Characterization of the omI 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 omI A 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 bioinformatics, 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 omI A gene in the biology of *A. pleuropneumoniae* and provide a new area of research into the OmlA protein.

Keywords: *Actinobacillus pleuropneumoniae*, omI A gene, phylogenetic reconstruction, porcine pleuropneumonia.

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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 omI A 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 omI A 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).
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% CO₂ 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

| Serotype | Strain | Accession number | 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 | 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.* | |
| 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 | MV235 | JF304621 | This study | Southern blotting |
| 5 | MV653 | - | This study | |
| 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 | |

1NCBI GenBank accession number. 2Unpublished. 3Not determined because of cross-reactivity in immunological tests.
kit™ (Promega, Madison, WI, USA) according to the manufacturer’s instructions. A pair of oligonucleotides, LF1 (5’-ATTGTAACCTTAGCCTTATT-3’) and LR1 (5’-ATTTAAGTTAAGCTATAACC-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 GoTag DNA polymerase (Promega) in a final volume of 50 µL of enzyme buffer containing 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.2 µM of each oligodeoxynucleotide and 50 ng of DNA in a C1000™ 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, JF304621 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 omlA 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’-CGGTTCAGTCGCAGTATTAG-3’) and omlAhR (5’-TCCTTACCCCTAAATTCTTAAGA-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 XbaI and BglII 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™ (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).

Phylogenetic molecular analysis

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).
Figure 1 - Characterization of the omfA gene and the OmlA protein from different *Actinobacillus pleuropneumoniae* serotypes. (A) Promoter region of the *omfA* 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 *omfA* 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 *omfA* 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 sequences (Figure 1A). 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%).

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 separately 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
if the group was analyzed separately from the others, given their almost identical \textit{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 \textit{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 \textit{omlA} gene in clinical isolates of \textit{A. pleuropneumoniae}

Since the nucleotide sequence of the \textit{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 \textit{omlA} gene; the resulting probe contained 372 bp. The organization of the \textit{omlA} gene was investigated in clinical isolates of \textit{A. pleuropneumoniae} obtained from seven farms in southeastern Brazil. Southern blotting revealed that the organization of the \textit{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

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Phylogenetic tree of 26 \textit{omlA} gene sequences from 15 serotypes of \textit{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.}
\end{figure}
serotype-specific immunity is generally observed (Nielsen, et al., 1997). Subsequent analyses took advantage of the variability 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 (Ochsner et al., 1999). 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.

Since the fragment size was > 10 kb the genome of this isolate most likely did not have the same recognition sequences for cleavage by XbaI and BglII 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).

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.

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 precise characterization of isolates present in a given area. In addition, many *A. pleuropneumoniae* isolates are nontypable 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.
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 omIA 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 omIA 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.

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References

Ashis SK, Torok VA, Percy NJ, Abimosleh SM and Howarth GS (2012) Microbial fingerprinting detects unique bacterial communities in the faecal microbiota of rats with experimentally-induced colitis. J Microbiol 50:218-225.

Baltes N, Hennig-Pauka I and Gerlach GF (2002) Both transferrin binding proteins are virulence factors in Actinobacillus pleuropneumoniae serotype 7 infection. FEMS Microbiol Lett 209:283-287.

Brinkman AB, Ettema TJ, de Vos WM and van der Oost J (2003) The Lrp family of transcriptional regulators. Mol Microbiol 48:287-294.

Bunka S, Christensen C, Potter AA, Willson PJ and Gerlach GF (1995) Cloning and characterization of a protective outer membrane lipoprotein of Actinobacillus pleuropneumoniae serotype 5. Infect Immun 63:2797-2800.

Cole C, Barber JD and Barton GJ (2008) The Jpred 3 secondary structure prediction server. Nucleic Acids Res 36:197-201.

Deslandes V, Nash JH, Harel J, Coulton JW and Jacques M (2007) Transcriptional profiling of Actinobacillus pleuropneumoniae under iron-restricted conditions. BMC Genomics 8:e72.

Fenwick B (2002) Porcine infectious pleuropneumonia. In: Bethel MA (ed) Pork Industry Handbook. Michigan State University Extension, East Lansing, 1178 pp.

Gerlach GF, Anderson C, Klashinsky S, Rossi-Campos A, Potter AA and Willson PJ (1993) Molecular characterization of a protective outer membrane lipoprotein (OmlA) from Actinobacillus pleuropneumoniae serotype 1. Infect Immun 61:565-572.

Gonzalez-Escalona N, Martinez-Urtaza J, Romero J, Espejo RT, Jaykus LA and DePaola A (2008) Determination of molecular phylogenetics of Vibrio parahaemolyticus strains by multilocus sequence typing. J Bacteriol 190:2831-2840.

Gottschalk M, Broes A, Mittal KR, Kobisch M, Kuhnert P, Lebrun A and Frey J (2003) Non-pathogenic Actinobacillus isolates antigenically and biochemically similar to Actinobacillus pleuropneumoniae: A novel species? Vet Microbiol 92:87-101.

Gram T and Ahrens P (1998) Improved diagnostic PCR assay for Actinobacillus pleuropneumoniae based on the nucleotide sequence of an outer membrane lipoprotein. J Clin Microbiol 36:443-448.

Gram T, Ahrens P, Andreasen M and Nielsen JP (2000a) An Actinobacillus pleuropneumoniae PCR typing system based on the apx and omIA genes-evaluation of isolates from lungs and tonsils of pigs. Vet Microbiol 75:43-57.

