Research Article

Characterization of the *oml*A gene from different serotypes of *Actinobacillus pleuropneumoniae*: A new insight into an old approach

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Abstract

The OmlA protein is a virulence factor of *Actinobacillus pleuropneumoniae*, an important pathogen in pigs. The polymorphisms present in the *omlA* gene sequence of 15 reference serotypes of *A. pleuropneumoniae* and non-serotypable isolates were assessed to determine the possible evolutionary relationship among them and to validate the importance of this gene as a molecular marker for the characterization of this bacterium. Divergence among the 15 serotypes of *A. pleuropneumoniae* probably resulted initially from two major evolutionary events that led to subsequent differentiation into nine groups. This differentiation makes it possible to characterize most of the serotypes by using bionformatics, thereby avoiding problems with immunological cross-reactivity. A conserved α-helix common to all the serotypes was most likely involved in connecting the protein to the outer membrane and acting as a signal peptide. A previously unknown gene duplication was also identified and could contribute to the genetic variability that makes it difficult to serotype some isolates. Our data support the importance of the *omlA* gene in the biology of *A. pleuropneumoniae* and provide a new area of research into the OmlA protein.

Introduction

Swine pleuropneumonia (SPP) is a significant respiratory disease and has been reported in all countries where pig farming is intensively practiced. The etiological agent of SPP is the Gram-negative coccobacillus *Actinobacillus pleuropneumoniae*, currently divided in 15 serotypes that are defined based on the antigenic properties of capsule polysaccharides. Although all of the serotypes are capable of causing SPP, differences in virulence make the serotyping of field isolates of *A. pleuropneumoniae* a key factor in the epidemiological study and control of this disease (Schuchert *et al.*, 2004).

Numerous assays have been developed for the serological characterization of *A. pleuropneumoniae* isolates. Although immunological assays are relatively fast, their main limitation is that they commonly show cross-reactivity (Jessing *et al.*, 2003). Various molecular techniques have also been used to study the molecular epidemiology of bacterial pathogens and are extremely important in monitoring the characteristics of a given population (Ashis *et al.*, 2012). Current approaches seek to develop molecular markers that can complement the sometimes inconclusive information obtained using serological techniques. Molecular phylogenies based on gene polymorphisms have been used to characterize and distinguish serotypes or isolates of microorganisms (Nightingale *et al.*, 2005; Gonzalez-Escalona *et al.*, 2008).

The objective of this study was to analyze polymorphisms of the *omlA* gene, which codes for an outer membrane protein, in isolates from different serotypes of *A. pleuropneumoniae*. This is the first in-depth study of the polymorphisms and phylogeny of the *omlA* gene in *A. pleuropneumoniae* and provides new insights on the structure and organization of this gene. This work provides additional molecular tools for genotyping *A. pleuropneumoniae*.

Materials and Methods

Microorganisms, culture conditions, DNA extraction and PCR

Clinical isolates of *A. pleuropneumoniae* and the reference strains used in this study were kindly provided by Microbiologia Veterinária Especial Ltda (MICROVET - Viçosa, MG, Brazil). The isolates were obtained from the lungs and tonsils of animals with clinical signs of pleuropneumonia from different areas of southeastern Brazil, most of them in the state of Minas Gerais (MG)

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(Table 1). The isolates of *A. pleuropneumoniae* were identified by biochemical tests (Gottschalk *et al.*, 2003), serotyped by immunodiffusion according to Williams *et al.* (2000) and genotyped by multiplex PCR (Gram *et al.*, 2000a). All isolates were grown at 37 °C for 24 h in a 5%

CO_2 atmosphere in brain-heart infusion (Oxoid, Hampshire, UK) supplemented with NAD (10 µg/mL; Sigma-Aldrich, Poole, UK).

Genomic DNA from *A. pleuropneumoniae* strains was obtained using the Wizard Genome DNA purification

Table 1 - Strains, clinical isolates and nucleotide sequences used in this study.

