Neoscardovia arbecensis gen. nov., sp. nov., isolated from porcine slurries

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ABSTRACT

Three Gram-positive, anaerobic, pleomorphic strains (PG10, PG18 and PG22), were selected among five strains isolated from pig slurries while searching for host specific bifidobacteria to track the source of fecal pollution in water. Analysis of the 16S rRNA gene sequence showed a maximum identity of 94% to various species of the family Bifidobacteriaceae. However, phylogenetic analyses of 16S rRNA and HSP60 gene sequences revealed a closer relationship of these strains to members of the recently described Aeriscardovia, Parascardovia and Scardovia genera, than to other Bifidobacterium species. The names Neoscardovia gen. nov. and Neoscardovia arbecensis sp. nov. are proposed for a new genus and for the first species belonging to this genus, respectively, and for which PG10 (CECT 8111T, DSM 25737T) was designated as the type strain. This new species should be placed in the Bifidobacteriaceae family within the class Actinobacteria, with Aeriscardovia aeriophilia being the closest relative. The prevailing cellular fatty acids were C₁₆:₀ and C₁₈:₀ 8c, and the major polar lipids consisted of a variety of glycolipids, diphasphatidylglycerol, two unidentified phospholipids, and phosphatidyl glycerol. The peptidoglycan structure was A1γ meso-Dpm­-direct. The GenBank accession numbers for the 16S rRNA gene and HSP60 gene sequences of strains PG10, PG18 and PG22 are JF319691, JF319693, JQ767128 and JQ767130, JQ767131, JQ767133, respectively.

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Microbial source tracking (MST) using host-specific bacteria is a promising tool for identifying the source of fecal contamination in water. A strong host-specificity has been described for several Bifidobacterium species, which has prompted the development of new methods for detecting these species [1,3,7,9,14,18,26]. The analysis of the 16S rRNA gene with denaturing gradient gel electrophoresis (DGGE) has been broadly used to study microbial diversity in different ecosystems [16,17]. A recent study analyzed the diversity of bifidobacterial populations from sewage, slurries and wastewater from different slaughterhouses using DGGE for identifying host-specific bacteria as MST markers. A specific DNA band was detected from pig DGGE profiles, which was subsequently sequenced and used to design a DNA probe to isolate the corresponding host-specific bacteria [1]. A total of five isolates growing on Beeren's agar [2], namely PG10, PG18, PG19, PG22 and PG30, were recovered by colony hybridization using a DIG-labeled probe (5′-GGGTGGGTAACCCGCTTTTGTGGGGAGCAAGG-3′) [1] from porcine slurries of a pig farm located in Arbeca (Lleida, Catalonia, Spain). The plates were incubated at 37°C for 48 h under anaerobic conditions (GasPak; BBL, Hampshire, United Kingdom) with CO₂ atmosphere generators (Anaerocult A; Merck, Darmstadt, Germany). The isolates were propagated on Columbia blood agar (CBA) (Difco, Le Pont de Claix, France) with the addition of L-cysteine hydrochloride at 0.5 g L⁻¹ (Merck, Darmstadt, Germany) and glucose at 5 g L⁻¹, incubated in RCM broth at 37°C, and kept frozen at −80°C with 10% glycerol until further analysis.

In the present study, phylogenetic reconstruction based on analysis of the 16S rRNA and HSP60 gene sequences affiliated the strains with the family Bifidobacteriaceae (see below). However, these reconstructions could not place the isolates in any recognized genus of the family. Given that two of the isolates showed clonal similarity, as revealed by the random amplification of polymorphic DNA (RAPD), the three strains showing different fingerprints were selected for further studies. Strain PG10 was deposited in two international culture collections under the numbers CECT 8111T, DSM 25737T, and represents the type strain of the first species of the novel genus Neoscardovia gen. nov. proposed here, which should be placed in the Bifidobacteriaceae family within the class Actinobacteria.

