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First Chromosomal Restriction Map of *Actinobacillus pleuropneumoniae* and Localization of Putative Virulence-Associated Genes

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Combined physical and genetic maps of the genomes of *Actinobacillus pleuropneumoniae* AP76 (serotype 7 clinical isolate) and of *A. pleuropneumoniae* ATCC 27088 (serotype 1 reference strain) were constructed by using the restriction endonucleases *Apa*I, *Asc*I, *Not*I, and *Sal*I. The chromosome sizes as determined by the addition of estimated fragment sizes were 2.4 Mbp, and both maps had a resolution of approximately 100 kbp. The linkages between the *Apa*I, *Asc*I, *Not*I, and *Sal*I fragments and their relative positions were determined by (i) fragment excision and redigestion and (ii) partial digests of defined fragments and Southern blot using end-standing probes. The single *Sal*I site within the chromosome of strain *A. pleuropneumoniae* AP76 was defined as position 1 of the map; for the map of *A. pleuropneumoniae* ATCC 27088, the corresponding *Sal*I site was chosen. Putative virulence-associated genes (*apx*, *omlA*, *sodA*, *tbpBA*, *ureC*, and a repeat element) and housekeeping genes (*glyA*, *metJ*, *recA*, and *rhoAP*) were positioned on the physical maps and located on the *Apa*I and *Not*I fragments of *A. pleuropneumoniae* serotype reference strains.

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a severe and economically important disease occurring worldwide (9). *A. pleuropneumoniae* isolates can be differentiated into 12 distinct serotypes (29) which are based on polysaccharide antigens of the slime capsule and the lipopolysaccharide of the bacteria (40). The serotypes have been described to be of clonal origin (27). The different serotypes have been also grouped based either on outer membrane protein profiles (32) or on antigenic similarities (28) which are most likely due to shared species-specific antigens such as lipopolysaccharide or membrane proteins (31). More recently, Chevallier and coworkers assessed the phylogenetic relationship among the different serotype reference strains based on restriction enzyme fragment polymorphisms after pulsed-field gel electrophoresis (PFGE) (7).

For *A. pleuropneumoniae*, several putative virulence-associated factors such as the RTX toxins ApxI, ApxII, and ApxIII (2, 6, 12, 19, 20, 24, 33, 37), the transferrin binding proteins TbpA and TbpB (13, 15, 25, 41), a superoxide dismutase (SodA [23]), a urease (4), an outer membrane lipoprotein (OmlA [5, 14, 17, 18]), and capsular antigens (16, 40) have been characterized. The RTX toxins are distributed in a serotype-specific manner with each serotype containing no more than two of the Apx toxins (12, 20), and the TbpB and OmlA proteins occur in three antigenically distinct isoforms (13, 17).

Despite this detailed information on serotype classification and individual virulence-associated factors no information on the genomic structure of *A. pleuropneumoniae* is available. As shown for other bacteria (22, 26, 34, 39), such information is required for a detailed understanding of virulence-associated mechanisms. Therefore, we have constructed the first physical and genetic maps of an *A. pleuropneumoniae* serotype 7 clinical isolate (AP76 [2]) and the *A. pleuropneumoniae* serotype 1 reference strain (ATCC 27088); these two strains were chosen since *A. pleuropneumoniae* ATCC 27088 is the strain most frequently investigated, and *A. pleuropneumoniae* AP76 is the only strain in which spontaneous chromosomal deletions have been shown to occur. In addition, we compared the presence and location of a panel of genes in the other *A. pleuropneumoniae* serotype reference strains.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and probes. The sources of strains used in this study are given in Table 1; the sources of DNA probes are listed in Table 2. *A. pleuropneumoniae* strains were cultivated in PPLO (pleuropneumonia-like organism) medium (Difco, Detroit, Mich.) with NAD (10 μg/ml) at 37°C in a 5% CO_2 atmosphere. Cultures used for DNA preparation were inoculated with 1/10 volume of an overnight culture, incubated with shaking, and harvested at an optical density at 600 nm of 0.3.

Sample preparation and PFGE. Embedded DNA samples were prepared for PFGE essentially as described by Bautsch (3). Briefly, bacteria were harvested by centrifugation, washed, and resuspended in 1/10 culture volume of PET IV (1 M NaCl, 10 mM Tris-HCl [pH 8], 10 mM Na_2EDTA). Suspensions were mixed 1:1 with 1.2% agarose at 60°C, and gel plugs were poured into molds. Gel plugs were treated 2 h at 37°C with lysis buffer (1 M NaCl, 10 mM Tris-HCl [pH 8], 0.2 M Na_2EDTA, 0.5% *N*-lauroylsarcosine, 0.2% deoxycholic acid) containing RNase (2 μg/ml) and lysozyme (1 mg/ml). Then plugs were treated overnight at 55°C with proteinase K (1 mg/ml) in ES buffer (0.5 M Na_2EDTA, 1% *N*-lauroylsarcosine). Proteinase K was inactivated by two washes with phenylmethylsulfonyl fluoride (1.5 mM) in TE buffer (10 mM Tris-HCl [pH 8], 1 mM Na_2EDTA). Phenylmethylsulfonyl fluoride was eliminated by repeated washing with TE buffer. Restriction enzyme digests were performed after equilibration in buffers supplied by the manufacturer (New England Biolabs, Schwalbach, Germany).