Serotype	Strain	Accession number[1]	Source	Application
Reference strains				
1	4074	AB007572	Ito (2008)	Phylogenetic analysis
1	AP37	L06318	Gerlach *et al.* (1993)	
1	SC-A	EU251513	Yuan and Guo[2]	
2	S1513	AB007573	Ito (2008)	
2	S1536	U86676	Gram and Ahrens (1998)	
2	4226	ZP_07339322	Zhan *et al.* (2010)	
3	S1421	AB007574	Ito (2008)	
4	M62	AB007575	Ito (2008)	
5a	K17	AB007576	Ito (2008)	
5b	L20	AB007577	Ito (2008)	
5a	NG-8	D28491	Ito *et al.* (1995)	
5	AP 213	Z48920	Bunka *et al.* (1995)	
6	Femo	AB007578	Ito (2008)	
7	WF83	AB007579	Ito (2008)	
7	AP76	NC010939	Tegetmeyer *et al.*[2]	
8	405	AB007580	Ito (2008)	
9	CVI13261	AB007581	Ito (2008)	
10	D13039	AB007581	Ito (2008)	
11	56153	AB007583	Ito (2008)	
12	8329	AB007584	Ito (2008)	
13	N273	JF311904	This study	
14	3606	JF304624	This study	
15	HS143	JF304622	This study	
Clinical isolates from Brazil				
8	MV512	JF304623	This study	
8	MV5237	JF304619	This study	
ND[3]	MV235	JF304621	This study	
5	MV653	-	This study	Southern blotting
8	MV433	-	This study	
8	MV512	-	This study	
8	MV513	-	This study	
8	MV573	-	This study	
8	MV5237	-	This study	
8	MV5651	-	This study	
ND	MV235	-	This study	
ND	MV452	-	This study	
ND	MV497	-	This study	
ND	MV718	-	This study	

[1]NCBI GenBank accession number. [2]Unpublished. [3]Not determined because of cross-reactivity in immunological tests.

kit[TM] (Promega, Madison, WI, USA) according to the manufacturer's instructions. A pair of oligonucleotides, LPF1 (5'-ATTGTAAACTTTAGAGCTTTATATT-3') and LPR1 (5'-ATTAAAAAGTAAAAAAGCTATCCC-3') (Gram and Ahrens, 1998), was used to amplify the *omlA* gene (the amplicon had an expected size of approximately 1270 bp). The PCR was done using 1.25 U of Go*Taq* DNA polymerase (Promega) in a final volume of 50 µL of enzyme buffer containing 1.5 mM $MgCl_2$., 0.2 mM of each dNTP, 0.2 µM of each oligodeoxynucleotide and 50 ng of DNA in a C1000[TM] thermal cycler (BioRad, Richmond, CA, USA). The DNA was initially denatured at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 45 s, and an extension step at 72 °C for 1.5 min, followed by a final extension step at 72 °C for 10 min. The reaction products were analyzed by electrophoresis in 1.0% agarose gels, purified using a Wizard SV gel and PCR clean-up system (Promega) and sequenced using the Sanger sequencing method.

Nucleotide sequences

The *omlA* gene nucleotide sequences used in this study were from *A. pleuropneumoniae* isolates from different serotypes and origins. The NCBI GenBank database accession numbers and the serotypes of their respective isolates are listed in Table 1. The nucleotide sequences of isolates from serotypes 13, 14 and 15 that were previously unavailable in the databases were obtained in the present study and deposited under accession numbers JF311904, JF304624 and JF304622, respectively. In addition, *omlA* genes from isolates with serotypes that could not be defined by immunological methods because of cross-reactivity were sequenced (accession numbers JF304619, JF304621 and JF304623).

Structural analysis of the *omlA* gene

The +1 point of translation and the termination codon of the *oml*A gene were predicted using the analysis tool ORF *finder* (Rombel *et al.*, 2002). Sequences corresponding to the promoter region (-10 and -35) of the *omlA* gene were predicted using the Bacterial Promoter Prediction Program BPROM, which was also used to predict possible *cis* elements for the recognition of transcription factors.

