Peptoniphilus duerdenii sp. nov. and Peptoniphilus koenoeneniae sp. nov., isolated from human clinical specimens

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Two previously uncharacterized strains of Gram-reaction-positive, anaerobic, coccus-shaped bacteria, designated strains WAL 18896T and WAL 18898T, were recovered from human wound specimens and characterized using phenotypic, chemotaxonomic and molecular taxonomic methods. Comparative 16S rRNA gene sequence analysis and chemotaxonomic and biochemical characteristics demonstrated that these organisms are genotypically and phenotypically distinct and represent previously unidentified sublines within the order Clostridiales in the phylum Firmicutes. Pairwise sequence analysis demonstrated that the novel organisms had 91.9% sequence similarity to each other and were most closely related to members of the genus Peptoniphilus. The major long-chain fatty acids of both strains were C16:0, C18:0, C18:1ω9c and C18:2ω6c,9c. Based on the phenotypic and phylogenetic findings, strains WAL 18896T (=CCUG 56065T = ATCC BAA-1640T) and WAL 18898T (=CCUG 56067T = ATCC BAA-1638T = DSM 22616T) represent two novel species, for which the names Peptoniphilus duerdenii sp. nov. and Peptoniphilus koenoeneniae sp. nov. are proposed, respectively.

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Gram-positive anaerobic cocci (GPAC) are part of the normal commensal flora in humans and animals and are commonly associated with a variety of human infections. Most clinical isolates of GPAC were originally classified as species of the genus Peptostreptococcus (Murdoch, 1998) but 16S rRNA sequencing has since confirmed the heterogeneity of GPAC and has subsequently led to a major taxonomic revision of this group of micro-organisms with many species being transferred to different genera, including Peptinophilus, Peptostreptococcus, Parvimonas, Finegoldia, Anaerococcus and Gallicola (Ezaki et al., 2001; Murdoch & Shah, 1999; Finegold et al., 2002; Tindall & Euzéby, 2006). The genus Peptoniphilus currently includes the following butyrate-producing, non-saccharolytic species that use peptone and amino acids as major energy sources: Peptoniphilus acascharolyticus (type species), Peptoniphilus lacrimalis, Peptoniphilus harei, Peptoniphilus indolicus, Peptoniphilus ivorii, Peptoniphilus gorbachii, Peptoniphilus methioninivorax and Peptoniphilus olseni (Ezaki et al., 2001; Rooney et al., 2011; Song et al., 2007a). In the present study, two isolates of GPAC were recovered from clinical specimens. Comparative 16S rRNA gene sequencing studies demonstrated that the two strains were genealogically heterogeneous and did not appear to correspond to any of the recognized species of GPAC. 16S rRNA gene sequence similarity values of 88–91 % between the novel strains and species of the genus Peptoniphilus demonstrated the phylogenetic separation of the novel strains from previously described species. Based on the biochemical, chemotaxonomic and phylogenetic findings presented here, the two novel strains represent two novel species of the genus Peptoniphilus.

Two clinical strains, designated WAL 18896T and WAL 18898T, were isolated from a vaginal abscess and from a buttock abscess, respectively, at the Olive View-UCLA

Abbreviation: GPAC, Gram-positive anaerobic cocci.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains WAL 18896T and WAL 18898T are EU526290 and EU526291, respectively.

A supplementary figure is available with the online version of this paper.
Medical Center. The specimens were processed and characterized using methods outlined in the Wadsworth-KTL Anaerobic Bacteriology Manual (Jousimies-Somer et al., 2002). The strains were cultivated on Brucella blood agar supplemented with 5% sheep’s blood, 5 μg haemin ml⁻¹ and 1 μg vitamin K₁ ml⁻¹ and incubated at 37 °C in an atmosphere of 90% N₂ : 5% H₂ : 5% CO₂. Strains WAL 18896ᵀ and WAL 18898ᵀ were co-isolated from human clinical specimens along with other strict anaerobes and/or aerobes, displaying heavy growth on primary isolation (≥10⁵ c.f.u. ml⁻¹). The mean numbers of accompanying anaerobic and aerobic species co-isolated with the novel strains were 7.7 and 1.5, respectively. Moderate to heavy growth was obtained for most of the accompanying isolates on primary isolation on blood agar plates. The majority of the accompanying isolates were present in counts of ≥10⁴ c.f.u. ml⁻¹. The novel strains grew anaerobically but did not grow following subculturing in air or in atmospheres of 2 or 6% O₂. Cells of the novel strains were Gram-reaction-positive and coccus-shaped with a typical cell size of ≥0.7 μm in diameter. Colonies of the novel strains were grey, flat or slightly convex, circular, entire and opaque with a diameter of 1–2 mm on Brucella blood agar plates after 5 days of growth. Both of the novel strains were sensitive to kanamycin (1000 μg) and vancomycin (5 μg) and were resistant to colistin sulfate (10 μg) and sodium polyanethol sulfonate (SPS); the strains were also sensitive to bile. The novel strains were catalase- and urease-negative and negative for nitrate-production and the spot indole test was variable. Both strains were asaccharolytic and did not produce acid from glucose or other carbohydrates tested.

