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1 **The genetic organisation of the capsule biosynthesis region of**
2 ***Actinobacillus pleuropneumoniae* serotypes 1, 6, 7, and 12**

3
4
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17 **Abstract**

18 The aim of the present study was to investigate the organisation of the genes (*cps*) involved in
19 biosynthesis the capsular polysaccharide (CPS) of *Actinobacillus pleuropneumoniae*
20 serotypes 6, 7, and 12 and to compare these to the corresponding genes previously described
21 in other *A. pleuropneumoniae* serotypes. In serotype 6 and 7 the sequenced DNA regions
22 comprised five and four open reading frames, respectively, designated *cps6ABCDE* and
23 *cps7ABCD*, whereas the sequenced DNA region in serotype 12 comprised only two open
24 reading frames designated *cps12AB*. At the amino acid level, CpsA, CpsB, and CpsC of *A.*
25 *pleuropneumoniae* serotypes 2, 6, 7, and 8 contained a high degree of homology. At the
26 amino acid level Cps6D revealed a high degree of homology to Cps8D, whereas Cps7D
27 contained a high degree of homology to the Cps2D. The deduced gene product of the partially
28 sequenced *cps6E* gene showed no homology to any deduced gene products of any *cps* genes
29 of *A. pleuropneumoniae* investigated so far. None of the deduced gene products of the *cps*
30 genes involved in encapsulation of *A. pleuropneumoniae* serotypes 2, 6, 7, and 8 revealed
31 homology to the deduced gene products of the *cps* genes of serotypes 1, 5A, and 12. For some
32 genes, a local homology was found to genes probably involved in teichoic acid synthesis in
33 other bacteria. The results obtained revealed a high degree of homology among the genes
34 involved in CPS biosynthesis for serotypes 2, 6, 7, and 8 and a different group of homologous
35 *cps* genes for serotype 1 and 12. In some serotype 7 strains, including the serotype 7 reference
36 strain, WF83, the *cps* genes were not located adjacent to the genes responsible for CPS export
37 (*cpx*), probably due to genetic rearrangements.

38

39

40 Introduction

41 *Actinobacillus pleuropneumoniae* is the etiologic agent of porcine pleuropneumonia, a
42 highly contagious and occasionally fatal respiratory disease in pigs (Shope, 1964). The
43 disease is found worldwide and causes large economic losses to the swine industry
44 (Gottschalk and Taylor, 2006). The virulence of *A. pleuropneumoniae* is not well understood,
45 but it seems clear that more than one virulence factor is involved (Haesebrouck et al., 1997;
46 Inzana, 1991). Factors responsible for virulence include extracellular toxins,
47 lipopolysaccharides, capsular polysaccharides (CPS), and membrane proteins.

48 The capsule provides protection of the bacterium from phagocytosis and complement-
49 mediated killing and is required for virulence (Inzana and Mathison, 1987, Inzana et al., 1988,
50 1993; Ward and Inzana, 1994; Ward et al., 1998; Rioux et al., 2000). The CPS of *A.*
51 *pleuropneumoniae* is the predominant serotype-specific antigen and 15 different serotypes
52 have been recognized on the basis of the CPS structure (Fodor et al., 1989; Perry et al., 1990;
53 Nielsen et al., 1997; Blackall et al., 2002).

54 The DNA region involved in CPS export (*cpx*) of *A. pleuropneumoniae* serotype 5a
55 has been identified and consists of four genes designated *cpxDCBA* (Ward and Inzana, 1997).
56 In addition, the DNA region involved in CPS biosynthesis (*cps*) in *A. pleuropneumoniae*
57 serotypes 1, 5a, and 8 has been reported (Ward et al., 1998; Bandara et al., 2003; Schuchert et
58 al., 2004). In all three serotypes this DNA region is located upstream and on the opposite
59 strand of the genes involved in export of the capsule.

60 In the present paper, we report the organisation of genes involved in biosynthesis of
61 the CPS in *A. pleuropneumoniae* serotypes 1, 6, 7, and 12. The genetic organisation and
62 homology to CPS biosynthesis genes reported from other *A. pleuropneumoniae* serotypes as
63 well as other bacterial species are discussed.