Organization of the *omlA* gene in *A. pleuropneumoniae* isolates

The copy number and organization of the *omlA* gene in the genomes of the different isolates was studied using Southern blotting (Sambrook *et al.*, 1989). We selected 11 *A. pleuropneumoniae* clinical isolates (Table 1) that were obtained between 2003 and 2010 from six farms located in southeastern Brazil and included four non-serotypable isolates. The primers omlAhF (5'-CGGTTTAGTCGCAGGTTTAGT-3') and omlAhR (5'-TCCTTAACCCCTAATTCCTTAAGA-3') were used

to synthesize a 372-bp probe for hybridization (Figure 1D). The probe was labeled using a PCR DIG Probe Synthesis kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. Five micrograms of total DNA from the isolates was digested for 16 h with the restriction enzymes *Xba*I and *Bgl*II to generate an expected fragment of 943 bp. The fragments were separated by electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane (Amersham Hybond N[+]; GE Healthcare, Chalfont St. Giles, UK). The hybridization was done under high stringency conditions using the DIG High Prime DNA Labeling and Detection Starter kit II[TM] (Roche) to ensure the high specificity of hybridization. The results were visualized and documented using L-Pix Chemi photodocumentation system (Loccus, São Paulo, SP, Brazil).

Structural analysis of the OmlA protein

The amino acid sequences coded by the *omlA* genes were predicted using the bacterial genetic code in the program Mega 5.03 (Tamura *et al.*, 2007). The putative secondary structure was predicted using the Phyre algorithm (Kelley and Sternberg, 2009) and the Jnet algorithm of Jpred3 (Cole *et al.*, 2008). The resulting structures were compared and only motifs with probability scores > 80% were used to construct the consensus structure model of the OmlA protein. The membrane protein topology prediction method TMHMM, based on the Markov model, was used to predict transmembrane helixes (Krogh *et al.*, 2001). Conserved domains were located using the PROSITE database (Sigrist *et al.*, 2010) and the Conserved Domain Database (NCBI).