Table 1. Key distinguishing characteristics of strains WAL 18896ᵀ and WAL 18898ᵀ and type strains of species of the genus Peptoniphilus

<table>
<thead>
<tr>
<th>Characteristic</th>
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<td>Alanine arylamidase</td>
<td>−</td>
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<td>Arginine dihydrolase</td>
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<td>Alkaline phosphate</td>
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<td>Glutamyl glutamic acid arylamidase</td>
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<td>−</td>
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<td>Tyrosine arylamidase</td>
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The novel strains were characterized biochemically by using a combination of conventional tests and the commercially available biochemical kits Rapid ID 32A and API ZYM (bioMérieux) (Song et al., 2007b). All tests were performed in duplicate. Profiles generated using the Rapid ID 32A kit were especially useful in differentiating the tested isolates. The Rapid ID 32A profiles for strains WAL 18896ᵀ and WAL 18898ᵀ were 0/2 0 0 0 0 1 6 0 0 0 and 2 0 0 0 0 1/3 7 1/5 0 4/5, respectively. Using the Rapid ID 32A kit, strain WAL 18896ᵀ gave positive reactions for arginine arylamidase, leucine arylamidase and pyroglutamic acid arylamidase activities and a weak reaction was obtained for alanine dihydrodrolase activity. According to the API ZYM system, the only positive reaction with strain WAL 18896ᵀ was for leucine arylamidase activity; all other enzyme tests were negative. Using the Rapid ID 32A system, strain WAL 18898ᵀ gave positive reactions for arginine dihydrodrolase, arginine arylamidase, leucine arylamidase, proline arylamidase, phenylalanine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase and serine arylamidase activities. Using the API ZYM system strain WAL 18898ᵀ was only weakly positive for acid phosphatase activity. The test results that were most useful for differentiating the novel strains from members of the genus Peptoniphilus using the Rapid ID 32A system are given in Table 1. It is of note that pyroglutamic acid arylamidase was produced by both the novel strains but was not produced by any of the type strains of species of the genus Peptoniphilus.

Carbohydrate fermentation tests were performed using pre-reduced, anaerobically sterilized peptone–yeast broth tubes (Anaerobe Systems) with the addition of different carbohydrates. The strains were grown in peptone–yeast (PY) broth and peptone–yeast–glucose (PYG) broth (Anaerobe Systems) for metabolic end product (short-chain volatile and non-volatile fatty acid) analysis by GLC as described previously (Jousimies-Somer et al., 2002). Strain WAL 18896ᵀ produced major amounts of propionic acid, butyric acid and acetic acid, a moderate amount of oxalic acid and trace amounts of valeric acid, isovaleric acid, isobutyric acid and succinic acid. Strain WAL 18898ᵀ produced moderate amounts of butyric acid and propionic acid and trace amounts of acetic acid, oxalic acid and pyruvic acid.

