The genetic organization of the capsular polysaccharide biosynthesis region of *Actinobacillus pleuropneumoniae* serotype 15

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**ABSTRACT.** Nucleotide sequence determination and analysis of the *cps* gene involved in the capsular polysaccharide biosynthesis of *Actinobacillus pleuropneumoniae* serotype 15 revealed the presence of three open reading frames, designated as *cps15ABC* genes. At the protein level, Cps15A and Cps15B showed considerably high homology to CpsA (67.0 to 68.7%) and CpsB (31.7 to 36.8%), respectively, of *A. pleuropneumoniae* serotypes 1, 4 and 12, revealing the common genetic organization of the *cps* among serotypes 1, 4, 12 and 15. However, Cps15C showed no homology to any proteins of *A. pleuropneumoniae* serotypes, indicating that *cps15C* may be specific to serotype 15. This study will provide the basic molecular knowledge necessary for the development of diagnostics and a vaccine for *A. pleuropneumoniae* serotype 15.

**KEY WORDS:** *Actinobacillus pleuropneumoniae*, serotype 15 capsule

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*Actinobacillus pleuropneumoniae* is a Gram-negative bacterium and the etiologic agent of porcine pleuropneumonia, which causes serious economic losses to the pig-rearing industry [6]. To date, 15 serotypes are recognized, mainly on the basis of the antigenic diversity of capsular polysaccharides in the organisms [3, 19, 21, 22]. Since virulence differs among serotypes [6] and vaccines for *A. pleuropneumoniae* are serotype-specific [20, 25], serotyping is important and should be widely performed in veterinary diagnostic laboratories. However, only a few laboratories can prepare a full set of serotype-specific antisera for serotyping. Furthermore, cross-reactions are often observed among different serotypes, such as between serotypes 1, 9 and 11; serotypes 4 and 7; serotypes 3, 6, 8 and 15; this prevents the accurate and rapid typing of field strains [5–7].

Prevalent serotypes differ from country to country [5]. For example, the predominant serotypes are serotypes 1 and 5 in North America [5, 6], serotype 2 in most of Europe [5, 6], serotype 15 in Australia [3, 5, 6] and serotypes 2, 1 and 5 in Japan [5, 13]. Serotypes 1, 2, 5, 9 and 11 have been generally found to be more virulent than other serotypes [6]. However, approximately 15% of fattening pigs in a herd died due to acute pleuropneumonia caused by serotype 15 [14], indicating that serotype 15, unlike serotypes 3 and 12, should not be considered as low pathogenic [6]. The development of reliable serotyping tests and vaccines for serotype 15 would be important, because isolation cases of serotype 15 have recently increased in Japan [13] and North America [7], because cross-reactions are often observed among serotypes 3, 6, 8 and 15 [5, 6] and because no commercial vaccine is fully effective against serotype 15 challenge [25].

In this study, we determined the nucleotide sequence of the gene involved in the capsular polysaccharide synthesis (CPS) of *A. pleuropneumoniae* serotype 15 (*cps15*). The first aim of this study was to obtain the basic molecular knowledge necessary for the development of *A. pleuropneumoniae* serotype 15 diagnostics, such as PCR serotyping tests, which have been developed in other serotypes [1, 4, 12, 15, 17, 24, 27]. The second aim of this study was to obtain a basic molecular knowledge necessary for the development of vaccine, such as a genetically modified capsule-deficient mutant vaccine [9].

