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Chromosome sizes and phylogenetic relationships between serotypes of *Actinobacillus pleuropneumoniae*

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Abstract

The genome size of *Actinobacillus pleuropneumoniae* was determined by pulsed field gel electrophoresis of *Asc*I and *Apa*I digested chromosomal DNA. The genome size of the type strain 4074^T (serotype 1) was determined to be 2404 ± 40 kb. The chromosome sizes for the reference strains of the other serotypes range between 2.3 and 2.4 Mb. The restriction pattern profiles of *Asc*I, *Apa*I and *Nhe*I digested chromosomes showed a high degree of polymorphism among the different serotype reference strains and allowed their discrimination. The analysis of the macrorestriction pattern polymorphism revealed phylogenetic relationships between the different serotype reference strains which reflect to some extent groups of serotypes known to cross-react serologically. In addition, different pulsed fields gel electrophoresis patterns also revealed heterogeneity in the chromosomal structure among different field strains of serotypes 1, 5a, and 5b, while strains of serotype 9 originating from most distant geographical places showed homogeneous *Apa*I patterns in pulsed field gel electrophoresis. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: *Actinobacillus pleuropneumoniae*; Chromosome size; Pulsed field gel electrophoresis

1. Introduction

During the last 20 years, porcine pleuropneumonia has been recognized as one of the major diseases in swine production world-wide [1]. The etiological agent of this contagious pulmonary disease is *Actinobacillus pleuropneumoniae* [2], a Gram-negative bacterium of the Pasteurellaceae family [3]. Twelve serotypes and two subtypes of *A. pleuropneumoniae* biotype 1 (NAD-dependent) and two serotypes of biotype 2 (NAD-independent) have been identified on the basis of capsular and lipopolysaccharide antigens [4–6]. Several reports have indicated that the differences in virulence among the various *A. pleuropneumoniae* serotypes are mainly related to the pro-

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duction of three APX exotoxins [7–9]. Serotypes 1, 2, 5a, 5b, 9 and 11 are frequently involved in severe outbreaks with high mortality and severe pulmonary lesions [2,9]. The other serotypes are less virulent but can be found in outbreaks with lower levels of mortality [2,9]. Serotyping of *A. pleuropneumoniae* strains is a valuable tool for epidemiological studies and provides important information for the decision taking in control programs aiming at the eradication of the virulent types of the pathogen. Epidemiological analyses showed that the prevalence of specific serotypes varies with geographic location [10,11]. However, cross-reactivity, between *A. pleuropneumoniae* serotypes 1, 9 and 11, between serotypes 3, 6 and 8, and between serotypes 4 and 7 has been described [12]. These cross-reactions were shown to be associated with common epitopes and common components of the cell wall antigens [12,13].

Molecular methodologies applied to the whole genome are becoming more and more relevant in providing means to estimate the genetic relationship between different biotypes and serotypes of a given bacterial species, and also for accurate bacterial typing and subtyping systems [14,15]. Restriction patterns of the whole bacterial chromosome, which could be resolved by pulsed field gel electrophoresis (PFGE) [16], offer a good method to understand the degree of genetic relatedness or variability among the different serotypes. In addition, PFGE of chromosomal DNA that was digested by restriction enzymes with recognition sites that occur infrequently in bacterial genomes presents a reliable and efficient method for estimating genome sizes and constructing macro-restriction maps of bacterial chromosomes [17]. The purpose of this study was to determine the chromosome sizes of the *A. pleuropneumoniae* type strain and all serotype reference strains and to study the genomic relationship between the different serotype reference strains and between strains of given serotypes.

2. Materials and methods

2.1. Bacterial strains

Reference strains of *A. pleuropneumoniae* representing serotypes 1–12 were S4074, S1536, S1421, M62, K17, L20, Femø, WF83, 405, CVJ13261, 13039, 56153, and 8329, and came from R. Nielsen (National Veterinary Laboratory, Copenhagen, Denmark). Field strains of serotypes 1 (21 strains), 5a (11 strains), 5b (10 strains), and 9 (9 strains) for biotype 1, and serotypes 2 (4 strains) and 9 (6 strains) for biotype 2 were isolated in France, Poland, The Netherlands, Canada, Czech Republic, and Italy.