The fragmented DNA was separated on 0.8% agarose (Appligene, Illkirch, France) in 0.5× TBE buffer (45 mM Tris-borate [pH 8], 1 mM Na_2EDTA) in a CHEF-DR III pulsed-field electrophoresis system (Bio-Rad Inc., Hercules, Calif.) as recommended by the manufacturer. Running conditions were 24 h at 12°C and 6 V/cm with linear ramped switch times from 5 to 20 s (for the resolution of fragments of 50 to 300 kbp), from 10 to 40 s (100 to 500 kbp), or from 20 to 80 s (500 to 1,000 kbp). DNA was stained with ethidium bromide (0.5 μg/ml) and visualized in an image documentation system (Gel Doc 1000/Multi-analyst; Bio-Rad).

Single bands were cut from PFGE gels and restriction cleaved like the original agarose plugs to identify products of double digestion by direct comparison in the same gel. The size of large fragments was determined as the sum of the subfragments by this procedure. Partial enzymatic cleavage was carried out by serial dilution of the appropriate enzyme.

DNA hybridization. DNA was transferred to a nylon membrane (Positive; Appligene) by capillary blotting (36). Probe DNAs were obtained by PCR or, where available, from recombinant *Escherichia coli* plasmids (Table 2) by cleavage with appropriate restriction endonucleases.

TABLE 1. *A. pleuropneumoniae* strains used and their sources

Strain	Description	Source
AP76	*A. pleuropneumoniae* serotype 7	Porcine lung isolate provided by Western College of Veterinary Medicine, Saskatoon, Saskatchewan, Canada
ATCC 27088	Reference strain serotype 1	
ATCC 27089	Reference strain serotype 2	
ATCC 27090	Reference strain serotype 3	
ATCC 33378	Reference strain serotype 4	
ATCC 33377	Reference strain serotype 5A	
ATCC 33590	Reference strain serotype 6	
WF83	Reference strain serotype 7	Provided by S. Rosendal, University of Guelph, Guelph, Ontario, Canada
405	Reference strain serotype 8	Provided by R. Nielsen, State Veterinary Serum Laboratory, Copenhagen, Denmark
CVJ13261	Reference strain serotype 9	Provided by R. Nielsen
D13039	Reference strain serotype 10	Provided by R. Nielsen
56153	Reference strain serotype 11	Provided by R. Nielsen
8329	Reference strain serotype 12	Provided by R. Nielsen

Small fragments were mapped by being used as probes in Southern blots with partially digested DNA. Fragments were obtained from the gel by adsorption to a silica matrix (Geneclean; Bio 101, Vista, Calif.) and labeled with biotin-dUTP or with [α-^{32}P]dATP by a random priming method (8). Biotin was detected with avidin-alkaline phosphatase conjugate and chemiluminescence substrate (CSPD; Tropix Inc., Bedford, Mass.) according to the manufacturer's recommendations.

RESULTS

Physical mapping. A physical and genetic map was constructed for the porcine lung pathogen *A. pleuropneumoniae*. The chromosome of *A. pleuropneumoniae* AP76 serotype 7 was mapped first since it had a single *Sal*I site which was defined as map position 1. The chromosome was found to have a size of approximately 2.4 Mbp containing 11 *Apa*I, 6 *Asc*I, and 8 *Not*I fragments with sizes between 9 and 900 kbp (Fig. 1; Table 3). The *Sal*I site was mapped to the fragments APA10, NOT2, and ASC2. The relative position of each fragment was determined by combining results of single and double restriction digests.

By recleaving with *Not*I, the large *Apa*I fragment APA1 (Table 3) was found to contain the three *Not*I fragments NOT4, NOT7, and NOT8 and two additional flanking fragments 68 and 215 kbp in size. The second large *Apa*I fragment (APA2) was found to contain NOT6 and two flanking fragments of about 157 and 64 kbp. APA3 was cut by *Not*I into two fragments of 172 and 106 kbp. The largest *Not*I fragment (NOT1) was cleaved by *Apa*I into fragments APA4, APA6, the small APA11, and two flanking fragments 172 and 215 kbp in size. The 794-kbp *Not*I fragment NOT2 contained the small *Apa*I fragments APA7, APA8, APA9, and APA10 and flanking fragments 64 and 220 kbp in size. Fragment NOT3 was cleaved by *Apa*I into two fragments of 157 and 68 kbp.

Fragments APA5 and NOT5 were shown to overlap by only 11 kbp. The sizes of the *Not*I-*Apa*I subfragments of fragment APA3 were identical to those of the flanking fragments from NOT5 cut with *Apa*I (106 kbp) and from NOT1 cut with *Apa*I (172 kbp) and therefore indicated an overlap. The overlap of fragment NOT1 with fragment APA1 ended with the congruence of the flanking fragments of 215 kbp. The region flanking fragments APA1 and APA2 was determined by the cleavage pattern of the linking fragment NOT3 with *Apa*I into two fragments of 157 and 68 kbp. The overlap from fragment APA2 to fragment NOT2 was determined by the ending linking fragment size of 64 kbp. The overlapping flanking fragment from NOT2 to APA5 was 220 kbp in size and therefore could be confounded with the 215-kbp fragment shared by APA1 and NOT1. This was resolved by Southern hybridization of an