For taxonomic comparisons, the type strains of representative species of the genera Scardovia (Scardovia inopinata B3109T, DSM 10107T and Scardovia wiggiae C1A_55T, DSM 22547T),
Parascardovia (Parascardovia denticolens B3028<sup>T</sup>, DSM 10105<sup>T</sup>) and Aeriscardovia (Aeriscardovia aeriaphila T<sup>6</sup>, DSM 22365<sup>T</sup>) were obtained from the DSMZ (Braunschweig, Germany) and were used as reference strains. Strains were grown on Bifidobacterium medium (BM) (DSMZ), except where otherwise stated.

DNA isolation was performed using the Wizard<sup>®</sup> Genomic DNA Purification Kit (Promega, Barcelona, Spain) following the manufacturer’s instructions. The clonality of the isolates was analyzed by random amplification of polymorphic DNA (RAPD) with the primers described in [5]: (A) 5’-CCGGCAACCA-3’, (B) 5’-AACCCGCAAC-3’, and (C) 5’-GCCGAAATAG-3’. The PCR reaction consisted of 0.25 µL dNTP mix (25 mM), 1 µL primer (10 µM), 2.5 µL 10× Taq polymerase buffer, 1.25 U Taq polymerase and 30 ng of chromosomal DNA used as template in a final volume of 25 µL. The PCR reaction was performed under the following conditions: 94 °C for 2 min; 6 cycles of 94 °C for 30 s, 36 °C for 1 min, and 72 °C for 90 s; 30 cycles of 94 °C for 20 s, 36 °C for 30 s, and 72 °C for 90 s; 72 °C for 3 min. As shown in Fig. S1, isolates PG18, PG19 and PG30 displayed identical RAPD profiles with the three primers used, indicating that the five isolates represented three strains, namely PG10<sup>T</sup>, PG18 and PG22, which were further studied.

The 16S rRNA gene was amplified using universal primers 27F and 1492R [27]. Partial HSP60 gene sequences were obtained using degenerate primers (H60F and H60R), as previously described [12]. Nucleotide sequencing of the corresponding amplicers was performed in duplicate with an automated DNA sequencer, model 377 (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany). Sequencing was carried out using the ABI PRISM Big Dye 3.1 Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Applied Biosystems, Spain). The almost complete 16S rRNA gene sequences of the isolates were added to the LTP release 106 database [28] and aligned using the ARB software package [13]. On the other hand, the sequences (both nucleotide and those translated to amino acids) of the HSP60 dataset were aligned using the Clustal X 1.83 program [25], and the alignments were improved by removing hypervariable positions using the available on-line program Gblocks (http://molevol.cimn.ca/cisc/est cavesana/Gblocks_server.html), under the default conditions [4]. In both cases where DNA sequences were used, the tree reconstructions were performed using the maximum likelihood algorithm RAxML version 7.0 [23] with the rate distribution model GTRGAMMA, and the bootstrap analyses were carried out by using 100 replicates. Also, the reconstructions were generated using all entries available from all members of the family Bifidobacteriaceae in the LTP release 106 database for the 16S rRNA gene sequences and the GenBank database for the HSP60 gene sequences. For 16S rRNA gene sequence reconstruction, different positional filters to remove phylogenetic noise were applied. The results presented in Fig. 1 show a consensus tree, where the multifurcation indicates branch instabilities. As mentioned above, the HSP60 alignment was sieved previously using the Gblocks program and, in this case, the total alignment with 556 homologous positions was reduced to 464 conserved positions. The resulting alignment contained 83% homologous positions of the original alignment. Similarly, for the protein sequences, the alignment of approximately 184 homologous positions was reduced to 146 residues, and the resulting alignment represented 79% of the original dataset. Similarities were calculated on the basis of a pair-wise comparison (ARB software) based on the alignments used for tree reconstruction. The 16S rRNA and HSP60 gene sequences determined in this study have been deposited in the GenBank database under accession numbers JF519691, JF519693 and JQ767128 for the 16S rRNA genes, and JQ767130, JQ767131 and JQ767133 for the HSP60 genes, from strains PG10<sup>T</sup>, PG18 and PG22, respectively.