2.2. Preparation of genomic DNA

Each bacterial strain was grown for 6 h at 37°C in 10 ml PPLO broth (Difco Laboratories, Detroit, MI, USA) containing 12.5 g l^{-1} yeast extract. After centrifugation at $1500\times g$ for 15 min at 4°C, the bacterial pellet was washed with 10 ml of cold TE buffer (10 mM Tris-(hydroxymethyl)-aminomethane, 1 mM EDTA, pH 8.0). After centrifugation at $1500\times g$ for 15 min at 4°C, the cells were resuspended in 1 ml of cold TE buffer supplemented with 3 mg ml^{-1} lysozyme. 1 ml of 1% agarose (Bio-Rad, Ivry sur Seine, France) in TE buffer, prewarmed at 60°C, was added and immediately poured into a block former (Bio-Rad). After solidification, agarose blocks were incubated for 6 h at 37°C in 10 ml TE buffer supplemented with 3 mg ml^{-1} lysozyme (Sigma-Aldrich, St Quentin Fallavier, France). Blocks were then transferred to 10 ml fresh TE buffer supplemented with 1% SDS and 0.25 mg ml^{-1} proteinase K (Eurobio, Les Ulis, France) and incubated overnight at 37°C. Blocks were transferred to 10 ml fresh TE buffer supplemented with 1% SDS and 0.25 mg ml^{-1} proteinase K and incubated for another 4 h at 37°C. They were then washed three times with TE buffer for 1 h at 37°C. The gel blocks were run for 1 h at 6.0 V cm^{-1} in 0.5×TBE buffer (45 mM Tris, 45 mM boric acid, 10 mM EDTA). This pre-migration step removed degraded or extrachromosomal DNA from the gel and thus strongly reduced the smear background in PFGE. Blocks were conserved at 4°C in 0.5×TBE for several months. It is important to note that it was important to use PPLO broth complemented with yeast extract as medium for growth of *A. pleuropneumoniae* for PFGE analysis in order to obtain clean and clearly interpretable results.