Phylogenetic molecular analysis

The 26 nucleotide sequences were initially aligned using Clustal W (Larkin *et al.*, 2007). Phylogenetic trees were built using the maximum parsimony (MP), maximum likelihood (ML) and neighbor-joining (NJ) methods. The neighbor-joining tree was inferred using the program MEGA 5.03 (Tamura *et al.*, 2007) and the remaining two were inferred using the program PAUP version 4.0b10 (Swofford, 2003). The ModelTest program version 3.7 (Posada and Crandall, 1998) was used to test 56 models and establish a DNA evolution model that would best fit the data for ML analysis. Subsequently, a heuristic search was done that was initiated using a NJ tree and the tree-bisection-reconnection (TBR) algorithm. The robustness of each internal knot of the trees was assessed statistically by using a combination of bootstrapping (1000 replications for the NJ and MP methods and 100 replications for the ML method) and the PAUP program. Because bootstrap values tend to be conservative, a Bayesian analysis was done using Mr. Bayes 3.0 (Huelsenbeck and Ronquist, 2001). The best model for the analysis was inferred using the program Mr. Modeltest 2.3 (Nylander, 2004).
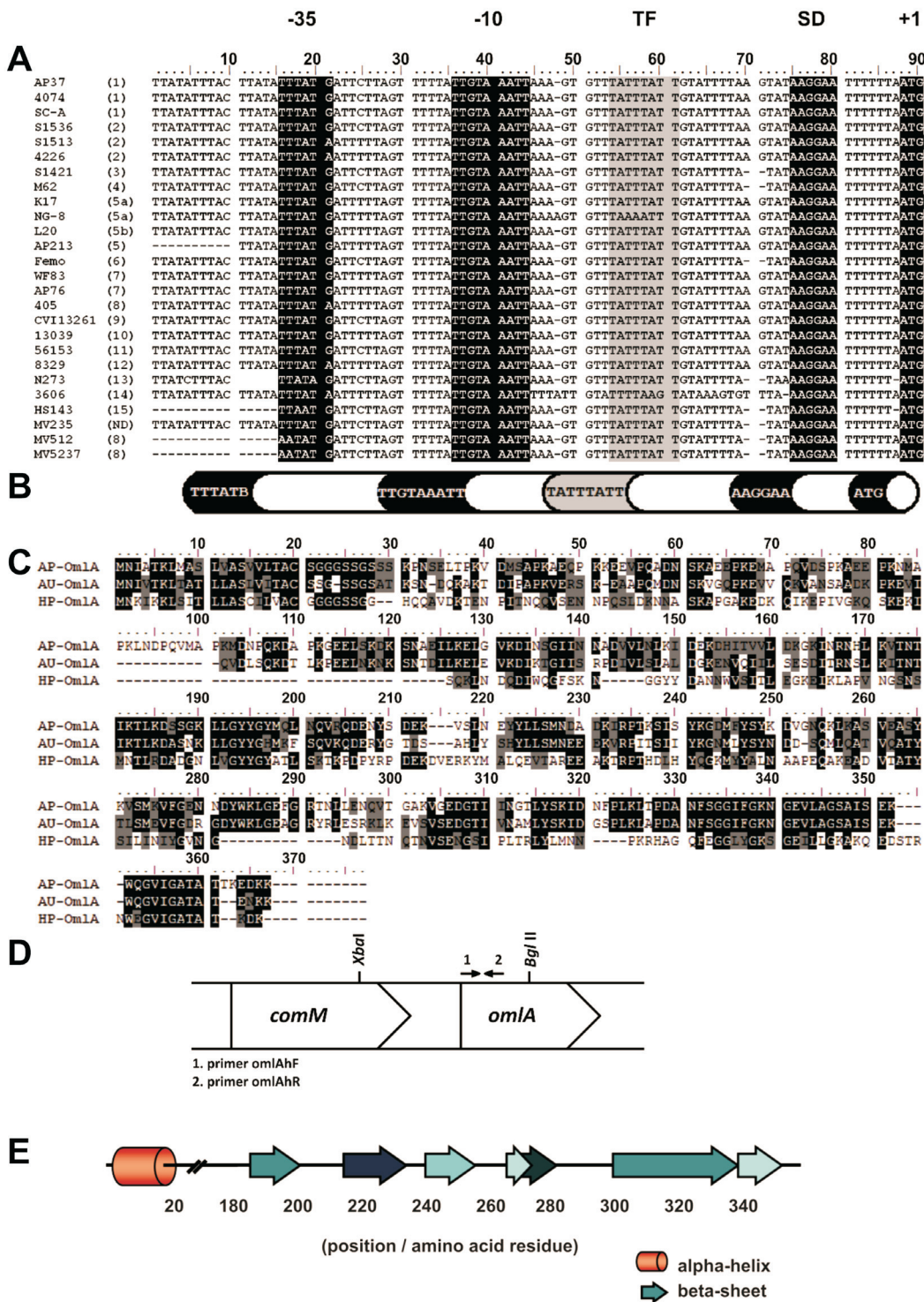
Figure 1 - Characterization of the *omlA* gene and the OmlA protein from different *Actinobacillus pleuropneumoniae* serotypes. (A) Promoter region of the *omlA* gene in the *A. pleuropneumoniae* isolates belonging to the 15 serotypes identified in this work. The putative transcription factor (TF) binding site, the -10, -35 and Shine-Dalgarno (SD) regions and the +1 point of translation are highlighted. (B) Schematic representation of the promoter region of the *omlA* gene of *A. pleuropneumoniae* based on the alignment shown in (A). The consensus sequences of the possible *cis* element for TF binding, the -35, -10 and Shine-Dalgarno regions and the translation initiation site are highlighted. B = adenine (17%) or guanine (83%). (C) Alignment of the OmlA sequences of *A. pleuropneumoniae* (AP)*, Actinobacillus ureae* (AU) and *Haemophilus parasuis* (HP). Identical sequences are highlighted in black while similar sequences are indicated in gray. (D) The region of the *omlA* gene used to synthesize the probes for Southern blotting and the cleavage sites for the restriction enzymes. (E) Putative secondary structure of the OmlA protein of *Actinobacillus pleuropneumoniae* as deduced using the algorithms Jpred3 and QuickPhyre. The cylinder represents the α-helix and the arrows represent the β-sheets. The intensities of the arrow colors reflect the prevalence of this structure in the 15 serotypes, with the lighter coloration indicating occurrence in 50% of the 15 serotypes and the darker coloration indicating occurrence in 100%.

Results

Sequence analyses

The sequences used in this study contained 1092-1125 base pairs and 506 variable sites (~43%) in the aligned positions. There were 450 parsimony-informative sites (39%) and the high variability in the nucleotide sequences resulted in 216 base substitutions, 77 (36%) in the first base of the codon, 84 (39%) in the second and 55 (25%) in the third (data not shown). Since most of the substitutions occurred in the first and second bases, there was a large number of variable sites in the amino acidic sequences deduced by Mega 5.03. Among the 383 aligned amino acids, 260 sites were variable (~68% of the total). The extensive number of alterations in the primary sequence of the OmlA protein resulted from transitions and transversions. Typically, transition rates are approximately two times higher than transversion rates (Zhang and Gerstein, 2003) since the latter are usually rapidly detected by DNA repair mechanisms. However, for the *omlA* gene, the transition/transversion ratio was ~0.99 (data not shown). Thus, the high transversion rate reflected the high genetic variability acquired during the evolution of different *A. pleuropneumoniae* serotypes.