64

65 **Materials and methods**66 *Bacterial strains and growth conditions*

67 Strains used in this study are listed in Table 1. All *A. pleuropneumoniae* strains were
68 grown in brain heart infusion broth (Difco Laboratories, Detroit, Mi.) supplemented with 5
69 µg/ml β-NAD (Sigma Chemical Co., St. Louis, Mo.). Serotyping was done according to Giese
70 et al. (1993), *Escherichia coli* strains DH5α (Invitrogen, Carlsbad, Calif) and XL1-Blue
71 (Stratagene, La Jolla, Calif.) were grown in Luria-Bertani broth (Difco Laboratories). *A.*
72 *pleuropneumoniae* capsule genes were cloned in the plasmid vectors pBluescript (Stratagene)
73 and pUC18 or pUC19 (Pharmacia, Uppsala, Sweden). Ampicillin (100 µg/ml) was used for
74 selection and maintenance of plasmids in *E. coli*.

75

76 *Recombinant DNA methods*

77 Genomic DNA was extracted using a QIAamp Tissue Kit (Qiagen ,Hilden, Germany).
78 Plasmid DNA was isolated from *E. coli* cells using QIAprep Spin Miniprep Kit (Qiagen.).
79 The method Easy Gene Walk was used as described elsewhere (Harrison et al., 1997) for
80 serotype 12. DNA was eluted from agarose gels using Qiagen Gel Extraction Kit (Qiagen.).
81 Restriction enzymes were obtained from New England Biolabs (Beverly, MA). Ligations
82 were performed with T4 DNA Ligase as described by the manufacturer (USB, Cleveland,
83 OH). Recombinant plasmids were used to transform competent cells according to the
84 supplier's instructions. The transformants were screened by colony hybridization with a
85 digoxigenin-labelled probe.

86 Digoxigenin-labeled probes, were prepared according to the manufacturer's
87 instructions (Boehringer, Mannheim, Germany). Southern blots of digested genomic DNA
88 were hybridized at 60°C or 65°C, and membranes were developed according to the Genius
89 system (GENIUS Technologies Inc., San Ramon, CA.) or using the DIG Wash and Block
90 buffer Set (Boehringer, Mannheim, Germany).

91

92 *PCR amplification*

93 Lysates of pure bacterial cultures were prepared for PCR by boiling a 10- μ l loopful of
94 bacteria in 200 μ l sterile water. PCR was performed in a total volume of 50 μ l. The reaction
95 mix contained 10 mM Tris-HCl (pH 8.3); 1.5 mM MgCl₂; 50 mM KCl; 0.005% Tween 20;
96 0.005% Nonidet P-40 detergent; 100 μ M (each) dATP, dCTP, dGTP, and dTTP and 0.5 unit
97 Taq polymerase (PerkinElmer, Boston, MA). Each primer (Table 2) was added to a final
98 concentration of 0.5 μ M and 1 μ l of bacterial lysate was added as template. Mineral oil was
99 added to prevent evaporation. The PCR conditions were an initial denaturation at 94°C for 3
100 min, followed by 30 cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at
101 52°C and 1 min of extension at 72°C. Amplification products were analysed by gel
102 electrophoresis, stained with ethidium bromide (10 μ g/ml), and photographed under UV light.

103

104 *DNA sequencing and analysis*

105 Cloned fragments were PCR amplified as described above using vector-specific primers and
106 purified with spin columns (Qiagen, Hilden Germany). Nucleotide sequencing was done with
107 an ABI377 automatic sequencer using the Prism BigDye terminator cycle sequencing kit
108 (Applied Biosystems, Foster city, CA) and vector- and fragment-specific primers (primer

109 sequences are available on request). Sequence data were assembled and analysed using the
110 programs Factura (Applied Biosystems), Autoassembler (Applied Biosystems), and DNASIS
111 (Hitachi Software Engineering Co, Ltd.). Phylogenetic analyses were conducted using MEGA
112 version 3.1 (Kumar et al., 2004). Homology searches of the GenBank databases were
113 performed using the BLAST server at the National Center for Biotechnology Information.