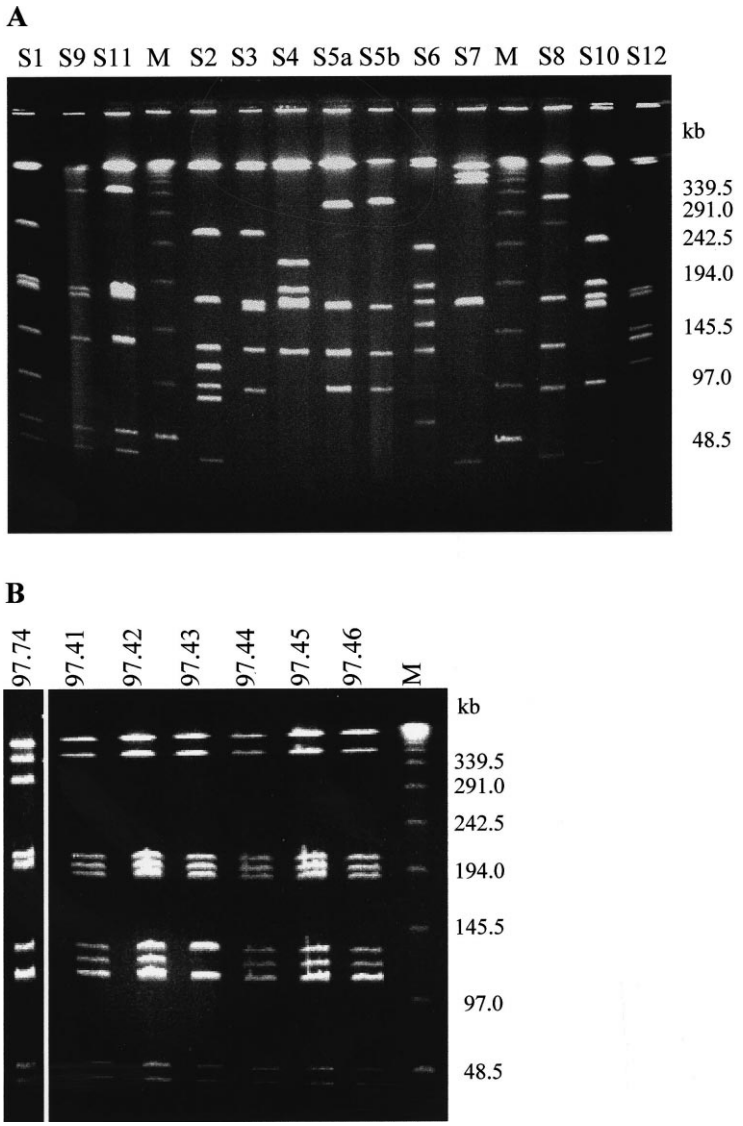

Fig. 1. A: Comparison of *A. pleuropneumoniae* type and reference strains by *Asc*I PFGE macrorestriction profiles. Electrophoresis was performed with pulse time range from 5 s to 20 s for 24 h at 200 V. The strain serotype is indicated at the top of each lane. B: Differentiation of different field strains from *A. pleuropneumoniae* serotype 5a by *Apa*I RFLP analysis using PFGE. The gel shown was made with a pulse time range from 5 s to 20 s for 24 h at 200 V. Panels A and B: Molecular masses of the size standard (lane M) made from polymers of bacteriophage λ genomes are indicated on the right of the gel.

2.3. Restriction digests and electrophoresis

Restriction enzyme digestion of genomic DNA embedded in agarose blocks was undertaken by a modification of procedures described by McClelland et al. [16]. Each restriction digest was done with half of a genomic DNA block. After 30 min in 1 ml of the restriction buffer, 800 μl was removed and the blocks were first incubated overnight with 30 U of restriction endonuclease at the temperature recommended by the manufacturer, then another 10 U was added for 1 h.

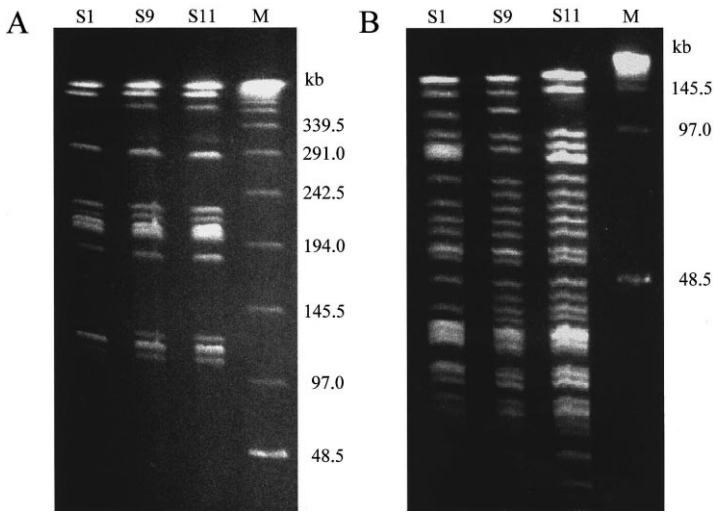

Fig. 2. PFGE of restriction enzyme digests of *A. pleuropneumoniae* DNA reference strains serotype 1, 9 and 11. The restriction enzymes used were *Apa*I in A and *Nhe*I in B. Molecular sizes (lane M) are indicated on the right of the gel. Pulse time conditions were 5 s to 20 s for 24 h at 200 V in A and 0.1 s to 4 s for 24 h at 200 V in B.