TABLE 2. Genes positioned in the map and origins of probes

Gene	Encoded	Source	Accession no.	Reference(s)
apxICA	Activator C and structural protein A of *apxI* operon	PCR, 5′-TTGCCTCGCTAGTTGCGGAT-3′ 5′-TCCCAAGTTCGAATGGGCTT-3′	X68595	11
apxIBD	Secretion proteins of *apxI* operon	PCR, 5′-GTATCGGCGGGATTCCGT-3′ 5′-ATCCGCATCGGCTCCCAA-3′	X68595	11
apxIICA	Activator C and structural protein A of *apxII* operon	Plasmid pCY76/504, *Nde*I fragment	M30602	2
apxIIICA	Activator C and structural protein A of *apxIII* operon	PCR, 5′-CCTGGTTCTACAGAAGCGAAAATC-3′ 5′-TTTCGCCCTTAGTTGGATCGA-3′	L12145	6
omlA	Outer membrane lipoprotein	PCR, 5′-TAAGGTTGATATGTCCGCACC-3′ 5′-TAGCACCGATTACGCCTT-3′	L06318	14
tolQ-tbpBA	Colicin transport protein, transferrin binding proteins	Plasmid pTF205/405; *Bam*HI-*Nsi*I fragment	Y17916	38, 41
ureC	Large subunit C of bacterial urease	PCR, 5′-GTAAGGATCCATTAACAATCCCACGCAGTCAGTAT-3′ 5′-TCATGTCGACTAGAACAAGAAATAACGCTGTGCAA-3′	U89957	4
sodA	Superoxide dismutase	PCR, 5′-ACGCTTATGATGCGTTAGAGC-3′ 5′-GTCCCAGTTTACCACGTTCCA-3′	U51441	23
glyA	Serine methylase	Plasmid p#4-213-804, *Hin*dIII-*Eco*RI fragment		This work
metJ	Met repressor	Plasmid p#4-213-84; *Xba*I fragment		This work
recA	Repair and recombination protein	PCR, 5′-GAAAARCAATTYGGTAAAGG-3′ 5′-GCYTTWCCTTGRCCAATTTT-3′	U32741	10
rho	Transcription termination factor	Plasmid pFR100; *Kpn*I-*Nsi*I fragment	Y17915	38
Repeat element	Transposon like element	Plasmid pCY76/102; *Bam*HI-*Bgl*II fragment	M74588	2

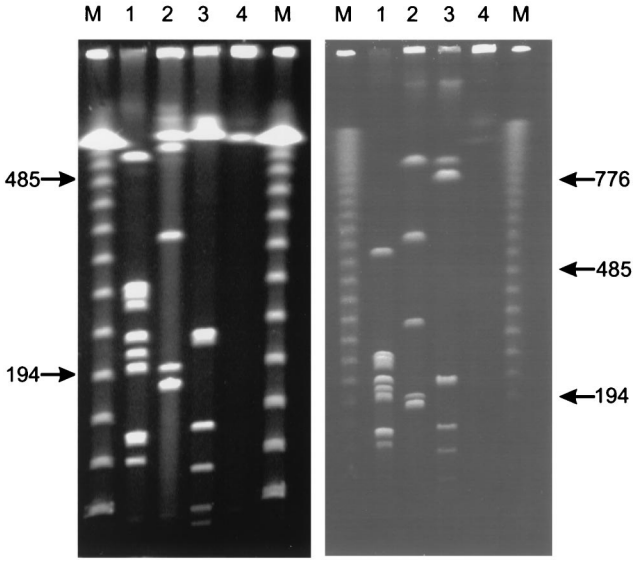

FIG. 1. PFGE separation of *A. pleuropneumoniae* AP76 DNA, using short (left) and long (right) running conditions. Agarose-embedded DNA was digested with *Apa*I (lanes 1), *Asc*I (lanes 2), *Not*I (lanes 3), and *Sal*I (lanes 4). Bacteriophage lambda concatemers were used as molecular size markers (M); the numbers indicate sizes of the corresponding bands in kilobase pairs.

omlA-specific probe to the 220-kbp fragment as well as to fragments NOT2 and APA5 (data not shown).

Using these double digests, we could not determine the order of small fragments in three regions. These were the *Apa*I fragments in NOT1 (APA4, APA6, and APA11) and NOT2 (APA7, APA8, APA9, and APA10) as well as the *Not*I fragments in APA1 (NOT4, NOT7, and NOT8). These ambiguities were investigated by partial cleavage and hybridization using probes for (i) a 172-kbp *Not*I-*Apa*I fragment, (ii) fragment APA11 (*sodA*), and (iii) fragment APA9 (*tolQ*). The resulting order of fragments in the map was confirmed by single and double digestion with *Asc*I and Southern hybridization analyses. The resulting physical map had a resolution of approximately 100 kbp (Fig. 2A).

The chromosome of *A. pleuropneumoniae* ATCC 27088 (serotype 1 reference strain) was also found to have a size of 2.4 Mbp containing eight *Apa*I, nine *Asc*I, seven *Not*I, and five *Sal*I fragments (Table 3). The initial information about the order of macrorestriction fragments in the *A. pleuropneumoniae* ATCC 27088 chromosome likewise was obtained by fragment excision and redigestion as well as by partial cleavage as described above. The results of these experiments were confirmed by cross-hybridization particularly using the *Asc*I fragments of *A. pleuropneumoniae* AP76 as probes for Southern blot analyses. The map was oriented such that the *Sal*I site corresponding to the single *Sal*I site of *A. pleuropneumoniae* AP76 was position 1. The resulting physical map also had a resolution of approximately 80 kbp (Fig. 2B).

