Bifidobacterium psychraerophilum sp. nov. and *Aeriscardovia aeriphila* gen. nov., sp. nov., isolated from a porcine caecum

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In a previous study that was based primarily on 16S rDNA sequencing, two groups of bifidobacteria that had been recovered from a pig caecum were proposed to belong to two novel species, termed '*Bifidobacterium psychraerophilum*' and '*Bifidobacterium aerophilum*'. In this study, based on DNA G + C content and partial heat-shock protein 60 (HSP60) gene sequences, the assignment of '*B. psychraerophilum*', corrected to *Bifidobacterium psychraerophilum*, to the genus *Bifidobacterium* was confirmed. The DNA G + C content of '*B. aerophilum*' was relatively low, which was consistent with its segregation into subcluster II of the 16S rDNA phylogenetic tree. Based on partial 16S rDNA and HSP60 gene sequences, the species was transferred to a novel genus and reclassified as *Aeriscardovia aeriphila* gen. nov., sp. nov. Biochemical profiles and growth parameters were established for both novel species. Interestingly, each had a high tolerance to oxygen and grew on agar media under aerobic conditions, a trait that may relate to their caecal habitat. Under aerobic growth conditions, the short-rod morphology of *A. aeriphila* lengthened considerably. This appeared to arise from incomplete cell division. In addition, *B. psychraerophilum* was unusual in that it grew at temperatures as low as 4 °C. On the basis of genetic, phylogenetic and phenotypic data, the identities of *Bifidobacterium psychraerophilum* sp. nov. (type strain, T16^T = LMG 21775^T = NCIMB 13940^T) and *Aeriscardovia aeriphila* gen. nov., sp. nov. (type strain, T6^T = LMG 21773^T = NCIMB 13939^T) are confirmed.

Bifidobacteria are Gram-positive, anaerobic bacteria that are prevalent within the human and animal gastrointestinal tract (GIT) (Langendijk *et al.*, 1995; Sghir *et al.*, 2000; Harmsen *et al.*, 2002). At the time of writing, the genus *Bifidobacterium* is represented by over 30 species and subspecies, which were mostly recovered from faecal, GIT and sewage samples (Biavati *et al.*, 2000; Hoyle *et al.*, 2002; Jian & Dong, 2002; Sakata *et al.*, 2002). In a previous study within our laboratory (Simpson *et al.*, 2003), 160 pig caecal isolates were assigned provisionally to the genus *Bifidobacterium* through detection of fructose-6-phosphate

phosphoketolase activity (Scardovi, 1986). The isolates were considered to represent 15 strains, based on distinct PFGE macrorestriction patterns; four species were identified, based primarily on 16S rDNA sequencing. Two were considered to be novel species and were provisionally termed '*Bifidobacterium psychraerophilum*' (which consisted of 23 isolates with the same PFGE macrorestriction pattern, termed PFGE type F) and '*Bifidobacterium aerophilum*' (which consisted of 77 isolates with three distinct PFGE macrorestriction patterns, termed PFGE types B, E and Ea) (Simpson *et al.*, 2003). '*B. psychraerophilum*' segregated in subcluster I of the 16S rDNA phylogenetic tree, with the majority of *Bifidobacterium* species. However, '*B. aerophilum*' grouped in subcluster II, which was considered previously to represent an undefined genus that was related closely to *Bifidobacterium*, but with a lower DNA G + C content (Miyake *et al.*, 1998). Indeed, based on heat-shock protein 60 (HSP60) gene sequencing, the two existing species within subcluster II, *Bifidobacterium inopinatum* and *Bifidobacterium denticolens*, were transferred to *Scardovia* gen. nov. and *Parascardovia* gen. nov., respectively (Jian & Dong, 2002).

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Abbreviations: FISH, fluorescent *in situ* hybridization; GIT, gastrointestinal tract; HSP, heat-shock protein.

The GenBank/EMBL/DDBJ accession numbers for the partial HSP60 gene sequences of LMG 21775^T (=NCIMB 13940^T), LMG 21773^T (=NCIMB 13939^T) and LMG 21774 are AY339132, AY339131 and AY339130, respectively.

Phase-contrast and whole-cell FISH images of strains studied in this paper are available as supplementary material in IJSEM Online.