PFGE was performed at 14°C with a DRII or DRIII CHEF system (Bio-Rad) in 0.5×TBE. Blocks were then cut in <1-mm slices and placed in 1.2 or 1.5% agarose (Bio-Rad Pulsed Field Certified Agarose) gel wells. Pulsed field gels were then run at various pulse ramps ranging from 0.1 to 4 s (5–100 kb), 5 to 20 s (20–300 kb), 10 to 40 s (200–800 kb) and 40 to 100 s (500–1300 kb), for 24–48 h at 200 V in order to obtain the best resolution of restriction fragments in different size ranges. Gels were stained for 1 h after electrophoresis with TBE buffer containing 0.5 μg ml^{-1} ethidium bromide, destained overnight with TBE buffer, examined over UV transillumination and photographed.

2.4. Data analysis

Gel images were scanned and analyzed using the BIO-GENE Software (Vilber Lourmat, Inc., Marne la Vallée, France). Sizes of restriction fragments were determined by comparison with standard lambda DNA concatemers (Pharmacia, Orsay, France). Dendrograms were created from a matrix of band matching using the Jaccard coefficient [18] and the unweighted pair group method of arithmetic averages (UPGMA) clustering fusion strategy [19].

3. Results

3.1. Genome size

In order to generate chromosomal digests with relatively few, clearly resolvable fragments, genomic DNA *A. pleuropneumoniae* which contains 42% G+C [20] was digested with restriction endonucleases with GC-rich recognition sequences such as *Asc*I (5′-GGCGCGCC-3′), *Apa*I (5′-GGGCCC-3′), *Ngo*MI (5′-GCCGGC-3′), *Sma*I (5′-CCCGGG-3′), *Sac*II (5′-CCGCGG-3′), *Sfi*I (5′-GGCCNNNNNGGCC-3′) and *Not*I (5′-GCGGCCGC-3′). Among these restriction enzymes, *Asc*I and *Apa*I were the most suitable since they cut the chromosomes of the *A. pleuropneumoniae* serotype reference strains into 6–12 fragments ranging from 11 kb to 1217 kb. Figs. 1A and 2A show photographs from PFGE runs with pulse parameters separating medium-sized (20–400-kb) fragments. For the determination of the chromosome sizes of the *A. pleuropneumoniae* type strain and the serotype reference strains, the sizes of the fragments from *Asc*I digested chromosomes were determined from PFGE using bacteriophage λ multimers as molecular mass standards. In each experiment three different gels with different pulse parameters were used in order to discriminate the

Table 1
Sizes of chromosomal *Asc*I restriction fragments from *A. pleuropneumoniae* reference strains