*A. pleuropneumoniae* serotype 15 strain HS143 was used to determine the nucleotide sequence of *cps15*. The organisms were cultivated with TSA agar (Difco, Sparks, MD, U.S.A.) supplemented with 5% defibrinated horse blood and 2% fresh yeast extracts at 37°C. In order to determine the nucleotide sequence of the *cps15*, internal region of *cpxD* of *A. pleuropneumoniae* serotype 15 (*cpxD15*) was PCR-amplified from the genomic DNA of serotype 15 strain HS143, which was prepared as described previously [10]. PCR primers were designed from data previously deposited in databases (5′-ACY TCA GGC CCT AGC CAT AST GC-3′ and 5′-CAC ACG ATA AAC CGT YGG TAC ATC-3′) [26]. The amplified PCR products were purified and sequenced as described previously [11]. Since *cps* is usually flanked by *cpxD* in *A. pleuropneumoniae* [16, 26], an inverse touch-down PCR was then performed to obtain DNAs flanked by *cpxD* with primers invF (5′-GCA GTA GGC GGA ACA ACG GAA AAC ATT-3′) and invR (5′-ATA TCC CGT GCC ACC TAC AGT ACC TAA AAA-3′), which were designed on the basis of the nucleotide sequence of *cpxD* determined...
in this study. Purified genomic DNA from serotype 15 strain HS143 was digested with restriction enzymes EcoRI and HindIII, religated with T4 DNA ligase in order to generate circular template DNAs and used for the following inverse touchdown PCR, respectively. The inverse touchdown PCR was performed in a total volume of 50 µl containing 1 X buffer (Toyobo, Otsu, Japan); 0.2 mM of each dNTP; 0.3 µM of each primer (invF and invR) and the template DNAs described above. The following amplification steps were used: 1 cycle at 94°C for 2 min (preheating); 5 cycles at 98°C for 10 sec and 74°C for 20 min (first step); 5 cycles at 98°C for 10 sec and 72°C for 20 min (second step); 5 cycles at 98°C for 10 sec and 70°C for 20 min (third step); 20 cycles at 98°C for 10 sec and 68°C for 20 min (forth step); 1 cycle at 68°C for 10 min (final step). Amplified DNAs (approximately 8 and 5 kilobase pairs) were purified by the QIA quick PCR amplification kit (Qiagen, Hilden, Germany) and submitted to nucleotide sequence determination with fluorescent dye terminators as described previously [11]. The nucleotide sequence determined has been deposited under accession number AB701753 in DDBJ/EMBL/GenBank. 

The nucleotide sequence of the DNA (8,551 bp) comprising cps15 was determined. Three open reading frames (ORFs) were located between the cpx15D and lysA genes (the CPS export gene and the diaminopimelate decarboxylase genes, respectively), which are conserved in A. pleuropneumoniae and flanked by the cps (Fig. 1). The ORFs were designated as cps15ABC genes (Fig.1) and encoded the Cps15A to Cps15C proteins, respectively. At the amino acid level, Cps15A showed considerably high homology to ORF1 of Actinobacillus suis [18] as well as did to Cps1A, Cps4A and Cps12A (CPS phosphotransferase) of A. pleuropneumoniae serotypes 1, 4 and 12, respectively [2, 16] (Table 1). Cps15B showed overall homology to a glycosyl tranferase of Mannheimia varigena and to ORF2 of A. suis [18] as well as did to Cps1B, Cps4B and Cps12B (glycosyl transferase family protein) of A. pleuropneumoniae [2, 16] (Table 1). Cps15C showed no homology to any proteins of A. pleuropneumoniae, whereas it showed homology to a hypothetical protein of Corynebacterium resistens [23] and to a protein involved in CPS biosynthesis of Neisseria meningitidis serogroup Z [8, 28] (Table 1). These findings suggested that a horizontal gene transfer of the cps gene across the taxonomically and phylogenetically unrelated bacterial classes, including Gram-positive bacteria C. resistens and N. meningitidis belonging to β-Proteobacteria, might have occurred during capsule evolution. The G+C contents of cps15A, cps15B and cps15C were 26.9, 26.8 and 34.2%, respectively (Table 1), which is lower than the 41% (overall G+C content of A. pleuropneumoniae) [26], indicating that the cps15ABC genes might have been acquired by horizontal gene transfer.

Serotype-specific enzymes that are involved in CPS biosynthesis are probably responsible for the dissimilarities among the CPS chemical structures [26]. However, it has been reported that the CPS structures produced by A. pleuropneumoniae serotypes 1 to 13 and 15 can be divided into three groups according to the basic differences in their chemical compositions and structures: Group I (serotypes 1, 4, 12 and 15), with CPS composed solely of repeating oligosaccharide units linked by phosphates; Group II (serotypes 5 and 10), with CPS composed of repeating oligosaccharide units; Group III (serotypes 2, 3, 6–9, 11 and 13), with CPS composed of teichoic acid polymers linked by phosphate diesters [16, 21, 26]. The genetic organization of the cps genes provided molecular evidence to support the CPS grouping of A. pleuropneumoniae serotypes [16, 26]. The present study...
Table 1. Identity of Cps protein of Actinobacillus pleuropneumoniae serotype 15 (Cps15) compared to those of A. pleuropneumoniae and other bacterial species