The DNA G+C content of the isolates was determined by HPLC (BCCM/LMG, Belgium) as described by Mesbah *et al.* (1989). Values reported are means of three measurements on the same DNA. Partial HSP60 gene sequences were obtained by PCR as described by Jian *et al.* (2001) with the following modifications: the PCR included each primer at 10 pM and MgCl₂ at 1 mM and was extended to 35 cycles. The amplicon (~590 bp) was extracted from an agarose gel and sequenced directly by using primers H60F and H60R. Analysis of HSP60 gene sequences and phylogenetic tree construction were done as described previously (Simpson *et al.*, 2003). Biochemical profiles for strains from both proposed species were obtained from triplicate tests with the API Rapid ID32A and 20A test strips (bioMérieux). Strains were cultured under anaerobic conditions in modified MRS medium (mMRS), which comprised Lactobacilli MRS medium (Difco) supplemented with 0.05 % (w/v) cysteine/HCl, at 37 °C. Growth at low

pH was determined by using pH-adjusted mMRS (with HCl). Growth on mMRS agar under aerobic conditions was determined by streaking cultures and/or spread-plating serial dilutions of overnight anaerobic mMRS broth cultures onto mMRS agar. Selected isolates were deposited with the LMG (BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Belgium) and NCIMB (National Collection of Industrial and Marine Bacteria, Aberdeen, UK) culture collections. High-molecular-mass DNA preparations and PFGE analysis were done as described previously (Simpson *et al.*, 2003), except that cells were removed from the surface of mMRS agar plates. Whole-cell fluorescent *in situ* hybridization (FISH) was performed on cells from colonies by using an FITC-labelled 16S rRNA probe that was specific for either the genus *Bifidobacterium* or all bacteria (Microscreen; Ribotechnologies). Images were viewed with an Olympus BX51 epifluorescence microscope and captured by using

Fig. 1. Phylogenetic tree based on partial HSP60 gene sequences (within the 250–840 nucleotide region). The tree was rooted with *Escherichia coli* and constructed by using the neighbour-joining method, with bootstrap values calculated from 1000 trees (represented as percentages and given at each branch-point). For each species, the GenBank accession number for their respective HSP60 gene sequence is shown in parentheses.

an Olympus DP50 digital camera (15 ms exposure for fluorescent images). Cell-size determinations were done as described previously (Simpson *et al.*, 2003).

Isolates T4, T6^T, T8 and T16^T were taken as representatives of PFGE types B, E, Ea and F, respectively. Isolate T16^T (= LMG 21775^T) had a DNA G + C content of 59.2 mol%, which was consistent with the reported range for the genus *Bifidobacterium* (55–67 mol%) (Crociani *et al.*, 1996). This concurs with the general grouping of the strain with other *Bifidobacterium* species within the 16S rDNA (Simpson *et al.*, 2003) and partial HSP60 gene (Fig. 1) phylogenetic trees. In both cases, inclusion of the strain resulted in separation of *Bifidobacterium minimum* from the three insect-derived species, *Bifidobacterium asteroides*, *Bifidobacterium indicum* and *Bifidobacterium coryneforme*. The highest similarity value for the partial HSP60 gene sequence was 83.58 %, which was shared with *B. minimum*, *B. asteroides* and *B. indicum*. These data support the previous assignment of the strain to the genus *Bifidobacterium* (Simpson *et al.*, 2003). In the current study, the specific epithet has been corrected to *pyschraerophilum*. Isolates T4 (= LMG 21774) and T6^T (= LMG 21773^T) had the same DNA G + C content (54.7 mol%); this relatively low value is consistent with their grouping with *Scardovia inopinata* and *Parascardovia denticolens* in subcluster II of the 16S rDNA phylogenetic tree (Simpson *et al.*, 2003). Based on partial HSP60 gene sequencing, both strains were again clearly related, as they shared 98.44 % sequence similarity. However, isolate T6^T was distinct from the genera *Scardovia*, *Parascardovia* and *Bifidobacterium*, having 71.41, 71.97 and 73.81 % (mean value of 70.85–76.82 %) similarity, respectively (Fig. 1). The latter value is comparable to those reported previously for *S. inopinata* and *P. denticolens* (Jian *et al.*, 2001) and we propose that '*B. aerophilum*' should be transferred to a novel genus. Given the grouping of the novel species in II of the 16S rDNA phylogenetic tree, we have termed the novel genus *Aeriscardovia* gen. nov., in keeping with the taxonomy proposed by Jian & Dong (2002) and to highlight the aerotolerance of the strains. In accordance with the gender of the proposed genus name, the specific epithet was changed to *aeriphila*.