Fragment	Serotype/Strain												
	1/S4074	2/S1536	3/S1421	4/M62	5a/K17	5b/L20	6/FemΦ	7/WF83	8/405	9/CVJ13261	10/13039	11/56153	12/8329
A	840.5 ± 5.5	832.6 ± 4.2	866.7 ± 0.3	911.8 ± 1.6	854.3 ± 0.6	1217 ± 14	830.3 ± 2.4	614.6 ± 4	852.2 ± 0.7	827.6 ± 1.1	878.7 ± 5.7	831.7 ± 3.1	841.5 ± 0.7
B	644.4 ± 8	665.5 ± 5.2	602.9 ± 6.4	599 ± 8.5	783.4 ± 5.6	333.9 ± 14	628 ± 21	593 ± 4	784.8 ± 3.3	649.3 ± 15	607.9 ± 11	640.5 ± 6.4	600.2 ± 7.6
C	266.4 ± 2.7	263.5 ± 5.1	271.3 ± 14	223.2 ± 4	316.4 ± 10	322 ± 6.7	235.8 ± 2.5	428.7 ± 1.6	329.7 ± 4.9	351.2 ± 9.2	249.9 ± 3.4	349.2 ± 7.8	181.3 ± 5.1
D	182.1 ± 3.8	176.6 ± 1.7	174.7 ± 1.8	188.6 ± 3.5	172.7 ± 3.2	178 ± 2.5	188.3 ± 1.1	390.7 ± 5.2	180 ± 4	186.9 ± 7.2	189.6 ± 3	181.7 ± 3.4	174.7 ± 3.7
E	175.1 ± 3.2	130.7 ± 2.1	170 ± 1.2	175.2 ± 2.3	127.9 ± 3.4	135.4 ± 3.7	173.3 ± 1.4	177.8 ± 4.8	135.8 ± 5.1	180 ± 7	176.1 ± 4.2	175.3 ± 2.4	141.3 ± 4.2
F	121.7 ± 11	114 ± 2.7	130.7 ± 2.1	175.2 ± 2.3	97.3 ± 3.8	97 ± 3.5	151.7 ± 1.1	177.8 ± 4.8	94.5 ± 0.4	137.9 ± 8.9	168.3 ± 2.6	130.9 ± 2.5	133.3 ± 4.2
G	86.9 ± 4.3	98.8 ± 3	96.1 ± 3.7	128.1 ± 2.4			129.2 ± 0.6	25.5 ± 2.3	31.6 ± 1	50.3 ± 1.2	97.7 ± 2.8	50.3 ± 1.2	133.1 ± 4
H	51.2 ± 0.3	86 ± 1.8					70.7 ± 3.1			32.8 ± 2.1	24.4 ± 2.8	32.8 ± 2.1	111.9 ± 4
I	35.8 ± 0.9	29.3 ± 0.9											39.7 ± 0.6
Total (kb)	2404 ± 40	2397 ± 27	2312 ± 30	2401 ± 25	2352 ± 17	2283 ± 44	2407 ± 33	2408 ± 27	2409 ± 19	2416 ± 52	2392 ± 36	2392 ± 29	2357 ± 34

Values are expressed as means ± standard deviation of three independent determinations from different gels.

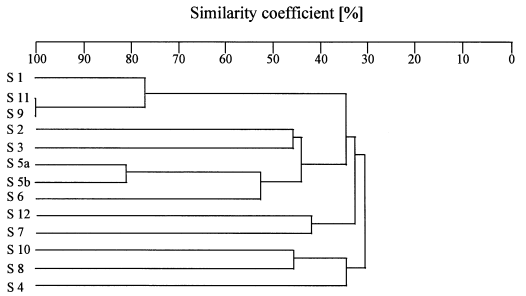

Fig. 3. The dendrogam of PFGE analysis of *A. pleuropneumoniae* serotype reference strains using *Apa*I restriction profiles. Serotypes are given on the left.

medium fragments (20–300 kb) (parameters: 5–20 s for 24 h), the large fragments (200–800 kb) (parameters: 10–40 s for 48 h) and the very large fragments (500–1300 kb) (parameters 40–100 s for 36 h). The chromosome sizes were calculated by addition of the sizes of the individual fragments from three independent experiments and are given in Table 1. The size of the chromosome of the *A. pleuropneumoniae* type strain 4074^{T} was determined to be 2404 ± 40 kb. The sizes of the different serotype reference strains differed only slightly from that of the type strain and ranged between 2283 kb and 2416 kb.

3.2. RFLP analysis

A. pleuropneumoniae reference strains gave clear differences in the restriction patterns after digestion with *Asc*I (Fig. 1) and with *Apa*I (data not shown), showing a high degree of polymorphism with 12 different profiles for 13 reference strains. Reference strains of serotypes 9 and 11 could not be distinguished using either *Apa*I or *Asc*I (Figs. 1A and 2A). However, these two serotypes could be differentiated using *Nhe*I (5′-GCTAGC-3′) and *Xba*I (5′-TCTAGA-3′), which had a higher resolution potential since they generated more than 30 genomic fragments ranging from 180 kb to less than 5 kb and produced complex banding patterns (Fig. 2B).