Genetic map. On the physical maps, several putative virulence-associated genes (*apxIA*, *-IIA*, and *-IIIA*, *apxIBD*, *omlA*, *sodA*, *tolQ-tbpBA*, *ureC*, and a repeat element) as well as a variety of housekeeping genes (*glyA*, *metJ*, *recA*, and *rho*) were located by Southern hybridization (Fig. 2C and D; Fig. 3). It was found that the *tolQ-tbpBA* region (38) and the urease operon mapped together on the 110-kbp fragment APA9 of strain AP76 and the 105-kbp fragment APA7 of strain ATCC 27088, respectively. The transposon-like repeat element mapped together with the *apxIICA* genes as expected (2). An additional repeat element mapped together with the *apxIBD* genes on the 194-kbp *Apa*I fragment APA7 of strain AP76. In *A. pleuropneumoniae* ATCC 27088, the complete *apxICABD* operon was present in a comparable region (on APA5) without an associated repeat sequence. No virulence-associated genes were mapped to approximately one-third of the genome rep-

TABLE 3. Sizes of macrorestriction fragments of the genomes of *A. pleuropneumoniae* AP76 and *A. pleuropneumoniae* ATCC 27088

A. pleuropneumoniae strain	*Apa*I		*Asc*I		*Not*I		*Sal*I	
	Designation	Size (kbp)	Designation	Size (kbp)	Designation	Size (kbp)	Designation	Size (kbp)
AP76	APA1	571	ASC1	886	NOT1	878	NA[a]	NA
	APA2	294	ASC2	591	NOT2	794		
	APA3	282	ASC3	369	NOT3	225		
	APA4	272	ASC4	190	NOT4	219		
	APA5	231	ASC5	170	NOT5	117		
	APA6	210	ASC6	170	NOT6	73		
	APA7	194			NOT7	40		
	APA8	116			NOT8	30		
	APA9	110						
	APA10	90						
	APA11	9						
	Sum	2,379		2,376		2,376		
ATCC 27088	APA1	858	ASC1	841	NOT1	970	SAL1	1,436
	APA2	503	ASC2	644	NOT2	795	SAL2	557
	APA3	286	ASC3	266	NOT3	270	SAL3	254
	APA4	220	ASC4	182	NOT4	209	SAL4	102
	APA5	201	ASC5	175	NOT5	81	SAL5	51
	APA6	196	ASC6	121	NOT6	40		
	APA7	105	ASC7	87	NOT7	30		
	APA8	28	ASC8	51				
			ASC9	36				
	Sum	2,397		2,403		2,395		2,400

[a] NA, not applicable (single recognition site only).