Isolates T4, T6^T, T8 and T16^T were Gram-positive, catalase- and oxidase-negative, non-spore-forming and non-motile; their respective biochemical profiles are presented in Table 1. For many *Bifidobacterium* species, carbohydrate profiles are known to vary between strains (Scardovi, 1986; Gómez Zavaglia *et al.*, 1998); this was also apparent for *Aeriscardovia aeriphila* gen. nov., sp. nov. In addition, given the relatively low number of strains (based on the number of distinct macrorestriction patterns) that are evident within the proposed species, the ability to identify the groups by biochemical profiling can not be stated. No *Bifidobacterium* species appear to grow at <20 °C (Scardovi, 1986; Biavati *et al.*, 2000). However, at 4 and 8 °C, isolate T16^T reduced the culture pH from ~6.2 to

Table 1. Biochemical profiles for strains LMG 21773^T, LMG 21774, LMG 21775^T and isolate T8

Taxa: 1, LMG 21773^T; 2, T8; 3, LMG 21774; 4, LMG 21775^T. All strains ferment glucose, salicin, arabinose and raffinose. None ferment rhamnose, trehalose, sorbitol, lactose, mannitol or glycerol. All strains are positive for α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, α -arabinosidase, arginine arylamidase, proline arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase activities. All strains are negative for β -galactosidase-6-phosphate, β -glucuronidase, *N*-acetyl- β -glucosaminidase, glutamic acid decarboxylase, alkaline phosphatase, leucyl glycine arylamidase, pyroglutamic acid arylamidase, alanine arylamidase, glutamyl glutamic acid arylamidase and urease activities and indole production. Gelatin is not liquified and nitrate is not reduced to nitrite. +, Positive reaction; (+), weakly positive reaction (negative reaction with the API Rapid ID32A test strip); –, negative reaction.

Characteristic	1	2	3	4
Acid from:				
Cellobiose	–	–	–	+
Maltose	+	+	+	–
Mannose	(+)	(+)	(+)	–
Melezitose	+	+	–	+
Sucrose	–	–	+	+
Xylose	–	–	+	+
α -Fucosidase activity	+	+	+	–

~5.91 and ~5.46, respectively, after 16 days incubation, compared to ~4.6 after 24 h at 37 °C. Cells cultured at 8 °C yielded a PFGE macrorestriction pattern that was characteristic for PFGE type F (data not shown). Although sensitivity to oxygen is known to vary between *Bifidobacterium* species (Scardovi, 1986; Shimura *et al.*, 1992; Meile *et al.*, 1997), no existing species have been reported to grow on agar media under aerobic conditions. However, isolates T4, T6^T and T16^T all had comparable colony counts following spread-plating on mMRS agar and incubation under aerobic or anaerobic conditions. Based on colony size, growth was clearly reduced in the presence of air. When the type strains for *B. minimum*, *S. inopinata* and *P. denticolens*, the nearest neighbours to the novel species (Fig. 1 and Simpson *et al.*, 2003) were streaked onto mMRS agar and incubated aerobically, only *B. minimum* showed signs of growth. However, growth was limited to the initial inoculation and no individual colonies were observed. Therefore, both novel species appear to be readily distinguished. It is not clear whether the proposed species represent food-associated bacteria that are passing through the GIT or are actual residents of the caecum. Given the relatively high concentration of dissolved oxygen in the pig caecum (Hillman *et al.*, 1993), either habitat could explain their distinctive aerotolerance. Indeed, ~25 % of anaerobes recovered from a human caecum were considered to be facultative anaerobes, compared to only ~0.1 % for faecal