*Apa*I patterns allowed a clear distinction between biotype 1 reference strains and biotype 2 strains from serotype 2 and from serotype 9. In addition analysis of different field strains from serotypes 1, 5a and 5b resulted in different, but related *Apa*I patterns as shown for strains of serotype 5a which generally differ in one or two *Apa*I fragments (Fig. 1B). In contrast, no differences in *Apa*I patterns at all could be detected in serotype 9 field strains isolated from most distant countries such as Australia, Italy, France, Poland, Czech Republic and Switzerland (results not shown).

The genetic relatedness between the different *A. pleuropneumoniae* serotype reference strains was evaluated using PFGE profiles produced by *Apa*I (Fig. 3). This analysis reveals the close relationship between serotypes 1, 9 and 11 (85% similarity) and between 5a and 5b (80% similarity) in particular, grouping also serotypes 2 and 3, serotypes 6, 5a and 5b, as well as 4, 8 and 10, at a similarity in the 40–50% range. When the patterns created with *Asc*I were used, slightly different groupings were obtained, due to the lower number of fragments produced by this enzyme.

4. Discussion

The sizes of the chromosomes of the *A. pleuropneumoniae* type and serotype reference strains were determined to range between 2.3 and 2.4 Mb using PFGE analysis of *Asc*I and *Apa*I digested chromosomal DNA. The size of the *A. pleuropneumoniae* chromosome is comparable to that of other species of the family Pasteurellaceae which includes *Actinobacillus actinomycetemcomitans* (2.3 Mb) [21], *Haemophilus parainfluenzae* (2.34 Mb), *Haemophilus influenzae* (1.83 Mb) [22] and *Haemophilus ducreyi* (1.76 Mb) [23]. The sizes of the chromosomes of the different *A. pleuropneumoniae* strains analyzed vary relatively little compared to other bacterial species. In contrast to the well conserved chromosome sizes, the different *A. pleuropneumoniae* serotypes and biotypes show polymorphism in their restriction enzyme patterns as revealed by PFGE fingerprinting, reflecting distinct genetic differences. Such differences were seen between the different serotypes and between biotypes of the same serotype, but also among strains of the same sero- and biotype. In particular serotypes 1, 5a and 5b showed several different, but serotype-related restriction patterns. The genomic differences between the two different biotypes of the same serotype, as deduced by PFGE analyses, must be more abundant than what can be expected

from a single genetic locus involved in NAD metabolism. Interestingly no differences were encountered in serotype 9 field strains isolated from most distant geographic areas. Serotype 9 hence seems to present a particularly stable widespread clonal line.

Analysis of the restriction enzyme polymorphisms by using the Jaccard coefficient and the UPGMA strategy for clustering showed the close genetic relationship of the serologically and toxigenetically related serotypes 1, 9 and 11 and of serotypes 5a and 5b. However, it clearly differentiates between serotypes 3, 6, and 8 and between 4 and 7 which are known to show serological cross-reactions which are known to interfere in serological subtyping. The genetic relationships between the different *A. pleuropneumoniae* serotype reference strains as determined by PFGE show a significant direct relationship with DNA:DNA homology values of the same strains as determined by free solution hybridization experiments [24]. From our experiments we estimate that PFGE has the same, or even a higher discriminatory potential than other molecular tools such as multilocus enzyme electrophoresis, restriction fragment length polymorphism using frequently cutting enzymes together with high resolution gels, or arbitrarily primed polymerase chain reaction [24–26]. PFGE, however, seems to be a method that can be well standardized in order to give highly reproducible results which allow comparative studies between different laboratories. PFGE therefore represents a powerful tool for studies on taxonomy and epidemiology of *A. pleuropneumoniae* in particular, as well as for many other bacteria where other, mostly phenotypic methods do not allow sufficient discrimination between different subtypes of a given species.

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