Pelistega suis sp. nov., isolated from domestic and wild animals

Ana I. Vela,1,2 Marta Perez Sancho,1 Lucas Domínguez,1 Hans-Jürgen Busse3 and Jose F. Fernández-Garayzábal1,2

Correspondence
J F. Fernández-Garayzábal
garayzab@vet.ucm.es

1Centro de Vigilancia Sanitaria Veterinaria (VISAVET). Universidad Complutense, 28040 Madrid, Spain
2Departamento de Sanidad Animal. Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain
3Institut für Bakteriologie, Mykologie & Hygiene, Veterinärmedizinische Universität Wien, A-1210 Wien, Austria

Biochemical and molecular genetic studies were performed on three novel Gram-stain-negative, catalase- and oxidase-positive, bacilli-shaped organisms isolated from the tonsils of two pigs and one wild boar. The micro-organism was identified as a species of the genus Pelistega based on its cellular morphological and biochemical tests. The closest phylogenetic relative of the novel bacilli was Pelistega indica HM-7T (98.2 % 16S rRNA gene sequence similarity to the type strain). groEL and gyrB sequence analysis showed interspecies divergence from the closest 16S rRNA gene phylogenetic relative, P. indica of 87.0.% and 69 %, respectively. The polyamine pattern contains predominantly putrescine and 2-hydroxyputrescine. The major quinone is ubiquinone Q-8 and in the polar lipid profile, phosphatidylethanolamine, phosphatidylglycerol, an unidentified aminolipid and an unidentified lipid are predominant. The novel bacterial isolate can be distinguished from P. indica by several biochemical characteristics, such as the production of L-pyrrolydonil arylamidase but not gamma-glutamyltransferase, and the utilization of different carbon sources. Based on both phenotypic and phylogenetic findings, the novel bacterium is classified as representing a novel species of the genus Pelistega, for which the name Pelistega suis sp. nov. is proposed. The type strain is 3340-03T (=CECT 8400T=CCUG 64465T).

The genus Pelistega, originally described by Vandamme et al. (1998), contains rod-shaped bacteria with variable morphological forms. At the time of writing, this genus embraces two species with validly published names, Pelistega europaea and Pelistega indica (Vandamme et al., 1998; Prakash et al., 2014). Isolates of P. europaea were obtained from diseased pigeons, while P. indica was isolated from human gut (Vandamme et al., 1998; Prakash et al., 2014). In this study, we report the phenotypic and phylogenetic features of three Pelistega-like organisms isolated from tonsil swab samples from two pigs (strains 3340-03T and DICM11-00060-1A) and one wild boar (strain ZTA10-00042-1C). Samples were collected, transported under refrigeration to the laboratory and processed for bacteriological analysis within 48 h. Samples were cultured on blood agar plates that were incubated at 37 °C for 24 h under aerobic and anaerobic [with 4–10 % CO2 using GasPak Plus (BBL) system] conditions. On the basis of the phenotypic and phylogenetic results, a novel species of the genus Pelistega is proposed.

For phylogenetic analysis, a large continuous fragment (approx. 1420 bases) of the 16S rRNA gene of the three isolates (3340-03T, DICM11-00060-1A and ZTA10-00042-1C) was determined from PCR-amplified products, derived from universal primers pA (5’-AGAGTTTGATCCTGCGAG-3’; positions 8–27, Escherichia coli numbering) and pH* (5’-AAGGAGGTATCCGAGGCAGC-3’; positions 1541–1522). The PCR was carried out in a volume of 100 μl, using 2.5 U Taq polymerase (Boehringer Mannheim), about 350 ng DNA, 500 nM of each primer and 200 μM dNTPs, in the appropriate buffer. After 2 min denaturation at 94 °C, the following cycle was repeated 30 times: 1 min denaturation at 94 °C, 65 seconds annealing at 55 °C and 90 seconds polymerization at 72 °C; the

Abbreviations: MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; PFGE, pulsed-field gel electrophoresis.

The GenBank/EMBL/DDJB accession numbers for the 16S rRNA gene sequence of strains 3340-03T, DICM11-00060-1A and ZTA10-00042-1C are LN830267, LN830268 and LN830269, respectively.