isolates (Marteau *et al.*, 2001). Similarly, bifidobacteria isolated from the chicken crop were considered to have aerotolerant properties (Petr & Rada, 2001). *S. inopinata* and *P. denticolens* have an atypical bifidobacterial morphology that is similar to that of isolates T4, T6^T and T8 (see Supplementary Figure in IJSEM Online, panels 2a and 4a). In addition, for each species, cell morphology changed to an elongated rod with enlarged ends when exposed to oxygen (Crociani *et al.*, 1996; Supplementary Figure, panel 5a). The morphology change was also evident when isolates T4, T6^T and T8 were cultured at 30 or 46 °C (data not shown). From whole-cell FISH with a *Bifidobacterium* genus-specific 16S rRNA probe (Ventura *et al.*, 2001), elongation appeared to be caused by incomplete cell division, resulting in compartmental distribution of the probe (Supplementary Figure, panel 5b). Incomplete cell division was previously considered to be the cause of elongation of cells of *Bifidobacterium longum* following exposure to oxygen (Ahn *et al.*, 2001). The weak signal observed with strains LMG 21773^T and LMG 21774 (Supplementary Figure, panel 2b) may relate to their non-*Bifidobacterium* status, although the signal was greater than that observed for the negative control culture, *Lactobacillus rhamnosus* (Supplementary Figure, panel 3b). It is not clear why, when elongated, cells of isolates T4, T6^T and T8 had a strong signal (Supplementary Figure, panel 5b). By using a universal 16S rRNA probe, both morphology types yielded equivalent signals (Supplementary Figure, panels 4b and 6b), indicating that the weak signal was not related to poor probe penetration into the cells. PFGE patterns obtained from colonies grown under aerobic and anaerobic conditions were identical and characteristic for each strain,

indicating that elongation represented an adaptation to stress. No change in cell morphology was seen when strain LMG 21775^T was cultured aerobically and an equally strong signal was observed with the *Bifidobacterium*-specific probe for both aerobically and anaerobically cultured cells. This is consistent with the assignment of the novel species to the genus *Bifidobacterium*.

The major characteristics that differentiate *Bifidobacterium psychraerophilum* sp. nov. and *Aeriscardovia aeriphila* gen. nov., sp. nov. from other closely related bacteria are summarized in Tables 2 and 3, respectively.

Description of *Bifidobacterium psychraerophilum* sp. nov.

Bifidobacterium psychraerophilum (psych.rae.ro'phi.lum. Gr. n. *psychros* cold; Gr. n. *aēr* air; N.L. adj. *philus* from Gr. adj. *philos* loving; N.L. neut. adj. *psychraerophilum* cold- and air-loving).

From PFGE analysis, 23 pig caecal isolates appeared to represent a single strain, termed PFGE type F. The following description is based on a single isolate of this strain. Cells are Gram-positive, catalase- and oxidase-negative, non-motile, non-spore-forming, short and irregularly shaped rods, approximately 0.7–1.0 µm wide and 0.8–1.5 µm long with occasional bifurcations, arranged singly or in pairs. Colonies on mMRS agar under anaerobic conditions are white, circular and convex with smooth edges and reach a diameter of up to 3 mm after 3 days incubation at 37 °C. Colonies are also formed under aerobic conditions, but attain a reduced diameter of ~1 mm after 3 days incubation. Optimal temperature for growth is 37 °C;

Table 2. Major characteristics that differentiate strain LMG 21775^T from phylogenetically related bacteria

Taxa: 1, strain LMG 21775^T; 2, *Bifidobacterium minimum*; 3, *Bifidobacterium asteroides*; 4, *Bifidobacterium coryneforme*; 5, *Bifidobacterium indicum*. Data are from this and earlier studies (reviewed by Scardovi, 1986). +, Positive; –, negative; v, variable; ND, not determined.

Characteristic	1	2	3	4	5
Growth on agar medium under aerobic conditions	+	+*	–	–	–
Temperature range for growth (°C)	4–42	22–40	21–42†	ND	ND
Acid from:					
Maltose	–	+	v	+	v
Mannose	–	–	v	–	v
Melezitose	+	–	–	–	–
Xylose	+	–	v	+	–
Salicin	+	–	+	+	+
Arabinose	+	–	+	+	–
Raffinose	+	–	+	+	+
Cellobiose	+	–	+	+	+
DNA G+C content (mol%)	59.2	61.5	59	ND	60

*Growth limited to the initial streak on mMRS agar medium.

†No growth at these temperatures.