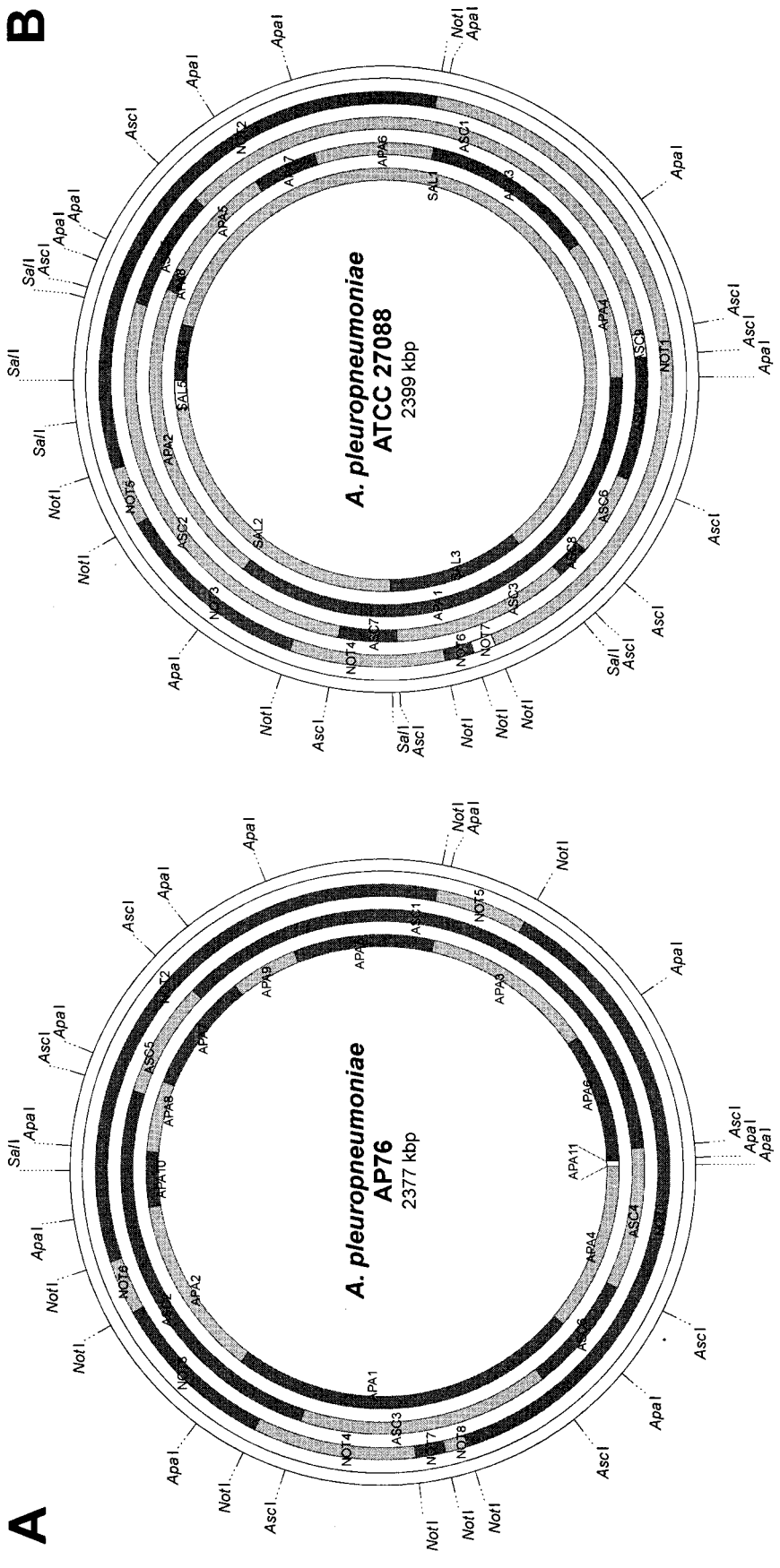

FIG. 2. Physical (A and B) and genetic (C and D) maps of the genomes of *A. pleuropneumoniae* AP76 and ATCC 27088 determined by using the restriction endonucleases *ApaI*, *AscI*, *NotI*, and *SalI*. The single *SalI* site of the chromosome of *A. pleuropneumoniae* AP76 and the corresponding site in *A. pleuropneumoniae* ATCC 27088 have been defined as position 1; sizes of the indicated fragments are listed in Table 2.