The almost complete sequence of the 16S rRNA gene was obtained for all strains (1342 bp). The 16S rRNA sequence identity between the different strains was 99.9% with only 1 bp difference. The maximum identity to the closest relatives was 94%, including several Bifidobacterium species, such as Bifidobacterium indicum JCM 1302<sup>T</sup> and Bifidobacterium breve DSM 20213<sup>T</sup>. Phylogenetic reconstruction was performed with these sequences, together with all Bifidobacterium species and those of related genera (nucleotide similarity indicated in brackets): A. aeriaphila T<sup>6</sup> (93%), P. denticolens B3028<sup>T</sup> (92%), Alloscardovia omnicolors CCUG 31649<sup>T</sup> (92%), Metascardovia criceti OMB105<sup>T</sup> (91%), Gardnerella vaginalis ATCC 14018<sup>T</sup> (91%), S. inopinata B3109<sup>T</sup> (90%) and S. wiggiae C1A,55<sup>T</sup> (89%). RAxML algorithm affiliated the novel species with the same phylogenetic branch as A. aeriaphila T<sup>6</sup>, as shown in Fig. 1.

Given the sequence dissimilarity of about 6% with the closest relative genera, and the isolation of the branch comprising our isolates, their classification as a new genus seemed plausible. In addition, and as an alternative to 16S rRNA gene sequence analyses, phylogenetic reconstructions based on partial sequences of the HSP60 gene have recently been shown to be an accurate tool for phylogenetic studies [12]. In this regard, the phylogenetic reconstruction based on the sequence of these strains with this gene showed tree
Table 1

<table>
<thead>
<tr>
<th>ANIB (%)</th>
<th>ANIm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC10T–PC18</td>
<td>98.7</td>
</tr>
<tr>
<td>PC10T–PC22</td>
<td>98.6</td>
</tr>
<tr>
<td>PC18–PC22</td>
<td>98.6</td>
</tr>
<tr>
<td>PC10T–A. aeriphila T6³</td>
<td>74.9</td>
</tr>
<tr>
<td>PC18–A. aeriphila T6³</td>
<td>76.5</td>
</tr>
<tr>
<td>PC22–A. aeriphila T6³</td>
<td>74.3</td>
</tr>
<tr>
<td>PC10T–S. wiggiae F0424</td>
<td>70.6</td>
</tr>
<tr>
<td>PC10T–S. inopinata F0304</td>
<td>69.8</td>
</tr>
<tr>
<td>PC10T–P. denticolens B3028®</td>
<td>71.3</td>
</tr>
<tr>
<td>PC10T–B. bifidum S17®</td>
<td>72.5</td>
</tr>
<tr>
<td>A. aeriphila T6³–S. wiggiae (F0424)</td>
<td>71.6</td>
</tr>
<tr>
<td>A. aeriphila T6³–S. inopinata (F0304)</td>
<td>71.3</td>
</tr>
<tr>
<td>A. aeriphila T6³–P. denticolens B3028®</td>
<td>73.0</td>
</tr>
<tr>
<td>A. aeriphila T6³–B. bifidum S17®</td>
<td>74.1</td>
</tr>
</tbody>
</table>

The novel isolates that were in accordance with our new organisms, since they formed an independent branch within Bifidobacteria. As shown in Fig. S2, the novel species was grouped accordingly within a cluster with the non-Bifidobacterium species together with A. aeriphila T6³.