Fatty acid methyl ester analysis was performed using the MIDI Sherlock Microbial Identification System as described previously (Kämpfer & Kroppenstedt, 1996; Miller, 1982; Sasser, 1990). Cells were grown for 3 days on Chocolate agar (Brain Heart Infusion agar, Difco 241830) under anaerobic conditions for 72 h at 37 °C. Analysis was carried out with a Hewlett Packard HP 5890 gas chromatograph equipped with a phenyl methyl silicone fused silica capillary column (HP-5; 25 m x 0.2 mm x 0.33 μm film thickness) and a
flame-ionization detector. Hydrogen was used as the carrier gas. The temperature program was initiated at 170 °C and increased by 5 °C min⁻¹ to a final temperature of 270 °C. Integration of peaks and further calculations were performed by using an HP 3396A integrator. After correction of the areas, the percentage compositions were determined and the retention time data were converted to equivalent chain-length (ECL) values. The quantitative fatty acid compositions of the two novel strains were as follows. Strain WAL 18896ᵀ contained major amounts of C₁₆ : ₀ (33.0 %), C₁₈ : ₀ (16.2 %), C₁₈ : ₁ο₉c (22.6 %) and C₁₈ : ₂ο₆,₉c/anteiso-C₁₈ : ₀ (21.1 %); minor amounts of C₁₀ : ₀ (2.8 %) and C₁₄ : ₀ (4.4 %) were also detected. Strain WAL 18898ᵀ contained major amounts of C₁₆ : ₀ (22.6 %), C₁₈ : ₀ (12.1 %), C₁₈ : ₁ο₉c (24.4 %) and C₁₈ : ₂ο₆,₉c/anteiso-C₁₈ : ₀ (20.6 %); minor amounts of C₁₄ : ₀ (3.1 %), C₁₆ : ₁ο₉c (3.3 %), iso-C₁₇ : ₁/C₁₆ : ₀ DMA (4.3 %), iso-C₁₇ : ₁ 3-ΟΗ (3.3 %) and C₁₈ : ₁ο₉c DMA (6.3 %) were also detected. Table S1 (available in IJSEM Online) shows the fatty acid compositions of the two novel strains as well as those of described type strains of species of the genus Peptoniphilus. The predominant diagnostic fatty acids detected were C₁₆ : ₀, C₁₈ : ₀, C₁₈ : ₁ο₉c and C₁₈ : ₂ο₆,₉c however, minor changes within the fatty acid profiles of each of the species listed were also useful in differentiating the novel strains from other members of the genus.

DNA was isolated using a MO BIO Ultraclean Microbial DNA Isolation kit or by a standard phenol/chloroform extraction. DNA G+C content (mol%) was determined by HPLC (Mesbah et al., 1989) using a Prevail C₁₈ reversed-phase column (Alltech) at room temperature with a mobile phase of 25 mM KH₂PO₄/CH₃CN (96 : 4), pH 2.5. The DNA G+C contents of WAL 18896ᵀ and WAL 18898ᵀ were 33.4 and 32.4 %, respectively.

The 16S rRNA genes were amplified by PCR using universal primers 8UA (positions 8–28, based on Escherichia coli numbering) and 1485B (positions 1485–1507) as described previously (Song et al., 2003). The amplified product was purified by using a QIAamp PCR purification kit (Qiagen) and directly sequenced with an ABI 3100 Avant Genetic System (Applied Biosystems). The closest relatives of the new isolates were determined by performing database searches using BLAST software (Benson et al., 1997). The corresponding sequences of related strains were retrieved from GenBank and aligned with the newly determined sequences of the novel strains using the program CLUSTAL W (http://www.genome.jp/tools/clustalw/) (Thompson et al., 1994). Phylogenetic analysis was performed by using the neighbour-joining algorithm in MEGA version 4 (Tamura et al., 2007).

Searches of GenBank and Ribosomal Database Project libraries for sequences of related species with validly published names revealed that the novel strains were members of the phylum Firmicutes and were most closely related to members of the genus Peptoniphilus. Pairwise comparisons between the sequences of the two novel strains (1406 bp) demonstrated a sequence similarity of 91 %. Between the type strain of the type species of the genus Peptoniphilus, P. asaccharolyticus, and the novel strains, sequences diverged by ~9 % for strain WAL 18896ᵀ and ~10 % for WAL 18898ᵀ. A phylogenetic tree, reconstructed by using the neighbour-joining method, depicting the phylogenetic position of the novel bacteria is shown in Fig. 1. All the major branching nodes were confirmed by using the maximum-parsimony method (Fig. S1). There was some rearrangement of the branching order within members of the genus Peptoniphilus due to the similar sequence divergence values between some of these species. Each of the novel strains represents a previously unreported subline within the genus Peptoniphilus. While previously assigned to the family Peptostreptococcaceae, in the latest edition of Bergey’s Manual of Systematic Bacteriology (Garrity et al., 2005) members of the genus Peptoniphilus, as well as a number of other genera, have