114

115 *Nucleotide sequence accession number*

116 The nucleotide sequences of the CPS biosynthesis genes of *A. pleuropneumoniae* serotypes 6,
117 7, and 12 have been deposited in GenBank under accession number AY534316, AY534317
118 and AY496881, respectively. A 3598 bp sequence of the of the *cps* operon in serotype 1 strain
119 4074^T is deposited in GenBank under accession number XY496882.

120

121 **Results**

122 *Cloning of cps genes*

123 For cloning the *cps* genes, the earlier published adjacent *cpxD* sequences of *A.*
124 *pleuropneumoniae* formed the basis for the PCR generated probes that were used for
125 identification of the homologous regions in *A. pleuropneumoniae* serotype 6, 7, and 12.
126 The *cps* region of *A. pleuropneumoniae* serotype 6 was identified by Southern blotting with a
127 probe specific to the conserved DNA region of the *cpxD* gene of *A. pleuropneumoniae*
128 serotype 5 (Ward and Inzana, 1997). In the reference strain Femø the probe hybridised to a
129 4.7 kb *EcoRI/HincII* fragment, which was subsequently cloned into pBluescript and
130 sequenced. A DNA probe specific for the 3' end of this sequence was designed and used to

131 identify an adjacent 3.6 kb *EcoRI/KpnI* fragment. In total 7.9 kb of DNA from the *cps* region
132 was sequenced.

133 A 3.5 kb *EcoRI/BamHI* fragment from the *A. pleuropneumoniae* serotype 7 reference
134 strain WF83 was cloned in pUC18 and sequenced as described above. The 5' end of the
135 sequenced DNA showed homology to the *cpxD* gene of serotype 5, but no homology was
136 found to genes involved in the capsule biosynthesis region of other *A. pleuropneumoniae*
137 serotypes or to other organisms. A portion of a DNA region adjacent to the *cpx7D* gene
138 revealed more than 97% homology to a sequence encoding a beta-lactamase of other
139 organisms. To confirm these results, genomic DNA from the reference strain of *A.*
140 *pleuropneumoniae* serotype 7 (WF83) and from five Danish field isolates of serotype 7 were
141 used as template in a PCR that amplified 623 bp of the *cpx7D* gene and the adjacent DNA
142 with similarity to the beta-lactamase homologous DNA in strain WF83. The reference strain
143 and one of the Danish field isolates were positive in this PCR (indicating that they have a
144 beta-lacamase gene adjacent to the *cpxD* gene), while the four remaining field isolates were
145 negative in this variant-specific PCR. The existence of two different DNA sequences within
146 serotype 7 was confirmed by Southern blot analysis using a part of *cpxD* as probe (data not
147 shown). Nonetheless, all the serotype 7 strains were indistinguishable by serotyping.

148 The *cpx* region of *A. pleuropneumoniae* serotype 7 field strain 72-14676 (one of the
149 PCR-negative strains described above) was identified by Southern blotting with a probe
150 specific to the *cpxD* gene of the conserved DNA region of *A. pleuropneumoniae* serotype 5.
151 The probe hybridized to a 4 kb *PstI* fragment that was cloned and sequenced. Subsequent
152 chromosome walking enabled the cloning of the complete *cps* region, which comprised 7.4 kb
153 of DNA.

154 The *cps* region of *A. pleuropneumoniae* serotype 12 was amplified by PCR, using
155 primers specific for the conserved *cpxD* gene. A DNA region of 2876 bp upstream of the
156 *cpxD* gene was determined using easy gene walking. A DNA probe specific for the 3' end of
157 this region was designed and used to identify a 5.2 kb *Bgl*III fragment, which was cloned into
158 the *Bam*HI site of the vector pBluescript and sequenced. A total of 6.8 kb were sequenced.