Structural analysis of the *omlA* gene

The open reading frame (ORF) of *omlA* was flanked by the codons ATG and TAA as the initiation and termination codons, respectively, and the alignment of these sequences showed that the initial region of the gene was very conserved, as also pointed by Gram and Ahrens (1998). Between the +1 point of translation and point +160, 87.5% of the nucleotides were identical in all the serotypes and differences were observed in only a few isolates. The BPROM program identified the probable positions of the -10 and -35 regions of the promoters in the *omlA* genes (Figure 1A). The sequence TATTTATT was prevalent in the promoter region of the gene and the BPROM program suggested that this might be a binding site for the regulatory protein Lrp, a global transcription regulator and member of a widely distributed family among Bacteria and Archaea (Brinkman *et al.*, 2003). The putative Shine-Dalgarno region (ribosome binding site) was inferred based on the composition and position of the consensus sequence. Figure 1B shows the schematic organization of the promoter region of the *omlA* gene based on analyses of all available sequences.

Structural analysis of the OmlA protein

The deduced sequence of the OmlA protein was 362-375 amino acid residues long and 80% of the first 58 residues were identical in all of the sequences analyzed. The primary sequence of the *A. pleuropneumoniae* OmlA protein was similar to OmlA in *Actinobacillus ureae* and *Haemophilus parasuis,* both belonging to the family Pasteurellaceae. Alignment of the primary sequences of this protein from these three species (Figure 1C) revealed conservation of the OmlA structure, including the N-terminal region, which reinforces the importance of this portion of the molecule. The secondary structure model of OmlA based on the profiles generated by Jpred3 and Phyre revealed that a large part of the protein is organized in β sheets (Figure 1E), the positions of which vary only slightly according to the serotype; there was only one α-helix and this was located between the 5th and 20th amino acids and was conserved in all serotypes. In most serotypes, this region contained the amino acid sequence KLIAGLVAGLVLTAC, with variations only in serotypes 1, 8, 9 and 11. In these cases, the third (isoleucine), fifth (glycine), ninth (glycine) and tenth (leucine) amino acids were replaced by methionine, serine (two) and valine, respectively, to yield the sequence KLMASLVASVVLTAC. This sequence variation probably does not significantly affect the formation of the α-helix since the substituted amino acids are from the same charge groups as the original residues. The TMHMM model showed that the α-helix did not have a transmembrane insertion, which suggested involvement in another function. In the PROSITE database, the first 20 amino acids of the sequence matched a lipid-binding site (profile PS51257) and a signal peptide. Hence, the α-helix of the OmlA protein may serve to anchor the protein to the external membrane, in addition to its function in directing the transport of the protein to its extracellular location. Analysis using the Conserved Domain Database indicated that the OmlA protein belonged to the lipoprotein 5 superfamily, which contains proteins that bind to transferrin and is distinctly related to other protein families that bind to solutes.

Phylogenetic analysis

The phylogenetic trees obtained with the different methods used had very similar topologies. Figure 2A shows a Bayesian tree and the nine distinct groups identified based on the microorganism serotype: group 1 – serotypes 1, 9 and 11, group 2 – serotypes 2 and 8, group 3 – serotypes 3, 4 and 6, group 4 – serotype 13, group 5 – serotype 15 and our clinical isolates, group 6 – serotype 7, group 7 – serotypes 5 and 10, group 8 – serotype 14 and group 9 – serotype 12. The bootstrap values were equally high for all of the methods used to construct the phylogenetic trees, as were the posteriori probability values obtained in Bayesian analysis (close to or equal to 100%).

