NOTE *Bacteriology*

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 H143, 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 touchdown 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 CGC ACC GCC TAC AGT ACC TAA AAA-3′), which were designed on the basis of the nucleotide sequence of *cpxD* determined

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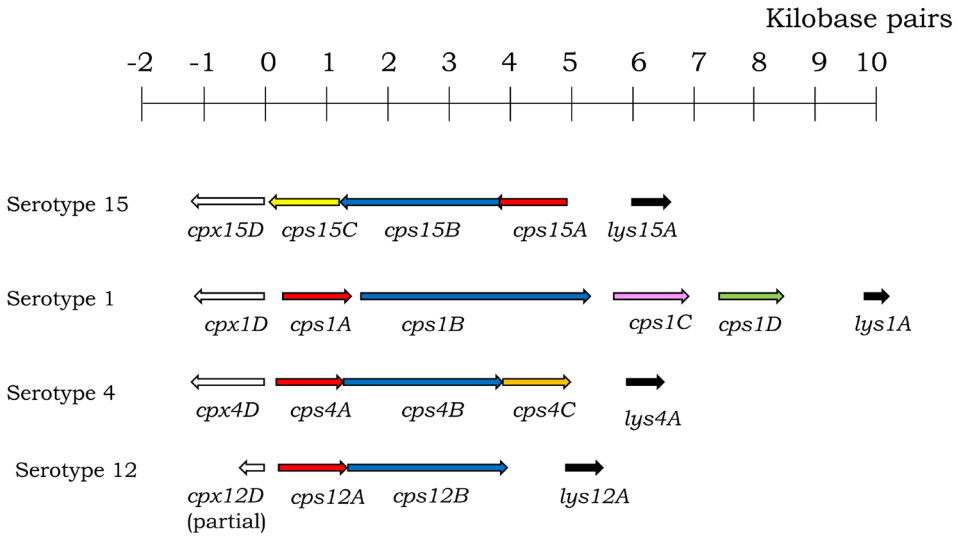

Fig. 1. Schematic diagram of the DNA region involved in CPS of *A. pleuropneumoniae* serotype 1 (accession numbers (nos.) AF518558 [2] and ADOD00000000 [26]), 4 (accession nos. GU585380 [unpublished] and ADOF00000000 [26]) and 12 (accession. nos. AY496881 [16] and ADOL00000000 [26]). Arrows of the same color indicate open reading frames of encoding proteins exhibit amino acid sequence homology.

in this study. Purified genomic DNA from serotype 15 strain HS143 was digested with restriction enzymes *Eco*RI and *Hind*III, 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 50μl 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 phylogeneticaly 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	G+C content (%) of *cps15* encoding Cps15	Bacterial species	Serotype	Homologous protein	Accession number	Reference	% Identity	Length over homologous aa
Cps15A	393	26.9	As[b]	K2	ORF1	EU048554	Unpublished	67.6	364
			Ap[c]	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	26.8	Mv[e]		Glycosyl tranferase	CP006953	Unpublished	38	829
			Ap	4	Cps4B	GU585380	Unpublished	36.8	862
			As	K2	ORF2	EU048554	Unpublished	37	862
			As	K?	ORF2	EU077419	Unpublished	37	862
			As	K1	ORF2	AF518558	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	AY496882[f]	[16]	33	221
Cps15C	380	34.2	Cr[g]		Hp[h]	CP002857	[23]	34.4	360
			Nm[i]	Z[j]	CapZD[k]	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) *A. suis*; c) *A. pleuropneumoniae*; d) Protein name was designated in this study as named in *A. suis* serotype K1 [Accession no. AY253301]; e) *Mannheimia varigena*; f) Partial sequence; g) *Corynebacterium resistens*; h) Hypothetical protein; i) *Neisseria meningitidis*; j) Serogroup; k) CapzZD is a synonym for CszD.

revealed that *A. pleuropneumoniae* serotype 15 carries a gene for the CPS phosphotransferase (*cps15A*) which may be involved in the chemical linkage of phosphates in the linear CPS backbone [26]. This, in turn, indicates that *A. pleuropneumoniae* serotype 15 belongs to Group I. This study also revealed that 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 *cpxD* and *lysA* 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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