159

160 *Genetic organisation of the cps region of A. pleuropneumoniae serotype 6 and 7*

161 In serotype 6 five open reading frames designated *cps6A*, *cps6B*, *cps6C*, *cps6D*, and
162 *cps6E* were found; the *cps6E* gene was only partially sequenced (Fig. 1). In comparison with
163 *cps2D* (1288 aa) and *cps7D* (1237 aa), *cps6D* (986 aa) was smaller. To confirm the stop
164 codon of the *cps6D* gene, the junction between the *cps6D* and *cps6E* gene was amplified by
165 PCR and sequenced in two Danish field isolates of *A. pleuropneumoniae* serotype 6. The
166 nucleotide sequences obtained from these two field isolates were identical to the nucleotide
167 sequence of the corresponding region in the reference strain Femø of *A. pleuropneumoniae*
168 serotype 6. All five open reading frames were located upstream and on the opposite strand
169 from the *cpxD* gene (Fig 1). The G+C content of the *cps6ABCDE* region ranged from 28.9%
170 for the *cps6E* gene to 36.4% for the *cps6B* gene, which is lower than the 42% overall G+C
171 content of the *A. pleuropneumoniae* genome. The deduced gene products of the four complete
172 open reading frames consisted of 378 aa, 142 aa, 380 aa, and 985 aa, respectively. The
173 partially sequenced *cps6E* gene encoded 290 aa.

174 In serotype 7, four complete open reading frames, designated *cps7A*, *cps7B*, *cps7C* and
175 *cps7D*, were identified in strain 72-14676. As in other *A. pleuropneumoniae* serotypes, all
176 open reading frames were located upstream and on the opposite strand from the *cpxD* gene
177 (Fig 1). The G+C content of the *cps7ABCD* region ranged from 31.1% for the *cps7D* gene to

178 36.4% for the *cps7B* gene, which is lower than the 42% overall G+C content of the *A.*
179 *pleuropneumoniae* genome. The deduced gene products of the four open reading frames
180 encoded 378 aa, 142 aa, 380 aa, and 1237 aa, respectively.

181

182 *Genetic organisation of the cps region of A. pleuropneumoniae serotypes 12 and 1*

183 Two open reading frames designated *cps12A* and *cps12B* were found in serotype 12
184 (Fig. 1). Both open reading frames were located upstream and on the opposite strand from the
185 *cpx12D* gene. Another ORF was found 954 bp further downstream from *cps12B*, showing a
186 strong homology (> 75% aa homology) to diaminopimelate decarboxylase (LysA) from
187 several closely related species, indicating that it should not be regarded as part of the *cps*-
188 genes in region 2. The G+C content of *cps12A* and *cps12B* were 29.5% and 24.6%
189 respectively, which is considerably lower than the overall G+C content of the *A.*
190 *pleuropneumoniae* genome (42%). The deduced gene products of the two open reading
191 frames consisted of 371 aa and 874 aa, respectively.

192 The organisation of the *cps12* genes differed from what has earlier been found in the
193 other Group I capsule, serotype 1, where three genes were identified (Bandara et al., 2003);
194 *cps1A* being homologous to *cps12A*, and *cps1B* and *cps1C* being homologous to the 3' and 5'
195 ends respectively of the *cps12B* gene. To address this discrepancy, further primers were
196 designed to amplify 668 bp of the 3' end of the *cps1B* gene and the 5' end of the *cps1C* gene
197 to confirm the existence of the stop codon and thus the existence of three *cps1* genes. DNA
198 was amplified from strain 4074, from three Danish field isolates as well as three Canadian
199 isolates of *A. pleuropneumoniae* serotype 1. None of these strains contained the putative stop
200 codon of *cps1B*. Further, we sequenced 3598 bp of the *cps* region of strain 4074. This
201 sequence contained two open reading frames, *cps1A* and *cps1B*, homologous to the genes

202 found in serotype 12 (Fig 1). We therefore conclude that the *cps* operon of serotype 1 only
203 consists of two genes, which will be referred to as *cps1A* and *cps1B* in the rest of this
204 presentation.

205

206 *Homology between cps genes of A. pleuropneumoniae serotypes*

207 The *cps* genes of serotypes 2, 3, and 8 were available from GenBank (from serotype 3
208 only *cps3A* and *cps3B* are yet available). At the nucleotide level, a high degree of homology
209 was found between the *cpsABC* genes of *A. pleuropneumoniae* serotypes 2, 3, 6, 7, and 8. The
210 *cps6E* gene showed no homology to *cps* genes of *A. pleuropneumoniae* serotypes 2, 7, and 8.