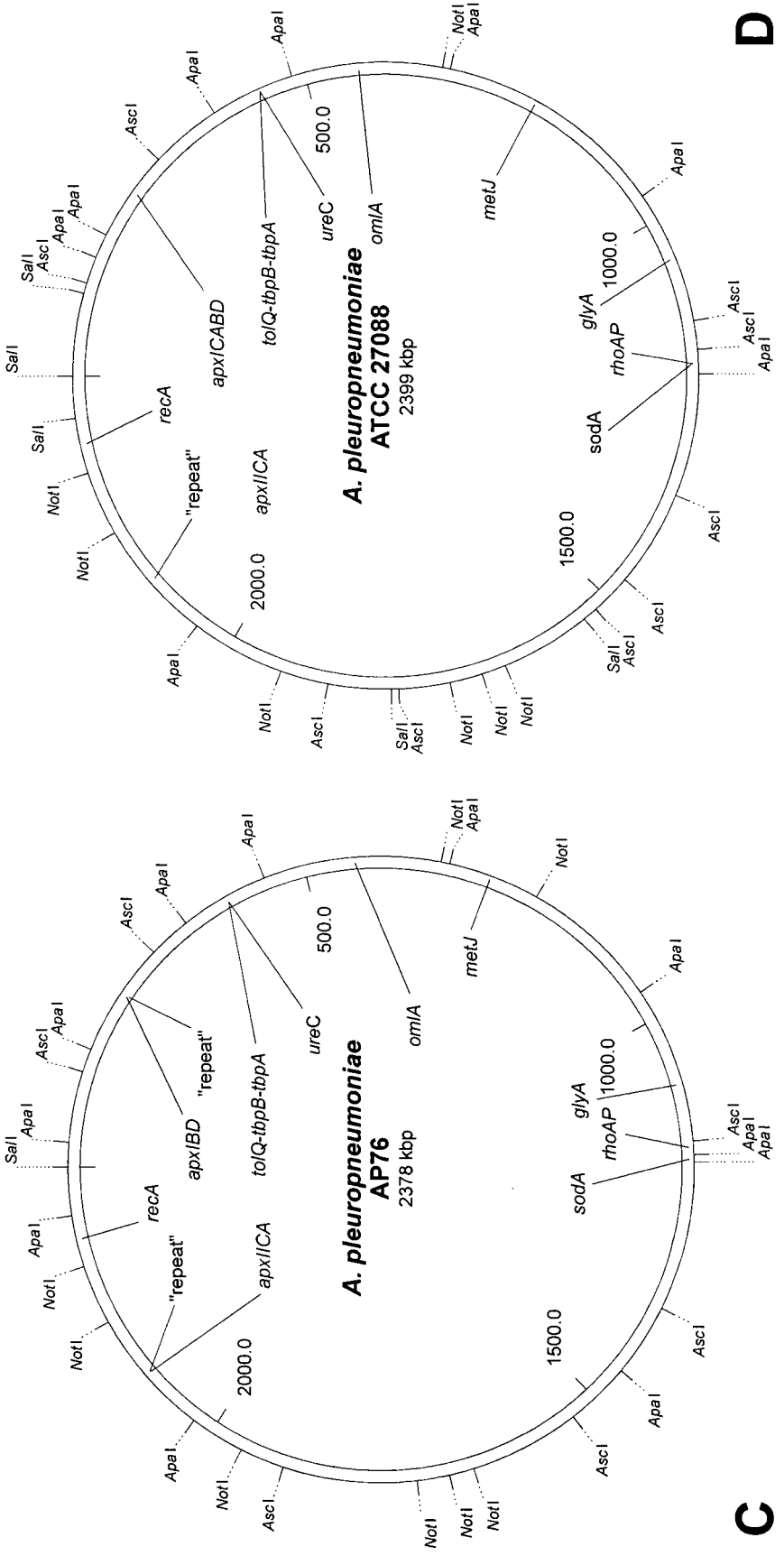

FIG. 2—*Continued.*

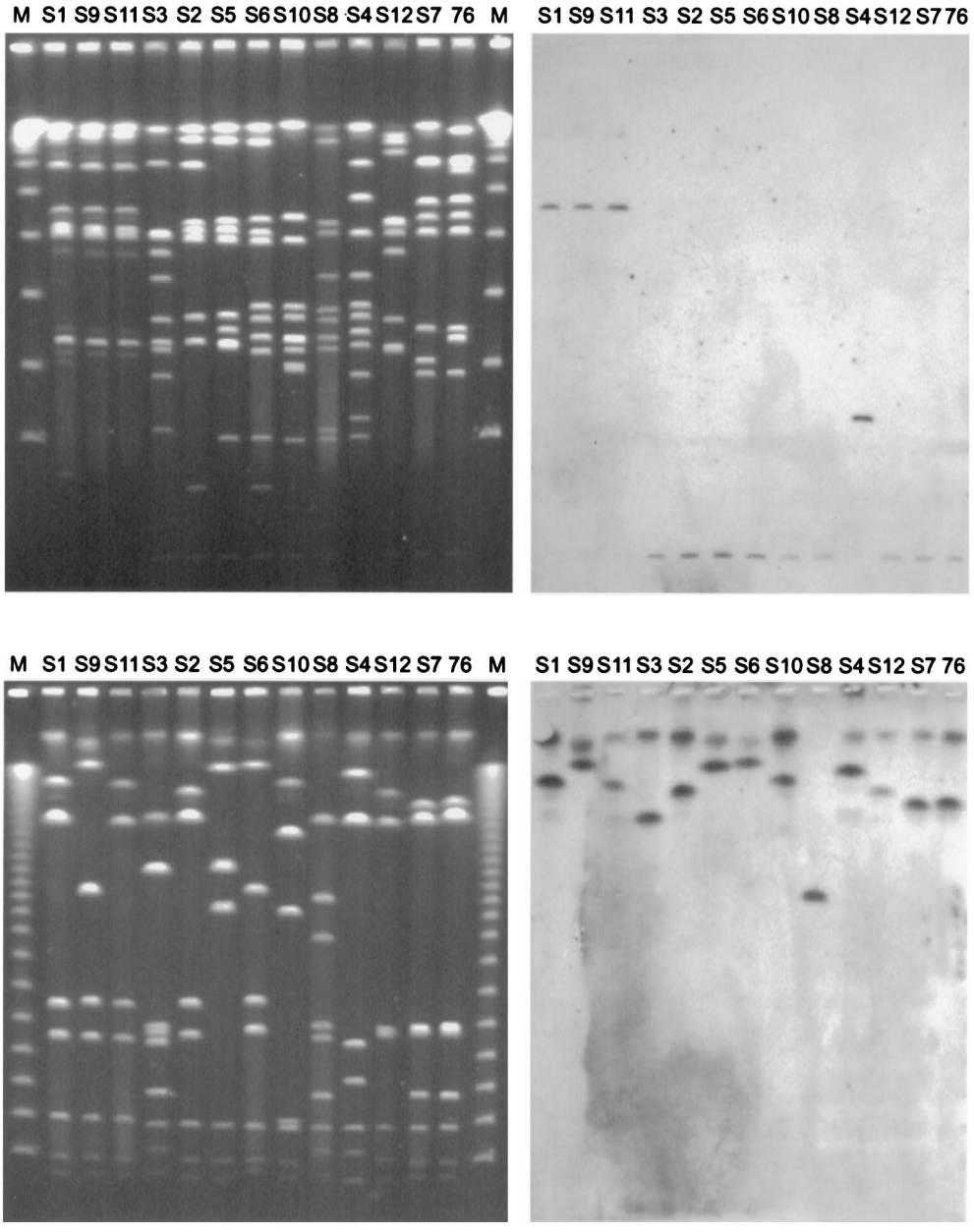

FIG. 3. PFGE separation (left) and exemplary Southern blots (right) of the *A. pleuropneumoniae* serotype reference strains, using the enzymes *Apa*I (top) and *Not*I (bottom) and a *sodA* probe. The top and bottom gels were generated by using short and long running conditions, respectively. The *A. pleuropneumoniae* serotype reference strains (S1 to S12) are grouped based on their phylogenetic relationship (7). The position of the mapped *A. pleuropneumoniae* clinical isolate AP76 is indicated by "76." Lambda concatemers were used as molecular size markers (M).

resented by fragments APA1 and APA4 (AP76) or APA1 (ATCC 27088). Therefore, the general genomic arrangement did not differ between the strains *A. pleuropneumoniae* AP76 (serotype 7) and *A. pleuropneumoniae* ATCC 27088 (serotype 1) (Fig. 2C and D).