Two supplementary figures are available with the online Supplementary Material.
last cycle was followed by 10 min elongation at 72 °C. The amplified product was sequenced bidirectionally using universal primers a (5'-AGAGTTTGATCCTGGCTCAG-3'; positions 8–27, E. coli numbering), pH+ (5'-AAGGAGGTG ATCCAGCGCCA-3'; positions'1541–1522), antiIKK (5'-CG TGCCAGACGGCGGAAT-3'; positions 517–537) and 3 (5'-GGTGCGCTGTTGGCGGACT-3'). Comparative analysis of the 16S rRNA gene sequences revealed 99.7 to 99.9 % sequence similarity between the isolates, thereby demonstrating their high genealogical relatedness. The identifications of phylogenetic neighbours and calculations of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (Kim et al., 2012; http://eztaxon-e.ezbiocloud.net/). Sequence searches revealed that the unknown bacilli were members of the genus Pelistega, being most closely related to Pelistega indica HM-77 (98.2 % 16S rRNA gene sequence similarity). The 16S rRNA gene sequence similarity of isolate 3340-03T with the type strain of the only other species of the genus Pelistega (Pelistega europaea DSM 27484T) was 96.5 %. These sequences and those of other related strains were retrieved from the GenBank database and aligned with the newly determined sequences using the program SeqTools (Rasmussen, 2002). Phylogenetic trees were reconstructed according to three different algorithms: neighbour-joining (Saitou & Nei, 1987) using the programs SeqTools and TreeView (Page, 1996; Rasmussen, 2002), maximum-parsimony using the software package MEGA version 4 (Tamura et al., 2007), and maximum-likelihood using the PHYML software (Guindon & Gascuel, 2003). Genetic distances for the neighbour-joining and maximum-likelihood algorithms were calculated by Kimura’s two-parameter method (Kimura, 1980), and close-neighbour-interchange (search level=2, random additions=100) was applied in the maximum-parsimony analysis. The stability of the groupings was estimated by bootstrap analysis (1000 replications). According to the analysis of the 16S rRNA gene sequences based on the neighbour-joining algorithm, the two currently recognized species of the genus Pelistega and isolate 3340-03T were not grouped in a single clade (Fig. 1). Isolate 3340-03T and P. indica HM-77 formed a separate clade to that represented by P. europaea DSM 10982T which clustered with the type strains of Taylorella asinigenitalis and Taylorella equigenitalis. This clustering was supported by significant bootstrap resampling values and was also obtained with the other two tree-making algorithms (Fig. 1). Therefore, based on 16S rRNA gene sequence data, the genus Pelistega seems to be paraphyletic.

Sequencing of multiple protein-encoding genes with higher resolution than 16S rRNA gene sequences has been proposed for delineation of prokaryotic species (Tindall et al., 2010). Sequence analysis of the genes gyrB (encoding the B-subunit of DNA gyrase) and groEL (encoding the 60 kDa heat-shock protein GroEL) has been demonstrated to be a useful tool for bacterial systematics (Yamamoto et al., 2000; Watanabe et al., 2001; Rasis et al., 2014; Li et al., 2015). In the present study, nucleotide sequences of gyrB and groEL genes were determined from isolate 3340-03T, P. indica HM-77, P. europaea DSM 10982T, Advenella incenata CCUG 45225T, Brackiella oedipodis CCUG 47103T, Oligella urethralis CCUG 13463T and Taylorella equigenitalis CCUG 10786T. Partial sequences of gyrB (690 bp) and groEL (575 bp), chosen in conserved regions, were amplified using primer pairs gyrBF (5'-ATGACCGA TGCCNGNGTAGNGA-3') and gyrBR (5'-ARYTGRYCKGGA TTCACTCCTCA-3'), and groELF (5'-ATGCARTTGGCG NGNNTA-3') and groELR (5'-ACRATRCCCTCTCNAC NGC-3'). The gyrB and groEL sequence similarities of strain 3340-03T and its closest 16S rRNA gene phylogenetic relative P. indica DSM 27484T were 69 % and 87.0 %,
respectively. Strain 3340-03\textsuperscript{T} exhibited a mean gyr\textit{B} sequence similarity of 61.5 % with other 16S rRNA gene phylogenetic relatives, ranging from 57.8 % (\textit{A. incenata} CCUG 45225\textsuperscript{T}) to 73.0 % (\textit{P. europaea} CCUG 39967\textsuperscript{T}). The mean gro\textit{EL} sequence similarity was 80.1 %, ranging from 77.1 % (\textit{A. incenata} CCUG 45225\textsuperscript{T}) to 87.1 % (\textit{P. europaea} CCUG 39967\textsuperscript{T}). Evolutionary distances were calculated using Kimura’s two-parameter method (Kimura, 1980) and the resulting tree was reconstructed using the neighbour-joining algorithm using the software package MEGA version 4 (Tamura et al., 2007). The phylogenetic trees inferred from gyr\textit{B} and gro\textit{EL} gene sequence comparisons revealed that \textit{P. indica} DSM 27484\textsuperscript{T} and \textit{P. europaea} LMG 10982\textsuperscript{T} grouped together with strain 3340-03\textsuperscript{T} in a single clade, with the later strain forming a separate branch from the two species of the genus \textit{Pelistega} (Fig. S1, available in the online Supplementary Material).