211 The homology of the amino acid sequences of the genes of the *cps* region of *A.*
212 *pleuropneumoniae* serotypes 2, 3, 6, 7, and 8 is shown in Fig.2. CpsB is identical in serotypes
213 2, 6, and 7; while the Cps8B and Cps3B sequences deviate in 4 and 5 of the 142 aa,
214 respectively. For the *cpsA* and *cpsC* gene products, serotypes 2 and 6 are closely related with
215 an increasing dissimilarity to serotype 7 and serotype 8, respectively. This pattern is
216 completely different when comparing the *cpsD* sequences, where serotypes 6 and 8 are
217 grouped together as are the sequences of serotypes 2 and 7 (Fig.2).

218 None of the *cps* genes or their deduced gene products of serotypes 2, 3, 6, 7, and 8
219 showed any homology to *cps* genes or their corresponding gene products found in the other *A.*
220 *pleuropneumoniae* serotype groups (1, 5a, and 12). At the amino acid level there was 93 %
221 identity between Cps1A and Cps12A. Between Cps1B and Cps12B there was much lower
222 identity, only 33%. Cps6E did not reveal any homology to deduced protein products of *cps*
223 genes from any *A. pleuropneumoniae* serotypes investigated so far.

224

225 *Homology with genes from other bacteria*

226 At the amino acid level, CpsA from both serotypes 6 and 7 showed no overall
227 homology to proteins of other organisms. The deduced protein sequence of Cps6B revealed
228 75% homology to CapZ from *Neisseria meningitides* and more than 70 % homology to
229 deduced coding sequences from several other organisms. The deduced protein products
230 Cps6C and Cps7C showed over 40% homology to CapZB from *N. meningitides*. Cps6D
231 revealed no overall homology to proteins from other bacteria. Approximately 34% local
232 homology was found to putative glycosyl/glycerophosphate transferases involved in teichoic
233 acid biosynthesis from a number of other bacteria. In contrast, Cps7D showed 63% homology
234 to CapZC from *N. meningitides* and 40% homology to a hypothetical protein from *E. coli*.
235 The deduced gene product of the partially sequenced *cps6E* gene revealed approximately 27%
236 local homology with glycosyltransferases involved in teichoic acid biosynthesis from a
237 number of other bacteria. *Cps12A* showed 89% homology to a putative capsular
238 polysaccharide phosphotransferase in *Actinobacillus suis* and more than 50% homology to
239 putative genes in several bacterial species. *Cps12B* showed 57% homology to putative acetyl
240 transferases from *N. meningitidis* serogroup L and 34% homology to capsular genes of *A. suis*
241 serotype 1.

242

243 Discussion

244 In the present investigation, a DNA region involved in CPS biosynthesis (*cps*) of *A.*
245 *pleuropneumoniae* serotype 6 strain Femø, serotype 7 isolate 72-14676 and serotype 12 strain
246 8326 was found adjacent to a DNA region (*cpx*) associated with export of CPS to the cell
247 surface. A similar genetic organisation of the *cpx* and *cps* genes has previously been found in
248 *A. pleuropneumoniae* serotypes 1 and 5a (Ward et al., 1998; Bandara et al., 2003). The
249 genetic organisation of the genes involved in encapsulation of *A. pleuropneumoniae* are also

250 similar to the organisation of capsule gene clusters in other Gram-negative bacteria, including
251 the K antigens of *E. coli* (Roberts et al., 1988), *H. influenzae* type b (Satola et al., 2003), *P.*
252 *multocida* A:1 (Chung et al., 1988), and *M. haemolytica* A1 (Lo et al., 2001). In these
253 bacteria, the gene clusters involved in encapsulation are divided into three distinct regions
254 (Roberts, 1996). Regions 1 and 3 are conserved among all species analyzed and encode
255 functions involved in export of the capsular polysaccharide. Region 2, which is located
256 between regions 1 and 3, is serotype specific and encodes proteins involved in synthesis of the
257 capsule. However, region 3 has not been reported to be adjacent to any of the *A.*
258 *pleuropneumoniae cps* genes investigated so far and was not found in the present study.