Given that the new isolates could represent a new genus within the family Bifidobacteriaceae, strain PC10T was designated as the type strain of the new species and genus. For this strain, the DNA guanine-plus-cytosine (G+C) mol% content was determined by enzymatically hydrolyzing the DNA and quantifying the resulting nucleosides by high-performance liquid chromatography as described in [24] with some modifications [21]. Commercial DNA from bacteriophage lambda (G+C content, 49.9 mol%; Sigma Chemical Co., St. Louis, MO) was used as a standard. The G+C content of strain PC10T was 57 mol%. In addition, and in order to observe the genomic coherence of the strains which could have belonged to the same species, the average nucleotide identity (ANI) was calculated as an alternative to DNA–DNA hybridization, as recommended in [20]. For this, partial sequencing was performed with the Genome Sequencer FLX System, 454 sequencing instrument (Life Sciences) by the Scientific and Technical Services of the University of Barcelona. For this purpose, the DNA extracted from the three strains PG10T, PG18 and PC22, and the type strain of A. aeriphila T6³ were tagged and pooled for the sequencing procedure. The analysis generated a total of 261,293 reads corresponding to 102.8 Mb (Table S4). The sequencing approach generated a total of 16.3 Mb for PG10T, 46.9 Mb for PG18, 14.3 Mb for PC22, and 25.3 Mb for the type strain of A. aeriphila. For each DNA set, the reads were assembled with the software Velvet (http://www.ebi.ac.uk/~zerbin/velvet/) using the basic parameters. A total of 12,708 contigs were generated for PG10T, 27,154 for PG18, 11,818 for PC22, and 16,284 for the type strain of A. aeriphila. The assembly finalized with a total of the non-redundant sequences that made up approximately 2.3 Mb for PG10T, 2.6 Mb for PG18, 2.3 Mb for PC22, and 2 Mb for the type strain of A. aeriphila. The current genome database lacks the genomes of all type strains of the genera belonging to the family Bifidobacteriaceae. However, we used the genome sequences of the reference genomes B. bifidum strain S17 [29], as well as the draft genomes of S. inopinata strain F0304 and S. wiggiae strain F0424 available at the “Scardovia Group Sequencing Project of the Broad Institute of Harvard and MIT (http://www.broadinstitute.org/)”.

ANIB and ANIm determinations were performed using the JSpecies program (http://www.imeida.uib.es/jspecies) as recommended in [20]. As can be seen in Table 1, ANI values of the three novel strains ranged from 99.1 to 99.2% using MUMmer, and 98.6 to 98.8% using BLAST. In all cases, the ANI results were far above the threshold of 94–96% that would correspond to the species

borderline [20], indicating that the three strains belonged to the same specific taxon. On the other hand, the genome of A. aeriphila DSM 22365 and the additional reference genomes used showed ANI values always far below the ANI species threshold, indicating a very low taxonomic relatedness. In addition, the differences in all parameters analyzed with our group compared to the reference genomes were in the same range as those observed for A. aeriphila T6³ and the other reference genomes. All these results were in accordance with the new group of strains representing a new genus within the family Bifidobacteriaceae.

As shown previously [15], MALDI-TOF profiles using whole cell extracts produce phenotypic clusters that reproduce a circumscription at the species level, and this was used to perform the analysis of the major proteins from our isolates. For this, biomass for each strain was picked from agar plates and placed onto a spot of a ground steel plate with a toothpick and air-dried at room temperature. Each sample was overlaid with 1 μL of matrix solution (saturated solution of α-cyan—4—hydroxy-cinnamic acid in 50% acetonitrile—2.5% trifluoroacetic acid) and air dried at room temperature. Measurements were performed on an Autoflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Leipzig, Germany) equipped with a 200 Hz Smartbeam laser. Spectra were recorded in the linear, positive mode at a laser frequency of 200 Hz within a mass range from 2000 to 20,000 Da. The IS1 voltage was 20 kV, the IS2 voltage was maintained at 18.7 kV, the lens voltage was 6.50 kV, and the extraction delay time was 120 ns. For each spectrum, approximately 500 shots at different positions of the target spot were collected and analyzed. The spectra were calibrated using the Bruker Bacterial Test Standard (Escherichia coli extract including the additional proteins RNase A and myoglobin). Calibration masses were as follows: RL29 3637.8 Da; RS32 5096.8 Da; RS34 5381.4 Da; RL33meth, 6255.4 Da; RL29, 7274.5 Da; RS19, 10300.1 Da; RNaseA, 13683.2 Da; myoglobin, 16952.3 Da. The analyses were performed in triplicate. MALDI-TOF analysis showed that all the isolates shared a very similar pattern of major molecules in accordance with the same species (Fig. S3).

