Olsenella scatoligenes sp. nov., a 3-methylindole- (skatole) and 4-methylphenol- (p-cresol) producing bacterium isolated from pig faeces

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Strain SK9K4T, which is a strictly anaerobic, non-motile, non-sporulating, Gram-stain-positive, saccharolytic coccobacillus, was isolated from pig faeces. SK9K4T metabolized indol-3-acetic acid to 3-methylindole (skatole), which is the main contributor to boar taint; it also produced 4-methylphenol (p-cresol) from p-hydroxyphenylacetic acid. Phylogenetic analyses, based on 16S rRNA gene sequences, revealed that the isolate represented a new lineage within the genus Olsenella of the family Atopobiacae. Strain SK9K4T was most closely related to the type strains of the three species of the genus Olsenella with validly published names; Olsenella profusa DSM 13989T (93.6%), Olsenella uli DSM 7084T (93.5%) and Olsenella umbonata DSM 22620T (92.7%). DNA–DNA relatedness values of strain SK9K4T with O. profusa, O. uli and O. umbonata were 28.3%, 69.1% and 27.2%, respectively. The genomic DNA G+C content was 62.1 mol% and the major cellular fatty acids (constituting >10% of the total) were C14:0 and C18:1ω9c. The major end product of glucose fermentation was lactic acid, with minor amounts of acetic acid and formic acid; no H2 was produced. Discrepancies in the fatty acid profiles, the MALDI-TOF mass spectra of cell extracts and the physiological and biochemical characteristics differentiated strain SK9K4T from other species of the genus Olsenella and indicate that the isolate represents a novel species within this genus. The name Olsenella scatoligenes sp. nov., is proposed and the type strain is SK9K4T (=JCM 19907T=DSM 28304T).

Abbreviations: IAA, indole-3-acetic acid; p-HPAA, p-hydroxyphenylacetic acid; MALDI-TOF MS, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry; ML, maximum likelihood; MP, maximum parsimony; NJ, neighbour joining; TRP, L-tryptophan.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of SK9K4T is JX905358.

Four figures and one table are available with the online Supplementary Material.

Skatole (3-methylindole) and p-cresol (4-methylphenol) are cytotoxic and malodorous compounds produced by microbial fermentation (Smith & Macfarlane, 1997; Whitehead et al., 2008). Skatole is the main contributing compound of boar taint, which is an offensive odour and flavour released upon heating meat from some pubertal or sexually mature male pigs. Eating ‘boar taint’ meat is disagreeable for most consumers (Jensen et al., 2014). Currently, the surgical castration of male piglets is common practice in order to prevent boar taint. However, due to animal welfare concerns, there is an increasing desire to stop such surgical castrations within the European Union by 2018. The search for alternatives to surgical castration is hampered by lack of knowledge of the specialized gastrointestinal skatole-producing bacteria. Production of skatole involves a two-step process in which the precursor L-tryptophan (TRP) is first deaminated to indole acetic acid (IAA), which is then further decarboxylated to skatole (Jensen et al., 1995; Whitehead et al., 2008; Yokoyama & Carlson, 1974). Whereas many bacteria are able to deaminate TRP to IAA (Patten et al., 2013), few bacteria have been reported to catalyse the step from IAA to skatole (Deslandes et al., 2001), and only two of these [Clostridium scatogenes (isolated from soil) and Clostridium drakei (isolated from sediment)] have been fully characterized and made available by culture collections (Whitehead et al., 2008). In the present study, we succeeded in isolating a skatole-producing bacterium from pig faeces that will allow further studies to be conducted on skatole metabolism, enabling elucidation of the processes and mechanisms that will reduce skatole production in swine gut and, consequently, the deposition of skatole in back fat. The significance of skatole is not limited to its contribution to boar taint, as it is also an aetiological agent for acute infections.
bovine pulmonary oedema and emphysema in ruminants (Deslandes et al., 2001), as well as being a putative pulmonary carcinogen (Weems et al., 2009) and a major halitosis-causing compound in humans (Codipilly & Kleinberg, 2008).

p-Cresol is produced by the degradation of L-tyrosine via the intermediate p-hydroxyphenylacetic acid (p-HPAA) (Yokoyama & Carlson, 1981). It is present in animal waste lagoons, contributing to malodorous emissions. It is a uraemic toxin and decreases the endothelial cell response to inflammatory cytokines (Dou et al., 2002).

