysaccharide biosynthesis proteins, Apx I toxin, and other genes related to virulence. The sequences and encoding proteins are shown in Supplementary Table 2. We identified 57 differential genes in the JL03 strain, which encode 25 hypothetical proteins and 32 putative proteins, including RacX proteins, DNA-binding proteins, integrases, Wzz-like proteins, various glycosyltransferases, oligosaccharide repeat unit polymerases, the Wzx flippase, UDP-galactopyranose mutases, polysaccharide biosynthesis proteins, teichoic acid biosynthesis proteins, Abi-like proteins, Apx III toxin, and other genes related to virulence. The sequences and encoding proteins are shown in Supplementary Table 3.

Confirmation of the 34 Differential Genes by PCR with Special Primers

After screening of differential between APP serotype 5b and serotype 3, 34 proven or putative virulence genes which were extremely useful on researching into typing detection and vaccine of APP were selected carefully and further confirmed by PCR with special primers. Genomic DNA from the reference strain L20 (serotype 5b) and JL03 (serotype 3) was used as a template for the PCR-based identification of these differential genes. The electrophoresis results showed that all of the selected differential genes (18) found in strain L20 were absent in JL03 strain, while all the selected differential genes (16) present in the *A. pleuropneumoniae* JL03 strain were absent in strain L20.

Analysis of the Distribution of the 34 Selected Differential Genes in 15 Serotypes

Before the application of these important differential genes fully in the future, it was very necessary to further characterize the distribution of the differential genes among the 15 serotypes of *A. pleuropneumoniae*. Therefore, the genomic DNA of 15 serotype reference strains were used as templates for PCR-based identification. The electrophoresis results showed that the 34 differential genes were differently distributed among the 15 serotypes (Supplementary Table 4). These data displayed clearly difference in genomes among the 15 serotypes, and they were very helpful to establish serotyping detection in gene level.

Analysis of the Expression of the 34 Selected Differential Genes by RT-PCR

To determine whether the differential genes were expressed, expression study of the 34 selected differential genes

was performed among the 15 serotypes of *A. pleuropneumoniae* by RT-PCR. The results showed that the transcription of the 34 differential genes was partially discordant with their distribution in genomes among the 15 serotypes, and some genes present in the genome did not transcribe. Only ten out of 34 differential genes were transcribed in all the strains which contained these related genes in their genomes among 15 serotypes, while 24 of 34 differential genes were only transcribed in some serotypes. 11 of 18 differential genes from serotype 5b did not transcribe in some serotypes containing these genes in genome and non-transcription times is 19, while 13 of 16 differential genes from serotype 3 did not transcribe and the times in total is 36. The genes and their transcription were showed in Supplementary Table 4.

Discussion

Currently, there are no commercial vaccines available that can provide cross-protection against the 15 serotypes of *A. pleuropneumoniae*. Comparison of the genomes of two closely related strains and identification of functional genes are effective approaches for elucidating bacterial pathogenic mechanisms and developing multivalent vaccines [5]. Although the complete genome sequences of the *A. pleuropneumoniae* strains L20 (serotype 5b) and JL03 (serotype 3) have been determined, the genomic differences between them have still not been elucidated in detail.

In this study, we used genome sequence alignment software to systematically compare the complete genome sequences of *A. pleuropneumoniae* serotype 5b and serotype 3 for screening of useful differential genes between them. The differential genes of these two strains encode different proteins, including various methylases, integrases, glycosyltransferases, lipopolysaccharide biosynthesis glycosyltransferases, Clp-like proteases, UDP-galactopyranose mutases, arabinose-5-phosphate isomerases, flp operon protein B, ABC transporters, capsular polysaccharide biosynthesis proteins, phage-related proteins, RacX protein, DNA-binding proteins, Abi-like proteins, Apx toxins, other proteins related to virulence factors, and so on. Although the reference strains and selected Canadian field isolates have been compared with the *A. leuropneumoniae* L20 strain (serotype 5b) by performing microarray analysis [3], the genomic differences between serotypes 5b and 3 have still not been elucidated. We only found six genes (3-29, 3-30, 3-31, 3-34, 3-36, and 3-37, listed in Supplementary Table 3) in our 141 genes had also been described as differential genes between *A. pleuropneumoniae* serotypes 1 and 3 [13], which could be associated with serotype strains, and others were first reported as differential genes from APP strains.