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**Fig. 1.** Phylogenetic dendrogram based on 16S rRNA gene sequences indicating the position of strains WAL 18896ᵀ and WAL 18898ᵀ within the genus Peptoniphilus. The tree was reconstructed using the neighbour-joining algorithm in the MEGA version 4 software package. Sedimentibacter saalensis was used as the outgroup. Bootstrap values >70 % (based on 1000 replications) are displayed on their relative branches. Bar, 1 % sequence divergence.
been placed within the family *Incertae Sedis* XI until their taxonomic status can been clarified (Ezaki *et al.*, 2001). While a DNA–DNA hybridization value of ~70% is generally recognized as the threshold for the delineation of bacterial species (Wayne *et al.*, 1987), no absolute values have been determined as thresholds for species and genus delineations based on 16S rRNA gene sequence similarities. While it is generally recognized that sequence divergence values of \(\geq 3\%\) are significant (Stackebrandt & Goebel, 1994), more recent data demonstrate that this value can be decreased to 1.3% without loss of resolution for strains to be recognized as separate species (Stackebrandt & Ebers, 2006). It appears to be more much of a difficult task when assigning strains to a novel genus based on sequencing data. For example, within the genus *Enterococcus*, a number of sub-clusters or groups are evident with 16S rRNA similarities between species as high as 99.8%. Despite these close relationships, these species have been differentiated based on the results of DNA–DNA hybridizations (Svec & Devries, 2009). Conversely, members of the genus *Porphyromonas* form a natural, but deep, phylogenetic group exhibiting 16S rRNA gene sequence divergence values of up to 15%. However, it appears that species of this more loosely associated grouping do possess a number of common biochemical and chemotaxonomic traits consistent with that of a genus. Similarly, many of the members of the genus *Peptoniphilus* share large sequence divergence values with the type strain of the type species *P. asaccharolyticus* but share consistent biochemical and chemotaxonomic (including fatty acid) profiles. If each species with a low sequence similarity were to be designated as a novel genus an explosion of novel taxa with few, if any, unique features would ensue.

GPAC are some of the most commonly isolated groups recovered from clinical specimens and rapid and reliable methods of identification are important not only for the recovery of these organisms but also for the evaluation of the importance of this group in the GI tract. Indeed, most members of the genus *Peptoniphilus* have been isolated from clinical sources where the use of 16S rRNA gene sequencing (Fontana *et al.*, 2005), cellular fatty acid profiling (Sasser, 1990) and a number of commercially available miniaturized biochemical systems (Song *et al.*, 2007a, 2007b; O’Hara *et al.*, 1993) are routinely used to identify known and novel isolates. In addition to data demonstrating their unique 16S rRNA gene sequences, the traits used for distinguishing the novel strains from other species of the genus *Peptoniphilus* are shown in Table 1, Table S1 and the species descriptions. Based on phenotypic, genotypic and chemotaxonomic evidence, strains WAL 18896\(^T\) and WAL 18898\(^T\) represent two novel species of the genus *Peptoniphilus*, for which the names *Peptoniphilus duerdenii* sp. nov. and *Peptoniphilus koenoeneniae* sp. nov., respectively, are proposed.

**Description of *Peptoniphilus duerdenii* sp. nov.**

*Peptoniphilus duerdenii* (duer.de’n.i.i. N.L. gen. masc. n. *duerdenii* of Duerden, to honour Dr Brian Duerden, a British microbiologist who has contributed much to our knowledge of anaerobic bacteriology).