Based on 16S rRNA sequence analysis, the novel skatole- and p-cresol-producing isolate was closely related to species of the genus Olsenella. The genus Olsenella was first proposed by Dewhirst et al. (2001) and has recently been reclassified to the family Atopobiaceae within the order Coriobacteriales and the class Coriobacteria (Gupta et al., 2013). At the time of writing, the genus comprises three species; Olsenella uli, Olsenella profusa and Olsenella umbonata (Dewhirst et al., 2001; Kraatz et al., 2011). Members of the genus Olsenella are Gram-staining-positive rods and occur singly, in pairs and in short or long serpentine chains. Cells are strictly anaerobic and lactic acid is the major fermentation product from glucose, with formic acid and acetic acid as minor products. The major fatty acid of the cell membrane is C_{18:1} cis 9 and the DNA G + C content ranges from 63 %–64 %. O. uli DSM 7084 (formerly Lactobacillus uli) (Olsen et al., 1991) is the type strain of the species O. uli, which is the type species of the genus Olsenella (Göker et al., 2010). The human oral cavity and gastrointestinal tract of homeothermic vertebrates are indicated as the habitats of Olsenella (Kraatz et al., 2011). To the best of our knowledge, no species of the genus Olsenella has so far been shown to produce either skatole or p-cresol.

Strain SK9K4T was isolated from pig faeces, as described by Jensen & Jensen (1998). Unless stated otherwise, the standard cultivation medium used was modified Peptone-Yeast extract with glucose (PYG-mod) (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium104.pdf). The anaerobic culturing methods used were essentially the same as those described by Miller & Wolin (1974), with cultivations performed at 37 °C. In this study, we describe the phylogenetic, morphological, biochemical and chemotaxonomic characteristics, as well as the metabolic profile of SK9K4T, whilst employing the most closely related type strains as reference strains in parallel tests: O. uli DSM 7084T, O. profusa DSM 13989T and O. umbonata DSM 22620T.

The genomic DNA of strain SK9K4T was extracted using a Maxwell 16S DNA purification kit (Promega) and automated DNA purification was performed on a Maxwell 16 Instrument (Promega) according to the technical manual provided by the manufacturer. The nearly complete 16S rRNA gene (1469 nt) of SK9K4T was amplified by PCR, as described by Mikkelsen et al. (2003), using the forward primer 5'-AGRRTTGTGATYMTGGCTAG-3' and the reverse primer 5'-GGYTACCTTGTAGGACTT-3'. The PCR products were purified using a QIAsquik PCR Purification kit (Qiagen) and sequenced commercially (Eurofins MWG Biotech) using the above primer pairs and the internal primers: 518R (5'-GTATTACCGGGCTGCTG-3'), 907F (5'-AACCTCAAAGGATCCGCG-3') and 1100R (5'-GGGTTCGGTCTGTTG-3'). Phylogenetic neighbours were identified and pairwise 16S rRNA gene sequence similarities were calculated using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012). Strain SK9K4T showed the highest 16S rRNA gene sequence similarity with O. profusa D315A-29T (93.6 %), followed by O. uli DSM 7084T (93.5 %), O. umbonata lac31T (92.7 %), and Atopobium parvulum DSM 20469T (91.0 %).

Based on the BLAST results, 16S rRNA gene sequences of the most closely related taxa were retrieved from the GenBank database and aligned using the CLUSTAL w tool implemented in MEGA6 (Tamura et al., 2013). The phylogenetic trees were reconstructed using the neighbour-joining (NJ) (Saitou & Nei, 1987), maximum-likelihood (ML) (Felsenstein, 1981) and maximum-parsimony (MP) (Fitch, 1971) methods using bootstrap values based on 1000 replications with the MEGA6 software package. Evolutionary distances were calculated using Kimura’s two-parameter model (Kimura, 1980). The resulting NJ tree (Fig. 1) revealed that strain SK9K4T clustered closely with sequences of the following bacterial isolates and clones: bacterium OL-1 (LK021119), originating from human stools; Olsenella sp. BS-3, originating from rumen (GU045476); uncultured bacterium clone OPEN_ROOT_34 (FJ982998), originating from the human oral cavity; uncultured Olsenella sp. clone J21 (DQ168838), originating from anaerobic sludge and uncultured bacterium clone BD02634 (JQ191023), originating from the human distal gut. They formed a distinct subclade within the family Atopobiaceae with a bootstrap value of 100 %. This relationship was supported further by the ML and MP tree algorithms (Figs S1.1 and S1.2, available in the online Supplementary Material).

DNA–DNA hybridization among the four strains was conducted by the DSMZ Identification Service, as described by De Ley et al. (1970), with some modifications described by Huß et al. (1983). Relative reassociation of the DNA of SK9K4T was highest with the DNA of O. uli (69.1 %) and approximately 30 % with respect to the DNA of O. profusa (28.3 %) and O. umbonata (27.2 %). The genomic DNA G + C (mol%) content of strain SK9K4T was determined at the DSMZ by using the HPLC method of Mesbah et al. (1989). The value of 62.1 mol% is close to the range (63 %–64 %) reported for other members of the genus Olsenella (Dewhirst et al., 2001; Kraatz et al., 2011) (Table 1).