| Cps15 protein | Length of aa\(^a\) of Cps15 (%) of cps15 encoding Cps15 | bacterial species | Serotype | Homologous protein | Accession number | Reference | % Identity | Length over homologous aas |
|---------------|----------------------------------------------------------|-------------------|-----------|-------------------|-----------------|----------|-----------|--------------------------|
| Cps15A        | 393                                                      | As\(^a\)          | K2        | ORF1              | EU048554        | Unpublished | 67.6       | 364                      |
|               |                                                          | Ap\(^e\)          | 4         | Cps4A             | GU585380        | Unpublished | 67         | 364                      |
|               |                                                          | As                 | K1        | ORF1              | AY253301        | Unpublished | 67.6       | 364                      |
|               |                                                          | Ap                 | 12        | Cps12A            | AY496881        | [16]      | 67.1       | 365                      |
|               |                                                          | Ap                 | 1         | Cps1A             | AF518558        | [2]       | 68.7       | 332                      |
|               |                                                          | As                 | K2        | ORF1\(^d\)        | CP003875        | [18]      | 69.3       | 329                      |
| Cps15B        | 845                                                      | As                 | K2        | ORF2              | EU048554        | Unpublished | 36.8       | 862                      |
|               |                                                          | As                 | K1        | ORF2              | EU077419        | Unpublished | 37         | 862                      |
|               |                                                          | As                 | K2        | ORF2\(^d\)        | CP003875        | [18]      | 37         | 862                      |
|               |                                                          | Ap                 | 1         | Cps1B             | AF518558        | [2]       | 36.4       | 803                      |
|               |                                                          | Ap                 | 12        | Cps12B            | AY496881        | [16]      | 31.7       | 878                      |
|               |                                                          | Ap                 | 1         | Cps1B             | AY496881\(^f\) | [16]      | 33         | 221                      |
| Cps15C        | 380                                                      | Cr\(^e\)          | Hp\(^g\)  |                   | CP002857        | [23]      | 34.4       | 360                      |
|               |                                                          | Nm\(^m\)          | Z         | CapZD\(^e\)       | AJ744766        | Unpublished | 24.4       | 376                      |
|               |                                                          | Nm                 | Z         | CszD              | HF562991        | [8]       | 24.4       | 376                      |
|               |                                                          | Nm                 | Z         | CapZD             | HQ437689        | [28]      | 24.2       | 376                      |

Homologous proteins whose amino acid sequences show significant alignments to A. pleuropneumoniae and other bacterial species are shown. Lanes for A. pleuropneumoniae proteins are shaded. a) Amino acid; b) Protein name was designated in this study as Cps15; c) Partial sequence; d) Hypothetical; e) Serogroup; f) Alternative name; g) Serogroup; h) Hypothetical; i) Neisseria meningitidis; j) Neisseria meningitidis. Serotypes 1–13 correspond to the CPS structural classification, as do serotypes 15–19. The genetic organization of the cps genes of A. pleuropneumoniae serotype 15 corresponds to the CPS structural classification, as do serotypes 1–13 [16, 21, 26].

As shown in Fig. 1, the genetic organization of the cps was essentially common among A. pleuropneumoniae serotypes 1, 4, 12 [16, 26] and 15 [this study]. However, the orientation of the cps15ABC gene against cpsD and lyaA genes was different from that of other A. pleuropneumoniae serotypes [16, 26] (Fig. 1). The different orientation between the cps15ABC genes and cps genes of other serotypes indicated that an inversion might have occurred only in A. pleuropneumoniae serotype 15.

In conclusion, the nucleotide sequence of the cps15 gene has been determined in this study. We believe that the present results will provide the basic molecular knowledge necessary to develop diagnostics and a vaccine for A. pleuropneumoniae serotype 15.