Cells are coccus-shaped and stain Gram-positive. Typical cells are \(\geq 0.7\) μm in diameter. Colonies are grey, flat or slightly convex, entire, circular, opaque and 1–2 mm in diameter on Brucella blood agar plates after 5 days of growth at 37°C. Cells are obligately anaerobic. Positive for indole production. Negative for catalase and urease activities and nitrate reduction. Cells are asaccharolytic; acid is not produced from glucose or other sugars. In peptone yeast broth and peptone yeast glucose broth, major amounts of propionic acid, butyric acid and acetic acid, a moderate amount of oxalic acid and trace amounts of valeric acid, isovaleric acid, isobutyric acid and succinic acid are produced. Using the Rapid ID 32A system, positive for arginine arylamidase, leucine arylamidase and pyroglycamic acid arylamidase activities; weakly positive for alanine dihydrolase activity; and negative for N-acetyl-\(\beta\)-glucosaminidase, alanine arylamidase, alkaline phosphatase, \(\alpha\)-arabinosidase, \(\alpha\)- and \(\beta\)-galactosidase, \(\beta\)-galactosidase-6-phosphate, \(\alpha\)- and \(\beta\)-glucosidase, \(\beta\)-glucuronidase, glutamic acid decarboxylase, glycine arylamidase, \(\alpha\)-fucosidase, glutamyl glutamic acid arylamidase, histidine arylamidase, phenylalanine arylamidase, proline arylamidase, leucyl glycine arylamidase, serine arylamidase and tyrosine arylamidase activities, and fermentation of raffinose and mannose. In the API ZYM system, positive for leucine arylamidase activity; negative for acid phosphatase, N-acetyl-\(\beta\)-glucosaminidase, alkaline phosphatase, \(\alpha\)-chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), \(\alpha\)-fucosidase, \(\alpha\)- and \(\beta\)-galactosidase, \(\beta\)-glucuronidase, \(\alpha\)- and \(\beta\)-glucosidase, lipase (C14), \(\alpha\)-mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase activities. Aesculin, starch and gelatin are not hydrolysed. The major long-chain fatty acids are \(C_{16:1}\), \(C_{18:0}\), \(C_{18:1}\) and \(C_{16:0}\). Sensitive to kanamycin (1000 μg), vancomycin (5 μg) but resistant to colistin sulfate (10 μg) and sodium polyanethol sulfonate (SPS) special potency identification disks.

The type strain, WAL 18896\(^T\) (=CCUG 56065\(^T\) = ATCC BAA-1640\(^T\)), was isolated from a vaginal abscess of a cervical cancer patient. The DNA G+C content of the type strain is 33.4 mol%.

**Description of *Peptoniphilus koenoeneniae* sp. nov.**

*Peptoniphilus koenoeneniae* (ko.e.noen.ni.i.ae. N.L. fem. gen. n. *koenoeneniae* of Köönen, to honour Dr Eija Köönen, Finnish microbiologist and dentist who has contributed a great deal to our knowledge of oral microbiology and anaerobic bacteriology).

Cells are coccus-shaped and stain Gram-positive. Typical cells are \(\geq 0.7\) μm in diameter. Colonies are grey, convex, entire, circular, opaque and 1–2 mm in diameter on Brucella blood agar plates after 5 days of growth at 37°C. Cells are obligately anaerobic. Positive for indole production.
Negative for catalase and urease activities and nitrate reduction. Cells are asaccharolytic; acid is not produced from glucose or other sugars. In peptone yeast broth and peptone yeast glucose broth, moderate amounts of butyric acid and propionic acid and trace amounts of acetic acid, oxalic acid, and pyruvic acid are produced. Using the Rapid ID 32A system, positive for alanine dehydrogenase, arginine arylamidase, leucine arylamidase, phenylalanine arylamidase, pyroglutamic acid arylamidase and serine arylamidase activities; weakly positive for glycine arylamidase, proline arylamidase and histidine arylamidase activities; and negative for N-acetyl-β-glucosaminidase, alanine arylamidase, alkaline phosphatase, α-arabinosidase, x- and β-galactosidase, β-galactosidase-β-phosphate, β-glucosidase, β-glucuronidase, glutamic acid decarboxylase, x-fucosidase, leucine arylamidase, glutamyl glutamic acid arylamidase, leucyl glycine arylamidase and tyrosine arylamidase activities, and fermentation of raffinose and mannose. In the API ZYM system, weakly positive for esterase (C₄) and acid phosphatase; negative for N-acetyl-β-glucosaminidase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, esterase lipase (C₅₀), x-fucosidase, x- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, leucine arylamidase, lipase (C₁₄₈), α-mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase activities. Aesculin, starch and gelatin are not hydrolysed. The major long-chain fatty acids are C₁₆:0, C₁₈:0, C₁₈:1ω9c, C₁₈:2ω6o6,9c. Sensitive to kanamycin (1000 μg) and vancomycin (5 μg) but resistant to colistin sulfate (10 μg) and sodium polyanethol sulfonate (SPS) special potency identification disks.

The type strain, WAL 18898<sup>T</sup> (=CCUG 5607<sup>T</sup> =ATCC BAA-1638<sup>T</sup> =DSM 22616<sup>T</sup>), was isolated from a human buttock abscess specimen. The DNA G+C content of the type strain is 32.4 mol%.

Acknowledgements

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References