Cell morphology, motility and spore formation were observed by phase-contrast microscopy at 625 × magnification (Fig. S2) and colony morphology was observed on PYG-mod agar after incubation for six days at 37 °C in...
an anaerobic cabinet (Fig. S3). Gram staining was determined using a Gram-colour staining set (Merck 111885). The strains were characterized biochemically by using API 20A, API Rapid ID 32A and API ZYM kits (bioMérieux), according to the manufacturer’s instructions. All inocula were prepared using centrifuged anaerobic young cultures (24 h-old) from PYG-mod broth. All biochemical tests were performed in triplicate. The morphological, physiological and biochemical characteristics of SK9K4T are given in the species description and the features that differentiate strain SK9K4T from the reference strains are shown in Table 1 and Table S1.

Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was conducted at DSMZ using bacteria in the exponential growth phase in PYG-mod medium. Sample preparation and instrumental conditions were applied, as described by Tóth et al. (2008). The data were analysed using BioTyper (version 3.1) software, and a MALDI Mass Spectral Profile-based dendrogram was created with the close neighbours of strain SK9K4T by using the same MALDI BioTyper software (Fig. S4). The MALDI-TOF assay showed that SK9K4T was not identified by any spectrum according to the latest update of the Bruker database and confirmed the differentiation of SK9K4T from the type strains of the species *O. uli*, *O. profusa* and *O. umbonata*. The dendrogram showed SK9K4T to be closer to *O. uli* and *O. profusa* than to *O. umbonata*, which supported the results of the 16S rRNA gene sequence analysis.

Cultures of the four strains in the exponential phase in PYG-mod medium were analysed by DSMZ for cellular fatty acids. Fatty acid methyl esters were extracted using the method of Miller (1982) with the modifications of Kuykendall et al. (1988) and the profile of cellular fatty acids was analysed by gas chromatography using the Sherlock Microbial Identification System (MIDI; Microbial ID; http://www.midi-inc.com/pdf/MIS_Technote_101.pdf). The predominant cellular fatty acids (>5% of the total) of strain SK9K4T were, from highest to lowest: C14:0 (25.9%), C18:1ω9c (25.7%), summed feature 1 (C12:0 3OH and/or C13:1 iso H) (20.7%), summed feature 4 (C17:1 iso I and/or C17:1 anteiso B) (7.7%) and C13:1 AT12-13 (6.6%). Differential cellular fatty acid profiles between strain SK9K4T and the three related species within the genus *Olsenella* are shown in Table 2. Dewhirst et al. (2001) and Kraatz et al. (2011) reported C18:0, anteiso-C14:0 and C14:0 to be the main cellular fatty acids in *O. uli*, *O. profusa* and *O. umbonata*, respectively, when grown in M2 liquid medium. Differences in the cellular fatty acid patterns between studies are probably due to the different cultivation and extraction conditions used.

![Fig. 1. NJ phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain SK9K4T and closely related members of the family Atopobiaceae. Bootstrap values (>50%) based on 1000 replicates are listed as percentages at branching points. Bifidobacterium breve ATCC 15700 was used as an outgroup. GenBank accession numbers are given in parentheses. Bar, 0.02 substitutions per nucleotide position.](image-url)
For metabolic end product analysis, Hungate tubes containing 10 ml PYG-mod medium without or with supplemented IAA, or p-HPAA, or IAA and p-HPAA (0.29 mM each) were inoculated with 72 h-old cultures (0.1 ml) of the four bacterial strains. The tubes were incubated for three days at 37 °C and samples were taken at the end of the incubation time. Samples were tested in triplicate. Headspace gas (0.5 ml) was collected through a syringe, and the H₂ concentration was immediately determined by gas chromatography using a GC 82-22 (Mikrolab) with He as the carrier gas. Data were analysed using PEAK359 software. Samples (1.0 ml) for organic acid and phenolic- and indolic compound analysis were taken and stored at -20 °C; acid concentrations were determined by GC as outlined by Canibe et al. (2007). The concentration of phenolic- and indolic compounds was determined by GC as outlined by Canibe et al. (2007).
Table 2. Cellular fatty acid contents of strain SK9K4<T> and the type strains of closely related species of the genus Olsenella

<table>
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<td>1.4</td>
<td>TR</td>
<td>4.5</td>
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*Summed features represent groups of two or three fatty acids that could not be separated by the Microbial Identification System. Summed feature 1 comprises C13:0 3OH and/or C15:1 iso H; summed feature 2 comprises C14:0 3OH and/or C16:1 iso 1 and/or unknown ECL 10.928; summed feature 3 comprises C16:1ω7c and/or C18:1ω9c; summed feature 4 comprises C17:1 iso 1 and/or C17:1 anteiso B.

analysed by HPLC as described by Knarreborg et al. (2002).