Whereas the initial regions of the genes were widely conserved, there was marked genetic variation in the internal and terminal regions among the serotypes. However, these differences in sequence homology largely disappeared when some of the groups in the phylogenetic trees were observed and analysed separated from the others. In group 1, the number of variable amino acid sites and variable nucleotide sites was 4 of 440 (0.9%) and 5 of 1320 (0.4%), respectively, which prevented their partition even
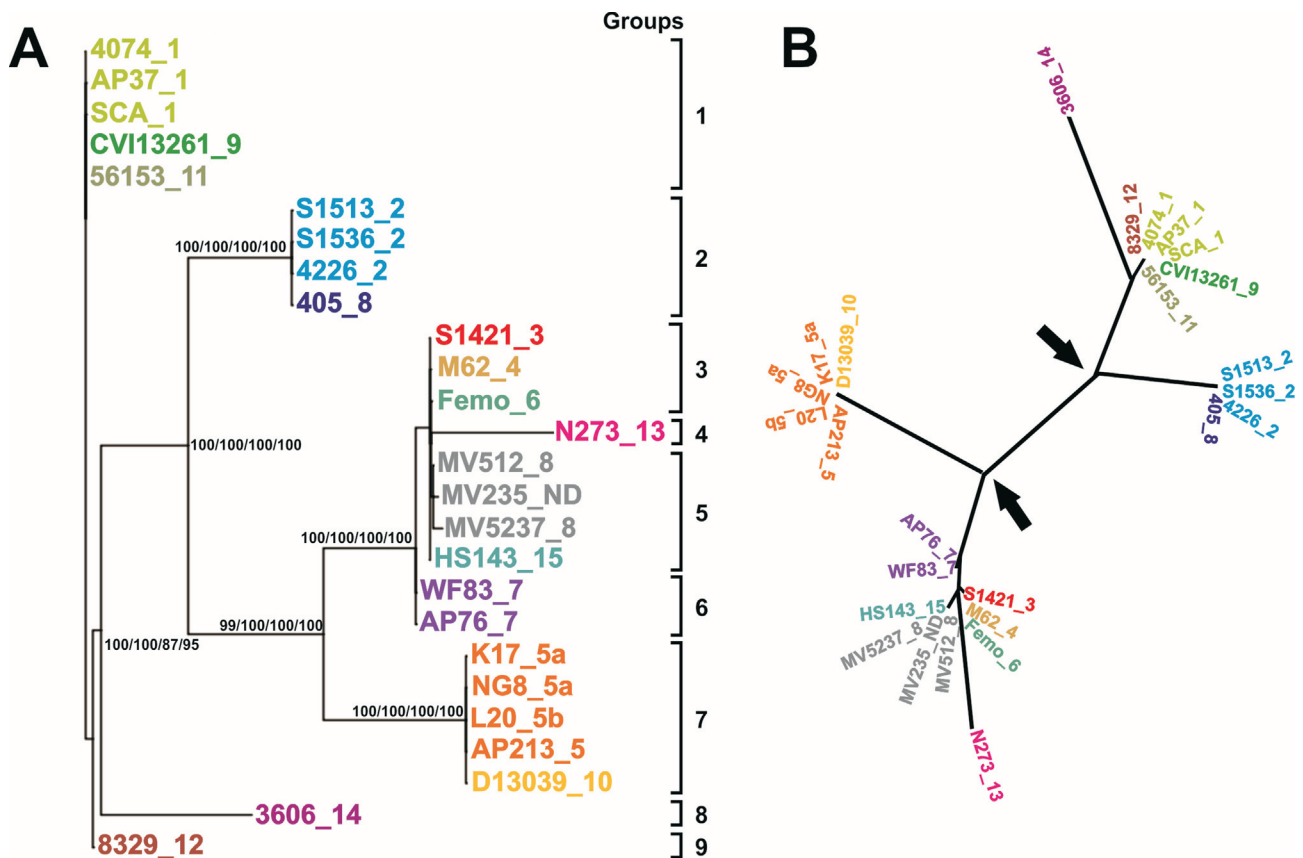
Figure 2 - Phylogenetic tree of 26 *omlA* gene sequences from 15 serotypes of *Actinobacillus pleuropneumoniae*. (A) Phylogenetic tree based on Bayesian analysis. Bootstrap values are shown next to the tree branches for each of the three phylogenetic methods used (neighbor-joining, maximum parsimony and maximum likelihood), along with the posterior probability values found for the consensus tree generated by Bayesian analysis. Branches show the strain name and corresponding serotype and are divided into nine groups. Different serotypes are indicated by different colors and clinical isolates are in gray. (B) Radial topology of the phylogenetic tree in (A). Arrows highlight the two main evolutionary events that may have divided the 15 serotypes into two large groups.