Phages play an important role in the horizontal transfer of virulence genes, and they are also important precursors during expression of the bacterial virulence process. They encode toxins as well as special proteins, thereby changing the bacterial resistance to host defense mechanisms. In addition, the interaction between phages and bacteria also allows bacteria to constantly acquire new virulence genes, leading to the emergence of new pathogenic strains [11]. Our results revealed that 9 phage-related genes (5-2, 5-22, 5-23, 5-27, 5-33, 5-35, 5-36, 5-37, and 5-38) were present in strain L20, but absent in JL03 strain (Supplementary Table 2). This may explain the high virulence potential of *A. pleuropneumoniae* serotype 5b. We also found that the distribution of the phage-related genes 5-2, 5-23, and 5-27 among the 15 serotypes was complex, but that all three genes were present in the most virulent serotypes (1, 5, 9, and 11). The different virulence potential in different serotypes may be the result of the horizontal transfer of virulence genes, which has significance for future studies.

The distribution of the gene encoding DNA methylase (5-15) shows significant differences among serotypes of *A. pleuropneumoniae* and is present in the highly virulent serotypes 1, 5b, 9, 10, and 11. A growing number of reports describe the role for DNA methylation in regulating the expression of various bacterial genes related to virulence in various pathogens, suggesting that DNA methylation may be a widespread and versatile regulator of the expression of virulence genes [4, 6]. Therefore, we reckon that this gene may be associated with highly virulent serotypes of *A. pleuropneumoniae*. However, this hypothesis needs to be studied further.

Notably, we discovered that a gene encoding the teichoic acid biosynthesis protein (3-44) is only present in serotype 3. We speculate that this gene could be used for the specific diagnosis of serotype 3 and may contribute to the development of serotyping-based methods for *A. pleuropneumoniae*. In addition, the distribution of some differential genes in *A. pleuropneumoniae* serotype 5a is not exactly the same as that in serotype 5b. Genes 5-9, 5-15, 5-23, 5-24, and 5-27 (listed in Supplementary Table 2) are present in *A. pleuropneumoniae* serotype 5b, but absent in 5a. While genes 3-19, 3-46, 3-49, and 3-50 (listed in Supplementary Table 3) are present in *A. pleuropneumoniae* serotype 5a, but absent in 5b. These differential genes may provide the basis for typing-based diagnostic methods to distinguish between the *A. pleuropneumoniae* serotypes 5a and 5b. Serological cross-reactivity between serotypes 1 and 9 [10] and serotypes 3, 6, and 8 [15] has been reported. Previous studies suggest that these cross-reactions can be primarily attributed to shared species-specific antigens, such as lipopolysaccharides or membrane proteins [8]. In our study, distribution analysis indicated that many differential genes of serotype 3 were

present in serotypes 3, 6, 8, and 15, including genes 3-19, 3-27, 3-33, 3-35, 3-49, 3-50, 3-56, and so on (listed in Supplementary Table 3). These differential genes (the encoded proteins are listed in Supplementary Tables 2 and 3) may be related to immune-protective antigens that are shared by some serotypes. Thus, these genes may serve as potential vaccine candidates for a multivalent vaccine that can provide cross-protection against multiple serotypes of *A. pleuropneumoniae*.

Whether the differential genes were expressed really was crucial for their functional research. So RT-PCR with special primers was used to test the transcripts of the 34 selected differential genes among the 15 serotypes of *A. pleuropneumoniae*. We list the transcription of 34 differential genes among 15 serotypes in Supplementary Table 4. For instance, Cps2A presented in serotypes 2, 3, 4, 7, 8, 9, 13; but mRNAs of Cps2A were only detected positively in serotype 4 and 13 by RT-PCR. These data showed that certain genes could be transcribed and might possess function among 15 different serotypes. These genes may also beproviding some novel targets that could be exploited in vaccine development and pathogenesis study.