259 The CPS structures produced by *A. pleuropneumoniae* serotypes 1 to 13 and 15, can
260 be divided into 3 three groups, based on structural differences (Perry et al., 1990, 2005;
261 MacLean et al., 2004). Serotypes 1, 4, 12 and 15 constitute Group I of CPS composed solely
262 of repeating oligosaccharide units joined through phosphate linkages. While serotypes 1 and
263 12 have been investigated, the *cps* genes of serotype 4 and 15 have not yet been reported. In
264 Group II with CPS composed of repeating oligosaccharide units only serotype 5a has been
265 sequenced, the structure of the *cps* genes of serotype 10 has still not been analysed. The third
266 group described by Perry et al. (1990, 2005), including serotypes 2, 3, 6, 7, 8, 9, 11, and 13,
267 produce CPS composed of teichoic acid polymers joined through phosphate diester linkages.
268 The *cps* genes of serotypes 2, 3, 6, 7, and 8 have been sequenced. The genetic organisation of
269 the *cps* genes of the *A. pleuropneumoniae* serotypes investigated so far corresponds to the
270 structural classification of the polysaccharide capsule described by Perry et al. (1990). It can
271 be expected that the *cps* genes of the serotypes not yet sequenced will show homology to the
272 *cps* genes of the serotypes belonging to the same CPS group.

273 Five genes, *cps6ABCDE*, were identified in the reference strain Femø of *A.*
274 *pleuropneumoniae* serotype 6, whereas four genes, *cps7ABCD*, were identified in the Danish
275 field isolate 72-14676 of *A. pleuropneumoniae* serotype 7. This organisation is in agreement
276 with the finding of genes in the earlier sequenced capsular Group III strains, serotype 2 and 8,
277 (Fig. 1). A high degree of homology was found between the *cpsABC* genes of *A.*
278 *pleuropneumoniae* serotypes 2, 6, 7, and 8 at both the nucleotide and the amino acid level,
279 and contained almost the same number of amino acid residues (Fig. 2). These results indicate
280 a similar function for CpsA, CpsB, and CpsC in the four *A. pleuropneumoniae* serotypes. A
281 high homology for the *cpsA* and *cpsB* genes in serotype 3 was also found to the corresponding
282 genes in serotypes 2, 6, 7, and 8. In contrast, the *cpsD* genes were more heterogeneous. The
283 complete sequences of the *cpsD* gene of serotypes 2, 6, and 7 revealed that Cps6D contained
284 only 986 aa compared to more than 1200 aa in both serotypes 2 and 7. The *cps8D* gene has
285 only been partially sequenced and the length of this gene is therefore not known. To
286 investigate whether the unexpected stop codon of the *cps6D* gene exists, the relevant region
287 was sequenced in two Danish field isolates of serotype 6. In all three strains the sequence was
288 identical, and the existence of an additional gene, *cps6E* was confirmed and partially
289 sequenced. This sequence showed no significant homology to any *cps* genes of other *A.*
290 *pleuropneumoniae* serotypes, but some local homology to putative glycosyltransferases of
291 other bacterial species, suggesting that a fifth gene actually could be involved in capsule
292 biosynthesis in serotype 6. Also noteworthy is, that in *cpsA*, *cpsB*, and *cpsC* the sequences of
293 serotypes 2 and 6 are very similar and more distantly related to serotypes 7 and 8. In the *cpsD*
294 genes much more variation was observed and the clustering was opposite; serotypes 2*cpsD*
295 and 7*cpsD* were similar and only distantly related to the same cluster in serotype 6 and 8.

296 In the reference strain WF83 of *A. pleuropneumoniae* serotype 7, the organisation of
297 capsule biosynthesis genes appeared different from what was observed in other strains with a
298 Group III capsule. A part of the DNA region adjacent to the *cpxD* gene in strain WF83 was
299 similar to sequences encoding a beta-lactamase of other bacteria. PCR and Southern blot
300 analysis showed that the *cpxD* genes of the tested field strains was similar to that of WF83,
301 while in the remaining four strains the *cps* genes were organised as in the serotype 6 reference
302 strain. These results showed that the genomic location of the *cps* genes can vary among
303 different, but phenotypically identical, strains of *A. pleuropneumoniae* serotype 7. Whether
304 this kind of genetic variation is widespread in other serotypes has yet to be determined.

305 All deduced gene products of the *cps* genes described in *A. pleuropneumoniae*
306 serotypes 6 and 7 revealed homology to putative glycosyl and/or glycerophosphate
307 transferases involved in teichoic acid biosynthesis in other bacteria, including the Gram-
308 positive bacterium *Bacillus subtilis* (Pooley et al., 1991).