Strain comparison. To investigate the genetic homogeneity within the species *A. pleuropneumoniae*, the hybridization patterns of *Apa*I- and *Not*I-restricted genomes of the *A. pleuropneumoniae* serotype reference strains were compared. The PFGE-separated fragments were hybridized with probes specific for genes positioned on the genetic maps of *A. pleuropneumoniae* AP76 and ATCC 27088 (Fig. 3 and 4). Using the more frequently cleaving enzyme *Apa*I, we found that predominantly fragments with comparable sizes were hybridized. In the *Not*I digest, hybridizing fragments were found to be more variable in size but the linkage of genes appeared to be consistent among the reference strains (Fig. 4). The repeat element was detected in all reference strains in up to three copies. Only the serotype 10 reference strain had no repeat element as assessed by Southern blotting and confirmed by a repeat-specific PCR (data not shown). The repeat element consistently mapped together on the same fragment with the *apxIICA* toxin genes in the serotype reference strains. The *apxIBD* genes were also found together on the same fragment with a repeat element in *A. pleuropneumoniae* serotypes 2, 7, and 12; in the other strains, *apxIBD* was not linked with a repeat element.

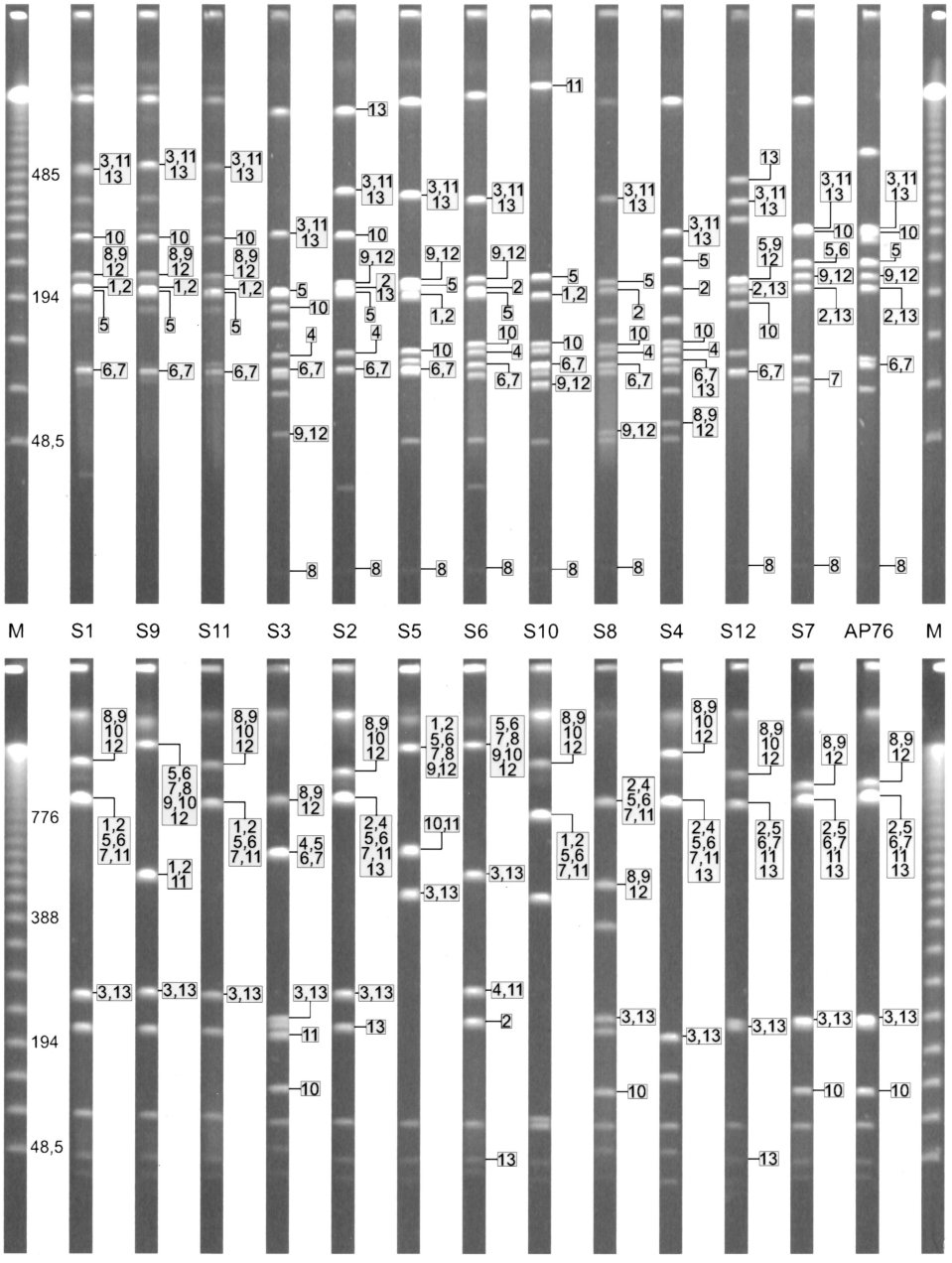

FIG. 4. Comparative locations of mapped genes on *Apa*I (top)- and *Not*I (bottom)-restricted genomes of the 12 *A. pleuropneumoniae* serotype reference strains. The top gel was run with linear ramped switch times from 5 to 20 s for 13 h and 35 to 70 s for 13 h. The bottom gel was run with switch times from 15 to 30 s for 13 h and 40 to 90 s for 17 h. The *A. pleuropneumoniae* serotype reference strains (S1 to S12) are grouped based on their phylogenetic relationship (7). Bacteriophage lambda concatemers were used as molecular size markers (M); the unboxed numbers indicate sizes of the corresponding bands in kilobase pairs; the boxed numbers indicate positions of the mapped genes: 1 (*apxICA*), 2 (*apxIBD*), 3 (*apxIICA*), 4 (*apxIIICA*), 5 (*omlA*), 6 (*ureC*), 7 (*tolQ-tbpBA*), 8 (*sodA*), 9 (*glyA*), 10 (*metJ*), 11 (*recA*), 12 (*rho*), and 13 (repeat element).