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REFERENCES

1. Angen, O., Ahrens, P. and Jessing, S. G. 2008. Development of a multiplex PCR test for identification of Actinobacillus pleuropneumoniae serovars 1, 7, and 12. Vet. Microbiol. 132: 312–318. [Medline] [CrossRef]

2. Bandara, A. B., Lawrence, M. L., Veit, H. P. and Inzana, T. J. 2003. Association of Actinobacillus pleuropneumoniae capsular polysaccharide with virulence in pigs. Infect. Immun. 71: 3320–3328. [Medline] [CrossRef]

3. Blackall, P. J., Klaasen, H. L., van den Bosch, H., Kuhnert, P. and Frey, J. 2002. Proposal of a new serovar of Actinobacillus pleuropneumoniae: serovar 15. Vet. Microbiol. 84: 47–52. [Medline] [CrossRef]

4. Bossé, J. T., Li, Y., Angen, Ø., Weinert, L. A., Chaudhuri, R. R., Holden, M. T., Williamson, S. M., Maskell, D. J., Tucker, A. W., Wren, B. W., Rycroft, A. N., Langford P. R., BRaDP1T consortium. 2014. Multiplex PCR assay for unequivocal differentiation of Actinobacillus pleuropneumoniae serovars 1 to 3, 5 to 8, 10, and 12. J. Clin. Microbiol. 52: 2380–2385. [Medline] [CrossRef]

5. Dubreuil, J. D., Jacques, M., Mittal, K. R. and Gottschalk, M. 2000. Actinobacillus pleuropneumoniae surface polysaccharides: their role in diagnosis and immunogenicity. Anim. Health Res. Rev. 1: 73–93. [Medline] [CrossRef]

6. Gottschalk, M. 2012. Actinobacillosis. pp. 653–669. In: Diseases of Swine, 10th ed. (Straw, B. E., Zimmerman, J. J., D’Allaire, S. and Taylor, D. I. eds.) Wiley-Blackwell, Oxford.

7. Gottschalk, M. and Lacouture, S. 2014. Actinobacillus pleuropneumoniae serotypes 3, 6, 8 and 15 isolated from diseased pigs
8. Harrison, O. B., Claus, H., Jiang, Y., Bennett, J. S., Bratcher, H. B., Jolley, K. A., Corton, C., Care, R., Poolman, J. T., Zollinger, W. D., Frasch, C. E., Stephens, D. S., Feavers, I., Frosch, M., Parkhill, J., Vogel, U., Quail, M. A., Bentley, S. D. and Maiden, M. C. 2013. Description and nomenclature of *Neisseria meningitidis* capsule locus. *Emerg. Infect. Dis.* 19: 566–573. [Medline] [CrossRef]

9. Inzana, T. J., Glindemann, G., Fenwick, B., Longstreth, J. and Ward, D. 2004. Risk assessment of transmission of capsule-deficient, recombinant *Actinobacillus pleuropneumoniae*. *Vet. Microbiol.* 104: 63–71. [Medline] [CrossRef]

10. Ito, H., Uchida, I., Sekizaki, T., Ooishi, E., Kawai, T., Okabe, T., Taneno, A. and Terakado, N. 1995. Molecular cloning of an *Actinobacillus pleuropneumoniae* outer membrane lipoprotein (OmlA) from serotype 5a. *Microb. Pathog.* 18: 29–36. [Medline]

11. Ito, H., Ishii, H. and Akiba, M. 2004. Analysis of the complete nucleotide sequence of an *Actinobacillus pleuropneumoniae* outer membrane lipoprotein (OmlA) from serotype 5a. *Microb. Pathog.* 36: 1704–1710. [Medline]

12. Ito, H. 2010. Development of a cps-based multiplex PCR for typing of *Actinobacillus pleuropneumoniae* serotypes 1, 2 and 5. *J. Vet. Med. Sci.* 72: 653–655. [Medline] [CrossRef]

13. Ito, H. 2013. Recent topics of serotypes/serotyping and host specificity of *Actinobacillus pleuropneumoniae*. *Proc. Jpn. Pig Vet. Soc.* 61: 14–21 (in Japanese).

14. Koyama, T., To, H. and Nagai, S. 2007. Isolation of *Actinobacillus pleuropneumoniae* serovar 15-like strain from a field case of porcine pleuropneumonia in Japan. *J. Vet. Med. Sci.* 69: 961–964. [Medline] [CrossRef]

15. Jessing, S. G., Angen, Ø. and Inzana, T. J. 2003. Evaluation of a multiplex PCR test for simultaneous identification and serotyping of *Actinobacillus pleuropneumoniae* serotypes 2, 5, and 6. *J. Clin. Microbiol.* 41: 4095–4100. [Medline] [CrossRef]