309 In serotypes 1 and 12, we found only two genes, *cps1AB* and *cps12AB* (Fig. 1). The
310 sequences of the *cps* genes of *A. pleuropneumoniae* serotype 1 have been published by
311 Bandara et al. (2003), who found three genes within the biosynthesis region. As this
312 organisation deviated from that of serotype 12, we sequenced a large portion of the *cps* region
313 of strain 4074 (3598 bp), and found only two genes as in serotype 12. To investigate this
314 sequence discrepancy further, we sequenced a 668 bp fragment from 3 field strains from
315 Denmark and from three strains from Canada. All of these sequences were identical to the
316 sequence we obtained from strain 4074. Thus, serotype 1 strains from Europe and from North
317 America are similar and the sequence AY496882 reflects the *cps* sequence of *A.*
318 *pleuropneumoniae* serotype 1. In serotypes 1 and 12 the amino acid sequences of CpsA are

319 very similar (92%), whereas in CpsB they are more distantly related, only showing 33%
320 homology.

321 The G+C content of the *cps6ABCDE*, *cps7ABCD*, *cps1AB*, and *cps12AB* regions was
322 considerably lower than the 42% overall G+C content of the *A. pleuropneumoniae* genome
323 (Kilian et al., 1978). The same results have been obtained for the *cps* regions of other *A.*
324 *pleuropneumoniae* serotypes and for the corresponding region in *H. influenzae* type b (Van
325 Eldere et al., 1995; Ward et al., 1998; Bandara et al., 2003). The low G+C content could
326 suggest a heterologous origin for the *cps* genes involved in biosynthesis of the CPS (Ochman
327 et al., 2000). It is an interesting observation that the first gene among strains having a Group I
328 capsule and similarly the first three genes among the strains having a Group III capsule are
329 very similar. On the other hand, there is considerable variation in the genes further
330 downstream in the operons. The existence of *cps* genes that are very similar while other *cps*
331 genes are not, could suggest that horizontal gene transfer has been part of the evolution of *A.*
332 *pleuropneumoniae* capsule forming genes.

333 In conclusion, DNA regions involved in the CPS biosynthesis of *A. pleuropneumoniae*
334 serotypes 6, 7, and 12 have been sequenced and characterized. A high degree of homology
335 was found at the nucleotide and amino acid levels between the *cpsA*, *cpsB*, and *cpsC* genes of
336 *A. pleuropneumoniae* serotypes 2, 6, 7, and 8 and between the *cpsA* and *cpsB* genes of
337 serotype 1 and 12. The results obtained in this study revealed a common genetic organisation
338 of the genes involved in CPS biosynthesis of *A. pleuropneumoniae* serotypes 2, 6, 7, and 8 and
339 serotype 1 and 12, respectively.

340

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348 **Legend to Figure 1:**

349 Schematic diagram of the genetic organisation of the DNA region involved in the capsular
350 polysaccharide of *A. pleuropneumoniae* serotypes 1 (GenBank accession no. AF518558,
351 corrected according to AY496882), 2 (GenBank accession no. AY357726), 5a (GenBank
352 accession no. AF053723), 6 (GenBank accession no. AY534316), 7 (GenBank accession no.
353 AY534317), 8 (GenBank accession no. AY356527), and 12 (GenBank accession no.
354 AY496881). The sequences were obtained from the reference strains of *A. pleuropneumoniae*
355 serotypes 1, 2, 5a, 6, 8, and 12, whereas the *cps* sequence of serotype 7 was obtained from the
356 Danish field isolate 72-14676. Serotype 3 is not included as only the two first genes of the
357 operon are yet available. The arrows indicate the location and direction of transcription of the
358 sequenced *cps* genes. The black arrows indicate complete open reading frames, whereas white
359 arrows indicate incomplete genes.

360

361 **Legend to Figure 2:**

362 Neighbour-joining tree showing the relationship between the deduced amino acid sequences
363 of CpsA, CpsB, CpsC and CpsD of *A. pleuropneumoniae* serotypes 2, 6, 7, and 8. For
364 serotype 3 only the sequences of CpsA and CpsB are yet available. The bar indicates
365 substitutions per site. Note the different scale in CpsD.