Gram T, Ahrens P and Angen O (2000b) Two Actinobacillus pleuropneumoniae serotype 8 reference strains in circulation. J Clin Microbiol 38:468.

Huelslneck JP and Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754-755.

Ito H (2008) Development of typing methods of Actinobacillus pleuropneumoniae based on the antigenic and genetic diversity of the protective outer membrane lipoprotein. Jpn Agr Res Q 42:261-266.

Ito H, Uchida I, Sekizaki T, Ooishi E, Kawai T, Okabe T, Taneno A and Terakado N (1995) Molecular cloning of an Actinobacillus pleuropneumoniae outer membrane lipoprotein (OmlA) from serotype 5a. Microb Pathog 18:29-36.

Jessing SG, Angen O and Inzana TJ (2003) Evaluation of a multiplex PCR test for simultaneous identification and serotyping of Actinobacillus pleuropneumoniae serotypes 2, 5, and 6. J Clin Microbiol 41:4095-4100.

Kelley LA and Sternberg MJ (2009) Protein structure prediction on the Web: A case study using the Phyre server. Nat Protoc 4:363-371.

Krogh A, Larsson B, von Heijne G and Sonnhammer EL (2001) Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. J Mol Biol 305:567-580.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al. (2007) Clustal W and Clustal X ver. 2.0. Bioinformatics 23:2947-2948.

Mittal KR and Bourdon S (1991) Cross-reactivity and antigenic heterogeneity among Actinobacillus pleuropneumoniae strains of Serotype-4 and Serotype-7. J Clin Microbiol 29:1344-1347.

Mittal KR, Higgins R and Lariviere S (1988) Quantitation of serotype-specifc and cross-reacting group-specific antigens by coagglutination and immunodiffusion tests for differentiating Actinobacillus (Haemophilus) pleuropneumoniae strains belonging to cross-reacting serotypes 3, 6, and 8. J Clin Microbiol 26:985-989.

Nielsen R (1984) Haemophilus pleuropneumoniae serotypes cross protection experiments. Nord Vet Med 36:221-234.
Nightingale KK, Windham K and Wiedmann M (2005) Evolution and molecular phylogeny of Listeria monocytogenes isolated from human and animal listeriosis cases and foods. J Bacteriol 187:5537-5551.

Nylander JAA (2004) MrModeltest ver. 2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Ochsner UA, Vasil AI, Johnson Z and Vasil ML (1999) Pseudomonas aeruginosa fur overlaps with a gene encoding a novel outer membrane lipoprotein, OmlA. J Bacteriol 181:1099-1109.

Osaki M, Sato Y, Tomura H, Ito H and Sekizaki T (1997) Genetic diversity of the genes encoding the outer membrane lipoprotein (omlA) of Actinobacillus pleuropneumoniae. J Vet Med Sci 59:213-215.

Paradis SE, Dubreuil JD, Gottschalk M, Archambault M and Jacques M (1999) Inhibition of adherence of Actinobacillus pleuropneumoniae to porcine respiratory tract cells by monoclonal antibodies directed against LPS and partial characterization of the LPS receptors. Curr Microbiol 39:313-30320.

Posada D and Crandall KA (1998) Modeltest: Testing the model of DNA substitution. Bioinformatics 14:817-818.

Rombel IT, Sykes KF, Rayner S and Johnston SA (2002) ORF-FINDER: A vector for high-throughput gene identification. Gene 282:33-41.

Sambrook J, Fritsch EF and Maniatis T (1989) Analysis of genomic DNA by Southern hybridization. In: Nolan C (ed) Molecular Cloning - A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, pp 9.32-9.58.

Schuchert JA, Inzana TJ, Angen O and Jessing S (2004) Detection and identification of Actinobacillus pleuropneumoniae serotypes 1, 2, and 8 by multiplex PCR. J Clin Microbiol 42:4344-4348.

Sigrist CJ, Cerutti L, de Castro E, Langendijk-Genevaux PS, Builliard V, Bairoch A and Hulo N (2010) PROSITE, a protein domain database for functional characterization and annotation. Nucleic Acids Res 38:D161-166.

Swofford DL (2003) Paup*: Phylogenetics analysis using parsimony (*and other methods) ver. 4.0b10. Sinauer Associates, Sunderland.

Tamura K, Dudley J, Nei M and Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software ver. 4.0. Mol Biol Evol 24:1596-1599.

Vanini MM, Spisni A, Sforca ML, Pertinhez TA and Benedetti CE (2008) The solution structure of the outer membrane lipoprotein OmlA from Xanthomonas axonopodis pv. citri reveals a protein fold implicated in protein-protein interaction. Proteins 71:2051-2064.

Wagner TK and Mulks MH (2007) Identification of the Actinobacillus pleuropneumoniae leucine-responsive regulatory protein and its involvement in the regulation of in vivo-induced genes. Infect Immun 75:91-103.

Williams JJ, Torres-León MA, Escheverria-Coelho P and Matos-Medina MC (2000) Aislamiento e identificación de Actinobacillus pleuropneumoniae en pulmones de cerdos con pleuroneumonia crónica sacrificados en el rastro municipal de Mérida, Yucatán, México. Rev Biomed 11:175-185.

Zhan B, Angen O, Hedegaard J, Bendixen C and Panitz F (2010) Draft genome sequences of Actinobacillus pleuropneumoniae serotypes 2 and 6. J Bacteriol 192:5846-5847.

Zhang Z and Gerstein M (2003) Patterns of nucleotide substitution, insertion and deletion in the human genome inferred from pseudogenes. Nucleic Acids Res 31:5338-5348.

Internet Resources

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

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