Phylogenetic analysis based on concatenated gyr\textit{B} and 16S rRNA gene sequences was also performed as genetic trees inferred from \textit{gyrB} and 16S rRNA gene sequences show- ing the position of strain 3340-03\textsuperscript{T} and its closest phylogenetic neighbours. Bootstrap values (expressed as a percentage of 1000 replications) >50 % are given at the branching points. Bar, 1 % sequence divergence.

The three novel isolates were Gram-stained and assessed for the presence of catalase. The haemolytic reaction was determined on Columbia agar containing 5 % desfibrinated sheep blood (bioMérieux) incubated aerobically at 37 °C for 24 and 48 h (Facklam & Elliott, 1995). Determination of the growth at 4, 15, 22, 30, 37 and 42 °C was performed in brain heart infusion broth (Difco) with the pH adjusted to 7.5 (Facklam & Elliott, 1995). The ability of the isolates to tolerate the presence of 3.5, 4.5 and 6.5 % NaCl in brain heart infusion broth (Difco) was assessed as recommended by Facklam & Elliott (1995). The isolates were biochemically characterized using the API 20E, API 20NE and API ZYM systems (bioMérieux) according to the manufacturer’s instructions. Bacterial suspensions were also analysed with GN cards using the VITEK system (bioMérieux) following the manufacturer’s instructions. The three isolates exhibited identical biochemical characteristics. The phenotypic characteristics that differentiate the proposed species from other species of the genus \textit{Pelistega} are shown in Table 1. Detailed descriptions of morphological, physiological and biochemical data for the novel species are given in the species description.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been considered a reliable identification method for species of the genus \textit{Pelistega} (Prakash et al., 2014) and it was therefore used in the present study to characterize the potential novel species. Colonies from strain 3340-03\textsuperscript{T} and from \textit{P. indica} DSM 27484\textsuperscript{T} were subjected to a protein extraction protocol according to the manufacturer’s instructions (SOP, Ethanol/Formic Acid Extraction Method; Bruker Daltonics) and were spotted in eight different spots [to test reproducibility and for the creation of a mass spectral profile (MSP)] on the MALDI-TOF MS sample plate and air-dried at room temperature. A 1 μl aliquot of matrix

Table 1. Characteristics useful in differentiating \textit{Pelistega suis} sp. nov. from the other two species of the genus \textit{Pelistega}

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tr>
<td>Growth on MacConkey agar</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>L-Pyrrolydonil arylamidase</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Gamma-glutamyl-transferase</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate (sodium)</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Alkalization of:</td>
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<td></td>
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<tr>
<td>L-Lactate</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Succinate</td>
<td>+</td>
<td>–</td>
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<td>Assimilation of:</td>
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<tr>
<td>L-Malate</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>L-Lactate</td>
<td>+</td>
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Fig. 2. Partial neighbour-joining phylogenetic tree inferred from concatenated gyr\textit{B}, gro\textit{EL} and 16S rRNA gene sequences showing the position of strain 3342-03\textsuperscript{3} and its closest phylogenetic neighbours. Bootstrap values (expressed as a percentage of 1000 replications) >50 % are given at the branching points. Bar, 1 % sequence divergence.
solution [saturated solution of \(\alpha\)-cyanohydroxycinnamic acid (\(\alpha\)-HCCA; Sigma-Aldrich)] dissolved in 50 % acetonitrile, 47.5 % water and 2.5 % trifluoroacetic acid (Fluka; Sigma-Aldrich) was added onto each sample spot and again allowed to dry (Liu et al., 2007; Böhme et al., 2010). Mass spectra acquisition was performed on a Bruker UltraflexXtrem platform (Bruker Daltonics) using a mass range of 2–20 kDa. The Bruker Bacterial Test Standard (Escherichia coli DH5) was used as an external protein calibration mixture (Alispaic et al., 2010). Mass spectra were smoothed, baseline corrected and peak detected using the FlexAnalysis software (version 3.4, Bruker Daltonics). Three biological replicates from each sample were created and analysed to test the reproducibility of the procedure. Generated profiles were compared with the bacterial database of Bruker Daltonics generated using type cultures of previously characterized bacteria. The results of MALDI-TOF for strain 3340-03\(^\top\) did not match with any bacterial species in the Bruker Daltonics database. MALDI-TOF data showed that the profile of both strain 3340-03\(^\top\) and \(P\). indica DSM 27484\(^\top\) contained 70 peaks in the range of 3000 to 12 000 Da. However, comparison of the MALDI-TOF mass spectra of both strains showed differences (Fig. S2) in terms of quantity of proteins (number of spikes) and amount of expressed protein (intensity of peaks). MALDI-TOF mass spectra of strain 3340-03\(^\top\) was also different to that of \(P\). europaea CCUG 39967\(^\top\) (data not shown). These MALDI-TOF data corroborate the phylogenetic results based on the 16S rRNA gene sequencing and indicate that strain 3340-03\(^\top\) is different from both currently recognized species of the genus \(P\). \(L\) bestega.