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456 3336.

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458TABLE 1. *Actinobacillus pleuropneumoniae* strains used in this study

Number of strains	Description	Source
1	4074 ^T = ATCC 27088. Type strain and reference strain of serotype 1	ATCC ^a
3	Danish field isolates of serotype 1	NVI ^b
3	Canadian field isolates of serotype 1	K.R. Mittal ^c
1	Femø. Reference strain of serotype 6	NVI
2	Danish field isolates of serotype 6	NVI
1	WF83. Reference strain of serotype 7	NVI
5	Danish field isolates of serotype 7	NVI
1	8329. Reference strain of serotype 12	NVI

459

^a ATCC, American Type Culture Collection, Manassas, Va.; ^b NVI, National Veterinary

460

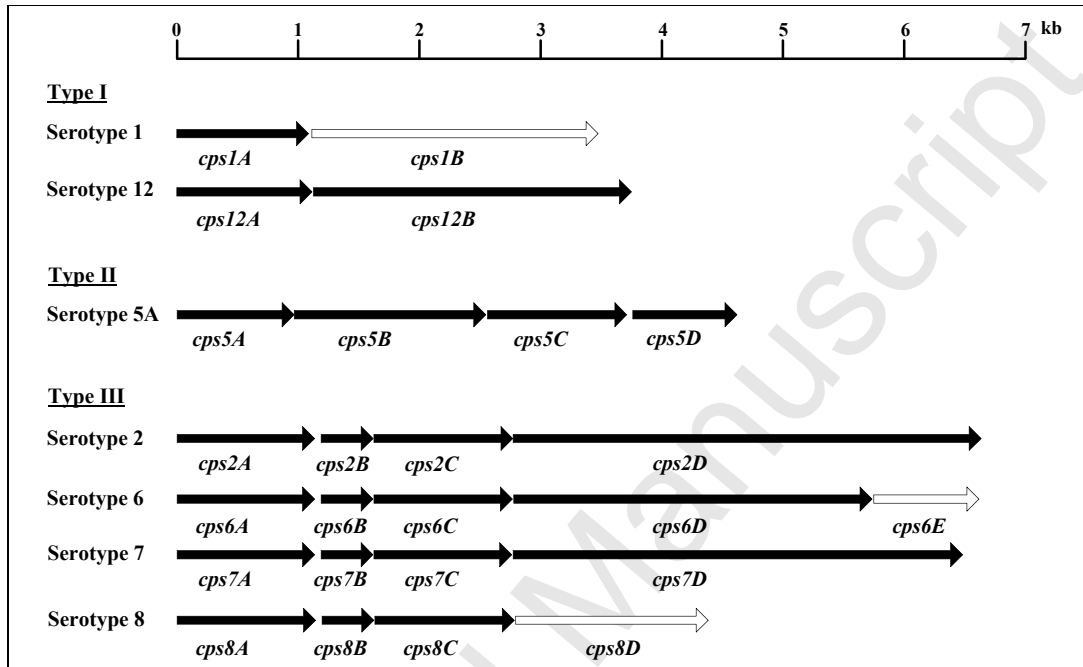
Institute, Copenhagen, Denmark; ^c Université de Montréal, Québec, Canada.

461 TABLE 2. PCR primers used in the present study

462 Serotype 1: *cps1B*–*cps1C* junction (773 bp)463 *cps1BF* 5'- GATGATTTTCTTAGCCTAA -3'464 *cps1CR* 5'- GCTTTTTCTCACGGTCAAT -3'465 Serotype 6: *cps6D*–*csp6E* junction (660 bp)466 Ap6 *cpsDF* 5'- CATAACTCTATTGATGAGGAA -3'467 Ap6 *cpsER* 5'- ACAAGGTACAAGAATCGAAA -3'468 Serotype 7: Junction between *cpxD* and the adjacent region (623 bp)469 Ap7 *cpxDF* 5'-GGTTACCGTCCCGTTTTGA -3'470 Ap7 *blaR* 5'- CGGTAGGAAGTACTCATCATT -3'471

472 FIG. 1.

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