if the group was analyzed separately from the others, given their almost identical *omlA* sequences. For group 2, the corresponding values for variable sites were 1.5% and 0.9%, and for groups 3 and 7, these values were 2.5% and 3.6%, respectively. Serotypes 13, 14 and 15 were distantly related and the clinical isolates grouped with serotype 15. The radial topology (Figure 2B) of the tree shown in Figure 2A strongly suggested that the differentiation of the 15 *A. pleuropneumoniae* serotypes may have involved two distinct evolutionary events (highlighted by arrows in Figure 2B). This radial analysis separated the isolates into two major clusters: the first comprising serotypes 1, 2, 8, 9, 11, 12 and 14 and the second, serotypes 3, 4, 5, 6, 7, 10, 13 and 15. These cluster profiles were very similar to those obtained when the reconstruction was done using the predicted amino acid sequences (data not shown), the only difference being that in the latter case, the serotype 4 organisms had distanced themselves from serotypes 3 and 6. This divergence most likely resulted from the duplication of a sequence present between positions +144 and +176

(GenBank accession number AB007575) and added 11 amino acids to the OmlA protein.

Organization of the *omlA* gene in clinical isolates of *A. pleuropneumoniae*

Since the nucleotide sequence of the *omlA* gene varies significantly among serotypes, the primer pair omlAhF/omlAhR was synthesized based on the conserved region located in the initial extremity of the *omlA* gene; the resulting probe contained 372 bp. The organization of the *omlA* gene was investigated in clinical isolates of *A. pleuropneumoniae* obtained from seven farms in southeastern Brazil. Southern blotting revealed that the organization of the *omlA* gene was conserved in most of the isolates from the farms, with an expected 943-bp fragment after the cleavage of genomic DNA (Figure 3). Two of the isolates diverged from the expected results. Isolate MV452 contained two copies of the gene and isolate MV 653 had a different organization from the other isolates because of the presence of a similar fragment at a different position. This isolate apparently contained only one copy of the gene, but
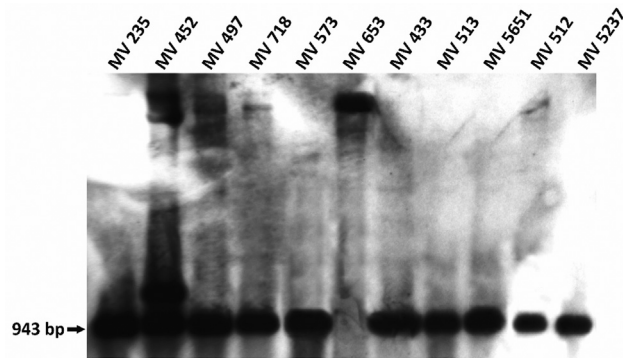
Figure 3 - Organization of the *omlA* gene in *Actinobacillus pleuropneumoniae*. Southern blotting was used to analyze clinical isolates from seven areas of southeastern Brazil. These isolates included non-serotypable (MV 235, MV 452, MV 497 and MV 718) and serotypable (MV 573, MV 653, MV 433, MV 513, MV 5651, MV 512 and MV 5237) forms.

since the fragment size was > 10 kb the genome of this isolate most likely did not have the same recognition sequences for cleavage by *Xba*I and *Bgl*II present in the other isolates.

Discussion

The OmlA protein belongs to a family of small, poorly characterized bacterial lipoproteins widely distributed in β and γ proteobacteria (Vanini *et al.*, 2008). OmlA protein is a virulence factor of *A. pleuropneumoniae* and has an important role in binding to transferrin to facilitate the acquisition of iron from the host (Baltes *et al.*, 2002).

The genetic diversity of the *omlA* gene was first observed when a 970-bp amplicon was digested with restriction enzymes and the resulting fragments then used to classify 12 serotypes into five groups (Osaki *et al.*, 1997). Subsequent analyses took advantage of the variability of the internal region of the *omlA* gene and used different techniques to try to distinguish the then known 12 serotypes, but could still only separate them into four or five groups (Gram and Ahrens, 1998; Gram *et al.*, 2000a). In the present study, part of the work by Gram and Ahrens (1998) was reassessed and the polymorphisms of the *omlA* gene in the 15 currently known serotypes were identified and used to build a phylogenetic tree based on more recent methods and their respective best fit models. This approach allowed us to separate the *A. pleuropneumoniae* serotypes into nine (when using the nucleotide sequences) or ten (when using the amino acid sequences) groups and also to infer possible evolutionary relationships between them. Additionally, the *omlA* genes from clinical isolates of *A. pleuropneumoniae* were sequenced and analyzed.