Metabolic end products from glucose, IAA or HPAA of the tested bacteria are shown in Table 3. All bacteria primarily metabolized glucose to lactic acid as the only major organic acid product and to minor amounts of acetic acid and formic acid, except for O. uli, which only produced trace amounts of formic acid (<1.0 mM). Only O. profusa produced H₂ from glucose fermentation. SK9K4<T> and O. uli metabolized IAA to skatole and p-HPAA to p-cresol in PYG-mod medium, but no skatole production by any of the strains was detected in PYG-mod medium containing TRP (data not shown). A previous study by Yokoyma & Carlson (1981) reported that the rumen Lactobacillus sp. strain 11201 was able to produce skatole and p-cresol from IAA and p-HPAA, respectively. Morphologically and physiologically, our isolate seems to be very similar to Lactobacillus sp. strain 11201, but to our knowledge this strain has not been further characterized or deposited in a culture collection. From the results of the present study, it could be speculated that production of skatole by SK9K4<T> may contribute to boar taint and that production of skatole by O. uli may contribute to halitosis in humans.

In summary, the low similarity values in 16S rRNA gene sequences (92.7%–93.6%), differentiation by MALDI-TOF mass spectra, as well as differences in physiological, biochemical and chemotaxonomic characteristics and metabolic end product patterns clearly demonstrate that strain SK9K4<T> merits recognition as the type strain of a novel species within the genus Olsenella, for which the name Olsenella scatoligenes sp. nov., is proposed.

Emended description of Olsenella uli

In addition to the results of Olsen et al. (1991), Dewhirst et al. (2001) and Kraatz et al. (2011), we found that after anaerobic incubation on PYG-mod agar for six days, colonies are granular in texture, creamy-white, opaque with semi-translucent margins and have diameters of 0.5 mm–1.0 mm with raised elevations (Fig. S3). Detailed biochemical and enzymic characteristics are given in Table 1. The major cellular fatty acid of cells grown in PYG-mod medium is C₁₈₋₁₉ω9c (Table 2). Metabolizes glucose to lactic acid as the only major organic acid with minor amounts of acetic acid, and produces trace amounts of formic acid. Produces skatole from IAA, but not from TRP, and produces p-cresol from p-HPAA.

Description of Olsenella scatoligenes sp. nov.

Olsenella scatoligenes [sca.to.li‘ge.nes. N.L. n. scatolum, skatole; N.L. suff. -genes, (from Gr. gennaio to produce) producing; N.L. part. adj. scatoligenes skatole-producing].

Description is based on a single strain. Cells are strictly anaerobic, non-motile, non-sporulating, Gram-stain-positive and coccobacillus-shaped. After anaerobic incubation on PYG-mod agar for six days, colonies are granular in texture, creamy-white, opaque with semi-translucent
Margins and diameters of 0.5 mm–1.0 mm with raised elevations. Cells are variously arranged, i.e. singly, in pairs or in short chains. Single cells are 1–2 μm in length and chains of cells are up to 5–6 μm long.

In API 20A, acid is formed from glucose, sucrose, maltose, mannose, lactose, salicin, cellobiose, and rhamnose, but not from mannitol, xylose, arabinose, glycerol, melezitose, raffinose, sorbitol and trehalose. Aesculin, but not gelatin is hydrolysed. Urease is not detected and acetoin is not produced. In Rapid ID32A, acid is produced from mannose and raffinose, indole is not formed, and nitrate is not reduced. Activity is detected for alkaline phosphatase, arginine-, leucyl glycine-, phenylalanine-, leucine-, tyrosine-, alanine-, glycine-, histidine-, serine-, proline-, pyroglytamic acid-, pyroglutamic acid- and glutamyl glutamic acid arylamidase, arginine dihydrolase, β-galactosidase, β-galactosidase-6-phosphate, α-glucosidase and β-glucosidase glucuronicidase glucuronicidase. No activity is detected for α-galactosidase, α-arabinosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-fucosidase and arginine dihydrolase. Using the API ZYM system, alkaline phosphatase, valine arylamidase, leucine arylamidase, cystine arylamidase, acid phosphatase, α-glucosidase, esterase lipase (C8), naphthol-AS-BI-phosphohydrolase, β-galactosidase and β-gluconidase are detected, but lipase, trypsin, α-chymotrypsin, α-galactosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase and esterase (C4) are not detected. The major end product of glucose fermentation is lactic acid, with minor amounts of acetic acid and formic acid; no H2 is produced. Produces skatole from IAA, but not from TRP, and produces p-cresol from p-HPAA. The major cellular fatty acids (>10% of the total present) are C14:0 and C18:1ω9c.

The type strain, SK9K4T (=ICM 19907T=DSM 28304T), was isolated from the faeces of pigs at Aarhus University, Denmark. The genomic DNA G+C content of the type strain is 62.1 mol%.

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References