With the development of bioinformatics, the genomes of an increasing number of microorganisms have been sequenced. In most cases, however, the differential genes among microorganisms should be identified to provide a basis for the research on pathogenic mechanisms in different serotypes of pathogenic bacteria as well as to develop serotyping-based diagnostic methods and new types of multivalent vaccines. In this study, we carried out a preliminary comparative analysis of differential genes in the genomes of *A. pleuropneumoniae* strains using informatics software, which provides much information for further research on microbial biological functions. Our results also established a platform for research on multivalent genetic vaccines for different pathogenic serotypes, thereby providing a new technique and method, and the foundation for the structural and functional research on multivalent genetic vaccines in the future.

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References

1. Blackall PJ, Klaasen HL, van den Bosch H, Kuhnert P, Frey J (2002) Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Vet Microbiol 84:47–52
2. Chiers K, De Waele T, Pasmans F, Ducatelle R, Haesebrouck F (2010) Virulence factors of *Actinobacillus pleuropneumoniae* involved in colonization, persistence and induction of lesions in its porcine host. Vet Res 41:65
3. Goure J, Findlay WA, Deslandes V, Bouevitch A, Foote SJ, MacInnes JI, Coulton JW, Nash JH, Jacques M (2009) Microarray-based comparative genomic profiling of reference strains and selected Canadian field isolates of *Actinobacillus pleuropneumoniae*. BMC Genomics 10:88
4. Heusipp G, Falker S, Schmidt MA (2007) DNA adenine methylation and bacterial pathogenesis. Int J Med Microbiol 297:1–7
5. Lei L, Sun C, Lu S, Feng X, Wang J, Han W (2008) Selection of serotype-specific vaccine candidate genes in *Actinobacillus pleuropneumoniae* and heterologous immunization with *Propionibacterium acnes*. Vaccine 26:6274–6280
6. Lobner-Olesen A, Skovgaard O, Marinus MG (2005) Dam methylation: coordinating cellular processes. Curr Opin Microbiol 8:154–160
7. Macinnes JI, Rosendal S (1988) Prevention and control of *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* infection in swine: a review. Can Vet J 29:572–574
8. Perry MB (1990) Structural analysis of the lipopolysaccharide of *Actinobacillus* (Haemophilus) *pleuropneumoniae* serotype 10. Biochem Cell Biol 68:808–810
9. Ramjeet M, Deslandes V, Goure J, Jacques M (2008) *Actinobacillus pleuropneumoniae* vaccines: from bacterins to new insights into vaccination strategies. Anim Health Res Rev 9:25–45
10. Rodriguez Barbosa JI, Gutierrez Martin CB, Tascon RI, Gonzalez OR, Mittal KR, Rodriguez Ferri EF (1996) Characterization of monoclonal antibodies that recognize common epitopes located on O antigen of lipopolysaccharide of serotypes 1, 9 and 11 of *Actinobacillus pleuropneumoniae*. FEMS Immunol Med Microbiol 16:173–181
11. Salgado CJ, Zayas M, Villafane R (2004) Homology between two different Salmonella phages: *Salmonella enterica* serovar Typhimurium phage P22 and *Salmonella enterica* serovar Anatum var. 15 + phageepsilon34. Virus Genes 29:87–98
12. Tascon RI, Vazquez-Boland JA, Gutierrez-Martin CB, Rodriguez-Barbosa I, Rodriguez-Ferri EF (1994) The RTX haemolysins ApxI and ApxII are major virulence factors of the swine pathogen *Actinobacillus pleuropneumoniae*: evidence from mutational analysis. Mol Microbiol 14:207–216
13. Xie F, Lei L, Du C, Li S, Han W, Ren Z (2010) Genomic differences between *Actinobacillus pleuropneumoniae* serotypes 1 and 3 and the diversity distribution among 15 serotypes. FEMS Microbiol Lett 303:147–155
14. Yang J, Wang J, Yao ZJ, Jin Q, Shen Y, Chen R (2003) GenomeComp: a visualization tool for microbial genome comparison. J Microbiol Methods 54:423–426
15. Zhou L, Jones SC, Angen O, Bosse JT, Nash JH, Frey J, Zhou R, Chen HC, Kroll JS, Rycroft AN et al (2008) PCR specific for *Actinobacillus pleuropneumoniae* serotype 3. Vet Rec 162:648–652