The existence of various serotypes and the range of antigenic differences among them has made effective vaccination against *A. pleuropneumoniae* difficult since only serotype-specific immunity is generally observed (Nielsen, 1984). This situation reinforces the importance of the pre-

cise characterization of isolates present in a given area. In addition, many *A. pleuropneumoniae* isolates are non-typable by currently used techniques but are nevertheless capable of causing disease (Fenwick, 2002). As shown here, the polymorphisms present in the nucleotide sequence of the *omlA* gene can be used to characterize isolates considered nontypable by conventional methods. In addition, the phylogenetic reconstruction described here for the *omlA* gene reinforced the characteristics noted elsewhere and confirmed the usefulness of this gene in distinguishing among *A. pleuropneumoniae* serotypes. For example, the clustering of serotypes 1, 9 and 11 agreed with the cross-reactivity observed amongst these serotypes (Paradis *et al.*, 1999). A similar conclusion is applicable to serotypes 3, 6 and 8 which also show similar tube agglutination, coagglutination and indirect hemagglutination (Mittal *et al.*, 1988). In this case, however, the molecular phylogeny separated serotype 8 from the other two. Cross-reactivity between isolates of serotypes 4 and 7 has also been observed (Mittal and Bourdon, 1991) and suggests evolutionary proximity between them. This conclusion was validated by their close clustering in the phylogenetic tree, although they were placed in monophyletic branches.

Together, these results indicate that the polymorphisms present in the nucleotide sequence of the *omlA* gene and in the amino acid sequence of the OmlA protein can be used as markers to distinguish among the serotypes of many isolates. This is a useful approach for understanding the characteristics and origin of isolates in a delimited region.

All of the clinical isolates examined here were grouped with serotype 15, even those designated by other molecular techniques as serotype 8, *i.e.*, some serotype 8 isolates of *A. pleuropneumoniae* in Brazil can show variation in the *omlA* gene that is actually closer to serotype 15. This finding agrees with Gram *et al.* (2000b) who identified some serotype 8 isolates in which the *omlA* gene was similar to serotypes 3, 4, 6 and 7. We also believe that although the isolate MV235 was not serotypable by commonly used methods, it was almost certainly a variation of serotype 8 because of its cluster position in the phylogenetic tree.

Although the expression of the *omlA* gene is constitutive in other organisms (Ochsner *et al.*, 1999), there is not much information on the expression of this gene in *A. pleuropneumoniae*. The structural model of the promoter region of the *omlA* gene suggests that there are possible binding sites for a transcription factor, the leucine-responsive regulatory protein (Lrp), that may control gene expression, particularly under stress (Wagner and Mulks, 2007), as has been observed in iron-restricted conditions (Deslandes *et al.*, 2007). Additionally, the predicted secondary structure of the OmlA protein indicates the existence of a conserved region that may be involved in the binding of this protein to the lipid region of the outer membrane of *A. pleuropneumoniae* and have a role as a signal

peptide. The tertiary structure of this protein cannot be predicted because of a lack of homologous proteins in the databases.

In contrast to the conserved organization of the *oml*A gene previously reported for other genomes (Gerlach *et al.*, 1993), we have shown that there are important variations in the organization of this gene in different *A. pleuropneumoniae* isolates obtained in Brazil, including a surprising duplication. Such variability has not been observed before and the duplication event may confer some advantage to this microorganism, *e.g.*, in adapting to new environments or even making it more virulent.

Although some aspects of the structural characterization of the *omlA* gene and the corresponding protein described here require additional experiments to confirm their functional relevance, we nevertheless believe that further detailed analysis of the genetic variability of this gene can yield important information on its role in the 15 serotypes of *A. pleuropneumoniae* identified in this work. The resulting information will improve our understanding of infection by *A. pleuropneumoniae*.

Acknowledgments

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Internet Resources

Bacterial Promoter Prediction Program BPROM, http://www.softberry.com (March 16, 2013).

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