The presence of catalase and oxidase activity was assessed with 3% hydrogen peroxide and N,N,N',N'-tetramethyl-p-phenylenediamine reagent, respectively, while the presence of spores was analyzed by malachite green staining. Phenotypically, all five isolates were Gram-positive anaerobic, catalase negative, oxidase negative, non-spore-forming bacteria. The colonies grown on CBA agar were white, approximately 1 mm in diameter after 72 h incubation at 37 °C, smooth, glistening, convex, and circular with entire edges. The morphology of the cells (PG10T) was assessed by scanning electron microscopy. As shown in Fig. 2, the cells were pleomorphic rods, sometimes producing a “Y” form. The growth temperature of these bacteria ranged between 15–C and 42 °C and they were able to grow between pH 5 and pH 8 at 37 °C. The lowest pH attained after growth was 4.39 ± 0.03.

Bifidobacterium medium (BM) (DSMZ, Braunschweig, Germany) was used to assess the ability of this species to grow at different pH values. The medium was adjusted to the different pH values using NaOH or lactic acid to achieve the desired pH. The corresponding media were inoculated with a 72 h culture of the corresponding strain (1:1000). Biochemical characterization was performed with the API 20A and API 50CHL strips supplemented with 0.025% cysteine (Biomerieux SA, Marcy, l’Etoile, France). The strains were incubated at 37 °C for 72 h under anaerobic conditions. The enzyme profile was determined using API ZYM (Biomerieux) as recommended by the manufacturer’s instructions. Testing for fructose-6-phosphate phosphoketolase was performed as described in [11]. Motility was investigated using BM medium containing 0.25% agar. The production of lactic and acetic acid from glucose was determined in peptone-yeast extract broth containing
1% glucose, as reported in [19], and the measurements were performed after 5 days incubation.

Biochemical characterization included detection of fructose-6-phosphate phosphoketolase as well as testing with API 20A, API 50CHL and API ZYM. The results are summarized in Table 2 (detailed results for each miniaturized test can be found in the supplementary material as Table S1, Table S2 and Table S3). Fructose-6-phosphate phosphoketolase is a key enzyme present in the microorganisms belonging to the Bifidobacterium genus and related genera, which have a characteristic metabolic pathway for hexasse degradation [6,22]. This enzyme was initially described as exclusive to the Bifidobacterium genus and distinguished this genus from other Gram-positive anaerobic bacteria that were morphologically similar (Lactobacillus spp., Arthrobacter spp., Propionibacterium spp. and Corynebacterium spp.). However, the enzyme was later detected in other genera, such as Gardnerella [10] among others, and therefore it cannot be exclusively linked to the Bifidobacterium genus. All the strains produced lactic acid and acetic acid, but with a different ratio ranging from 1:10 to 1:2. All three strains gave positive reactions in the API ZYM for leucine arylamidase, acid phosphatase, naphthol-AS-Bi-phosphohydrolase, α-and-β-galactosidase, and α-and-β-glucosidase, although with variable intensity. The members of this new group could be differentiated from those of the closest relative genera Scardovia, Aeriscardovia and Parascardovia by the fermentation of l-fucose and potassium gluconate.