16. Jessing, S. G., Ahrens, P., Inzana, T. J. and Angen, Ø. 2008. The genetic organisation of the capsule biosynthesis region of *Actinobacillus pleuropneumoniae* serotypes 1, 6, 7, and 12. *Vet. Microbiol.* 129: 350–359. [Medline] [CrossRef]

17. Lo, T. M., Ward, C. K. and Inzana, T. J. 1998. Detection and identification of *Actinobacillus pleuropneumoniae* serotype 5 by multiplex PCR. *J. Clin. Microbiol.* 36: 1704–1710. [Medline]

18. MacInnes, J. L., Mackinnon, J., Bujold, A. R., Ziebell, K., Kroppinski, A. M. and Nash, J. H. 2012. Complete genome sequence of *Actinobacillus suis* H91-0380, a virulent serotype O2 strain. *J. Bacteriol.* 194: 6686–6687. [Medline] [CrossRef]

19. MacLean, L. L., Perry, M. B. and Vinogradov, E. 2004. Characterization of the antigenic lipopolysaccharide O chain and the capsular polysaccharide produced by *Actinobacillus pleuropneumoniae* serotype 13. *Infect. Immun.* 72: 5925–5930. [Medline] [CrossRef]

20. Nielsen, R. 1984. *Haemophilus pleuropneumoniae* serotypes—cross protection experiments. *Nord. Vet. Med.* 36: 221–234. [Medline]

21. Perry, M. B., Altman, E., Brisson, J.-R., Beynon, L. M. and Richards, J. C. 1990. Structural characteristics of the antigenic capsular polysaccharides and lipopolysaccharides involved in the serological classification of *Actinobacillus (Haemophilus) pleuropneumoniae* strains. *Serodiagn. Immunother. Inf. Dis.* 4: 299–308. [CrossRef]

22. Perry, M. B., MacLean, L. L. and Vinogradov, E. 2005. Structural characterization of the antigenic capsular polysaccharide and lipopolysaccharide O-chain produced by *Actinobacillus pleuropneumoniae* serotype 15. *Biochem. Cell Biol.* 83: 61–69. [Medline] [CrossRef]

23. Schröder, J., Maus, I., Meyer, K., Wördemann, S., Blom, J., Jaelnicke, S., Schneider, J., Trost, E. and Tauch, A. 2012. Complete genome sequence, lifestyle, and multi-drug resistance of the human pathogen *Corynebacterium resistent* DSM 45100 isolated from blood samples of a leukemia patient. *BMC Genomics* 13: 141. [Medline] [CrossRef]

24. Schuchert, J. A., Inzana, T. J., Angen, Ø. and Jessing, S. 2004. Detection and identification of *Actinobacillus pleuropneumoniae* serotypes 1, 2, and 8 by multiplex PCR. *J. Clin. Microbiol.* 42: 4344–4348. [Medline] [CrossRef]

25. Tumamao, J. Q., Bowles, R. E., van den Bosch, H., Klaasen, H. L., Fenwick, B. W., Storie, G. J. and Blackall, P. J. 2004. Comparison of the efficacy of a subunit and a live streptomycin-dependent porcine pleuropneumonia vaccine. *Aust. Vet. J.* 82: 370–374. [Medline] [CrossRef]

26. Xu, Z., Chen, X., Li, L., Li, T., Wang, S., Chen, H. and Zhou, R. 2010. Comparative genomic characterization of *Actinobacillus pleuropneumoniae*. *J. Bacteriol.* 192: 5625–5636. [Medline] [CrossRef]

27. Zhou, L., Jones, S. C. P., Angen, Ø., Bossé, J. T., Nash, J. H. E., Frey, J., Zhou, R., Chen, H. C., Kroll, J. S., Rycroft, A. N. and Langford, P. R. 2008. Multiplex PCR that can distinguish between immunologically cross-reactive serovar 3, 6, and 8 *Actinobacillus pleuropneumoniae* strains. *J. Clin. Microbiol.* 46: 800–803. [Medline] [CrossRef]

28. Zhu, H., Wang, Q., Wen, L., Xu, J., Shao, Z., Chen, M., Chen, M., Reeves, P. R., Cao, B. and Wang, L. 2012. Development of a multiplex PCR assay for detection and genotyping of *Neisseria meningitidis*. *J. Clin. Microbiol.* 50: 46–51. [Medline] [CrossRef]