Preparation of high-molecular-mass DNA and DNA–DNA hybridization experiments were performed by the Identifi- cation Service of the Leibniz Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). Genomic DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA–DNA hybridization was carried out in 2 \(\times\) SSC at 68 °C by the Identification Service of the DSMZ using the method described by De Ley et al. (1970) under consideration of the modifications described by Huss et al. (1983) with a model Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostated 6 \(\times\) 6 multicell changer and a temperature controller with \(\textit{in situ}\) temperature probe (Varian). The DNA–DNA reassociation value between isolate 3340-03\(^\top\) and \(P\). indica DSM 27484\(^\top\) was 10.4 %, below the recommended threshold value of 70 %, which confirms that this taxon merits its separate species status (Wayne et al., 1987).

Biomass subjected to chemotaxonomic analyses was grown in 3.3 \(\times\) PYE broth (1 % peptone from casein, 1 % yeast extract, pH 7.2). Quinones and polar lipids were extracted from biomass harvested at the stationary growth phase and analysed applying the integrated procedure reported by Tindall (1990a, b) and Altenburger et al. (1996). Polyamines were extracted and analysed from cells harvested at the late exponential growth phase as recommended by Busse & Auling (1988). HPLC analysis was carried out using the equipment described by Stolz et al. (2007) slightly modified in the gradient as reported by Busse et al. (1997). The quinone system of strain 3340-03\(^\top\) consisted predominantly of ubiquinone Q-8 (97 %) and minor amounts of Q-9 (2 %) and Q-7 (1%). The polar lipid profile contained the major lipids phosphatidylethanolamine, phosphatidylglycerol, an unidentified aminolipid (AL1) and an unidentified lipid (L1) only visible after staining for total lipids. Furthermore, moderate to minor amounts of another aminolipid (AL2) and four polar lipids (L2–L5) were detected (Fig. 3). The polyamine pattern of strain 3340-03\(^\top\) was composed of putrescine [31.6 \(\mu\)mol (g dry weight)\(^{-1}\)], 2-hydroxypu- trescine [31.3 \(\mu\)mol (g dry weight)\(^{-1}\)] and minor amounts of 1,3-diaminopropane [0.2 \(\mu\)mol (g dry weight)\(^{-1}\)], cadaverine [0.1 \(\mu\)mol (g dry weight)\(^{-1}\)], spermi- dine [3.1 \(\mu\)mol (g dry weight)\(^{-1}\)] and spermine [1.7 \(\mu\)mol (g dry weight)\(^{-1}\)]. These chemotaxonomic traits are in line with those of other members of the family \(A\). \(A\). \(c\)aceae (Kämpfer et al., 2006, 2010; Blümel et al., 2001; Busse et al., 1992; Stolz et al., 2005; Zhang et al., 2012).

The three strains from domestic and wild pigs were characterized by pulsed-field gel electrophoresis (PFGE) profiling of their genomic DNAs, after digestion with the restriction enzyme \(Bsp\)1201, according to previous specifications (Garcia et al., 2011). Similarities between restriction endonuclease digestion profiles were based on visual comparisons of the band patterns of strains run in the same gel. Strains differing in at least one band were considered

Fig. 3. Total polar lipid profile of strain 3342-03\(^\top\) after staining with ethanolic molybdatophosphoric acid. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL1–AL2, unidentified aminolipids; L1–L5, unidentified polar lipids only detectable after applying molybdatophosphoric acid.
different. The strains displayed three different PFGE restriction profiles (data not shown).

Overall, the results of the present polyphasic study demonstrate that the characteristics of the novel strains from pigs and wild boar match those described for the genus Pelistega, and the strains should be assigned to this genus. The phylogenetic, genotypic and phenotypic differences clearly demonstrate that the strains merit classification as a novel species of the genus, for which the name Pelistega suis sp. nov. is proposed.

Description of Pelistega suis sp. nov.

Pelistega suis (su’is. L. gen. n. suis of the hog).

Cells are Gram-stain-negative, 0.75 μm wide by 1 μm long, non-spor-forming, non-motile and bacilli-shaped. Facultatively anaerobic and catalase- and oxidase-positive. Colonies are circular, smooth, entire and approximately 1 mm diameter on Columbia blood agar after incubation for 24 h at 37 °C. Growth does not occur on MacConkey agar. Cells are not able to grow in the presence of 3 % NaCl. Produces acetoin at 37 °C. Colonies of the species are DICM11-00060-1A and ZTA10-00042-1C, isolated from a pig and a wild boar, respectively.

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proposal of *Castellaniella defragrans* gen. nov., comb. nov. and *Castellaniella denitrificans* sp. nov. *Int J Syst Evol Microbiol* 56, 815–819.