DISCUSSION

A restriction map with a mean resolution of about 100 kbp has been established for the *A. pleuropneumoniae* AP76 (serotype 7 clinical isolate) and ATCC 27088 (serotype 1 reference strain). These two strains were chosen since *A. pleuropneumoniae* ATCC 27088 is the internationally most relevant reference strain and *A. pleuropneumoniae* AP76 is the only strain in which spontaneous genetic rearrangements have been shown to occur (2). The maps were used to position putative virulence-associated genes as well as some housekeeping genes to their genomic surroundings and to compare their relative locations among the *A. pleuropneumoniae* serotype reference strains.

The genome size and *Asc*I restriction fragment pattern determined for *A. pleuropneumoniae* ATCC 27088 corresponded to results presented previously (7). Also, we could confirm the *Apa*I restriction fragment patterns of the serotype 1, 9, and 11 reference strains (7). Faint bands in the *Apa*I cleavage pattern of strain ATCC 27088 (7) (Fig. 3 and 4) we considered to be products of incomplete digestion.

In the *Not*I restriction pattern of *A. pleuropneumoniae* AP76, no differences from the serotype 7 reference strain *A. pleuropneumoniae* WF83 were found. Comparing the *Apa*I restriction patterns, we identified two regions of variation. One variation is most likely due to a loss of the *Apa*I restriction site in strain AP76 at position 1450 kbp in the map (Fig. 2A), thus joining the two fragments APA1 and APA4 to form a markedly larger fragment (Fig. 4). Another, more complex heterogeneity involves the position of the *ureC* gene mapping together with the *tolQ-tbpBA* region (Fig. 2C). In strain WF83, *ureC* maps to a different fragment, where it is located together with *omlA* (Fig. 4). Whether this is due to changes of at least two *Apa*I restriction sites or caused by a small inversion of this part of the chromosome could not be determined from our data. The four variations in fragment sizes in the pattern obtained with *Asc*I are most likely due to the loss and acquisition of singular restriction sites.

The fragment sizes and macrorestriction maps of the two *A. pleuropneumoniae* strains analyzed showed more differences than similarities (Fig. 2A and B). The arrangements of the genes, however, were found to correspond (Fig. 2C and D). This result confirms the hypothesis of a clonal origin of the *A. pleuropneumoniae* serotypes proposed by Musser et al. based on multilocus enzyme electrophoresis (27). Thus, only a single difference between the two genetic maps was detected at the position of the genes *apxIBD*. In *A. pleuropneumoniae* AP76, a repeat element mapped on the same fragment, whereas in *A. pleuropneumoniae* ATCC 27088 a complete *apxICABD* gene cluster was found in this position.

It was found that the *apxIICA* sequence consistently mapped together with the repeat element as had been described for *A. pleuropneumoniae* serotype 7 (2). This supports the function of the repeat element as a transposable DNA element and indicates acquisition of the ApxII toxin late in evolution by horizontal gene transfer. In addition, the *apxIBD* sequence required for toxin transport was found on the same fragment about 200 kbp in size together with another repeat element in serotypes 2, 7, and 12, whereas no association was found in other strains. This is interesting since it might suggest a different evolutionary development for the reference strains carrying the genotype *apxIBD apxIICA apxIIICABD* without association (serotype 4, 6, and 8) and with possible association (serotype 2) between *apxIBD* and the repeat element. Thus, the pathogenic *A. pleuropneumoniae* serotype 2 may have developed from a serotype 3 ancestor with low pathogenicity by acquiring the mobilizable *apxIBD*. In contrast, serotypes 4, 6, and 8 may have developed from an ancestor with *apxICABD* by deletion of *apxICA* as proposed by Frey (12). In this model, *A. pleuropneumoniae* serotype 10 would be the evolutionarily oldest strain with only *apxICABD*. The youngest strains in evolutionary terms would be serotypes 7 and 12 carrying only *apxIBD* and *apxIICA*, both accompanied by a repeat element.

The results presented here document a high degree of genetic homogeneity and stability within the species *A. pleuropneumoniae*. Gross rearrangements as described for different serovars of *Vibrio cholerae* (22, 39) or closely related isolates of *Pseudomonas aeruginosa* (34) were not observed. Also, there is no indication for the presence of large discrete unstable regions on the *A. pleuropneumoniae* genome which might be indicative for pathogenicity islands as described for members of the family *Enterobacteriaceae* (21, 26, 30, 35) and *Helicobacter pylori* (1). A comparison of our initial genetic map with that of *Haemophilus influenzae* Rd based on the complete genomic sequence (10) revealed clear differences in the relative order of the genes positioned by us (not shown). This shows that *H. influenzae* Rd sequences, though useful for identifying homologous *A. pleuropneumoniae* sequences by PCR or Southern blotting, do not allow a prediction of the relative locations of the corresponding *A. pleuropneumoniae* genes.

In conclusion, the physical maps presented here will facilitate localization of newly detected genes by Southern hybridization with DNA restriction patterns from single digests with the four restriction endonucleases *Apa*I, *Asc*I, *Not*I, and *Sal*I. Particularly in the absence of extensive genomic sequence data, such a completed genetic map will represent a valuable tool for the investigation of *A. pleuropneumoniae* pathogenicity and for the development of attenuated live vaccines.

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