Analyses of cellular fatty acids, polar lipids and the peptidoglycan structure of strain PG10T was performed by the Identification Service and Dr. Brian Tindall (DSMZ, Braunschweig, Germany) using the methodology previously described [8]. The morphology of the cells was studied with scanning electron microscopy (20 kV). The major cellular fatty acids of strain PG10T were C16:0 and C18:1ω9c, which were the same as those previously described for the genus Scardovia (Table S5). The major polar lipids consisted of a variety of glycolipids, diphosphatidyl glycerol, two unidentified phospholipids, and phosphatidyl glycerol (Fig. S4). The peptidoglycan structure was A1γ meso-Dpm-direct.

From the above results, the three strains PG10T, PG18 and PG30 formed a homogeneous group that was clearly genetically and phenotypically distinct from any other recognized genera. Therefore, we propose a novel genus Neoscardovia gen. nov. with the species Neoscardovia arbecensis sp. nov. as the first species of this genus.

**Description of Neoscardovia gen. nov.**

Neoscardovia (Ne.o.sacr.do’vía.a. Gr. pref. neo- (from Gr. adj. neos), new; N.L. fem. n. Scardovia, a bacterial generic name; N.L. fem. n. Neoscardovia, a new Scardovia).

The cells of the genus are Gram-positive, catalase negative, oxidase negative, non-motile, non-spore-forming, irregular shaped rods occasionally forming a "Y" shape. Lactic and acetic acid are produced from glucose fermentation. Fermentation of l-fucose and potassium gluconate clearly distinguishes this genus from the closest related genera Scardovia, Aeriscardovia and Parascardovia. The G+C content of the type strain is 57 mol%. The prevailing cellular fatty acids are C16:0 and C18:1ω9c, and the major polar lipids consist of a variety of glycolipids, diphosphatidyl glycerol, two unidentified phospholipids, and phosphatidyl glycerol. The peptidoglycan structure is A1γ meso-Dpm-direct. The type species of the genus is N. arbecensis, which is represented by the type strain PG10T deposited in the culture collections CECT and DSM under the culture accession numbers CECT 8111T and DSM 25737T, respectively.

**Description of N. arbecensis sp. nov.**

N. arbecensis (ar.be.ćen’sis. N.L. fem. adj. arbecensis, of or belonging to Arbeca).

Obligate anaerobe Gram-positive, non-spore-forming bacilli. The cells are pleomorphic with occasional bifurcations, sometimes arranged in a "Y" form. Colonies grown on CBA agar are white, approximately 1 mm in diameter after 72 h incubation at 37°C, smooth, glistening, convex, and circular with entire edges. The maximum temperature for growth is 42°C and the minimum is 15°C. This species can be differentiated from those of the related genera Aeriscardovia, Scardovia, and Parascardovia on the basis of its ability to grow at lower temperatures, and from the most closely related A. aeriphila for its low tolerance to oxygen. The minimum initial pH for growth is 5.0 and the maximum is 8.0. All three strains of N. arbecensis form a homogenous group able to ferment l-arabinose, d-ribose, d-xylene, d-galactose, d-glucose, d-fructose, salicin, d-cellobiose, d-maltose, d-lactose, d-saccharose, d-raffinose, starch, glycerogen, d-turanose, l-fucose, and potassium gluconate. All three strains have the following activities: fructose-6-phosphate-phosphoketolase, leucine arylamidase, acid phosphatase, naphthol-AS-Bi-phosphohydrolase, α-and-β-galactosidase, and α-and-β-glucosidase. Lactic and acetic acid are produced as end products of glucose fermentation in a variable ratio.
ranging from 1:10 to 1:2. The G+C content of the type strain PG105 is 57 mol%. Isolated from pig slurry, this species might be useful to track the source of fecal contamination of porcine origin. The type strain has been deposited in the culture collections CECT and DSM under the culture accession numbers CECT 8111 and DSM 25737, respectively.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2012.06.007.

References