Table 3. Major characteristics that differentiate strains LMG 21773^T and LMG 21774 from phylogenetically related genera

Taxa: 1, strain LMG 21773^T; 2, strain LMG 21774; 3, *Bifidobacterium*; 4, *Scardovia*; 5, *Parascardovia*. Data are from this and earlier studies (Scardovi, 1986; Gavini *et al.*, 1991; Crociani *et al.*, 1996; Jian & Dong, 2002). +, Positive; (+), weakly positive; –, negative; V, variable; ND, not determined.

Characteristic	1*	2	3	4	5
Growth on agar medium under aerobic conditions	+	+	(+) [†]	–	–
Temperature range for growth (°C)	30–46	30–46	25–46	27–44	27–44
Elongated cell morphology on exposure to oxygen	+	+	V	+	+
Acid from:					
Maltose	+	+	V	+	+
Mannose	(+)	(+)	V	–	–
Melezitose	–	+	V	V	–
Sucrose	+	–	+	+	+
Xylose	+	–	V	+	–
Salicin	+	+	V	V	+
Arabinose	+	+	V	–	V
Raffinose	+	+	V	V	V
Trehalose	–	–	V	–	V
Sorbitol	–	–	V	ND	ND
Lactose	–	–	V	V	+
Mannitol	–	–	V	–	–
Cellobiose	–	–	V	–	+
DNA G+C content (mol%)	54.7	54.7	55–67	45	55

*Isolate T8 (PFGE type Ea) had the same characteristic profile as strain LMG 21773^T (PFGE type E).

[†]*Bifidobacterium minimum* grows at the initial inoculation point on mMRS agar.

maximum temperature for growth is 42 °C, with no growth at 46 °C. Growth occurs at 4 °C, although it is considerably reduced. Lowest pH attained is 4.0, with a minimum initial pH for growth of 4.5. DNA G+C content is 59.2 mol%. Biochemical characteristics are shown in Table 1.

Type strain is T16^T (PFGE type F) (=LMG 21775^T = NCIMB 13940^T). Isolated from a pig caecum (contents and epithelium) in Fermoy, Ireland. Previously termed '*Bifidobacterium psychroaerophilum*'.

Description of *Aeriscardovia* gen. nov.

Aeriscardovia (Aer'i.scar.do'vi.a. L. masc. n. *aer*, *aeris* air; N.L. fem. n. *Scardovia* a bacterial generic name to honour Vittorio Scardovi, an Italian microbiologist; N.L. fem. n. *Aeriscardovia* cells similar to the genus *Scardovia* that can grow in air).

Gram-positive, catalase- and oxidase-negative, non-motile, non-spore-forming, short and irregularly shaped rods. Optimal growth occurs under anaerobic conditions, with aerobic growth yielding elongated cells. DNA G+C content of the type species is 54.7 mol%. Isolated from a porcine caecum. According to 16S rDNA and HSP60 gene sequence comparisons, *Aeriscardovia* belongs to the family *Bifidobacteriaceae*. Only one species, the type species *Aeriscardovia aeriphila*, has thus far been described.

Description of *Aeriscardovia aeriphila* sp. nov.

Aeriscardovia aeriphila (L. masc. n. *aer*, *aeris* air; Gr. adj. *philos* loving; N.L. fem. adj. *aeriphila* air-loving).

From PFGE analysis, 77 pig caecal isolates appeared to represent three strains, termed PFGE types B, E and Ea. The following phenotypic description is based on a representative isolate for each strain. Cells are Gram-positive, catalase- and oxidase-negative, non-motile, non-spore-forming, short and irregularly shaped rods, approximately 0.6–0.9 µm wide and 0.8–1.5 µm long, that are arranged singly or in pairs, but not in chains. Colonies on mMRS agar under anaerobic conditions are grey-white, circular and flat to convex with entire edges and reach a diameter of up to 3 mm after 3 days incubation at 37 °C. Colonies are also formed under aerobic conditions, but a diameter of only ~1 mm is attained after 5 days incubation. Under aerobic conditions, cell morphology includes elongated cells of approximately 4–6 µm in length. Optimum temperature for growth is 37 °C, with a maximum of 46 °C and minimum of 30 °C. No growth occurs at 48 or 25 °C. In mMRS, the lowest pH value attained is 4.2; minimum initial pH for growth is 4.5. DNA G+C content of strains of PFGE types B and E is 54.7 mol%. Biochemical characteristics are shown in Table 1. A single plasmid of 30 kbp may be present in isolates of PFGE type Ea, based on macrorestriction pattern data.

Type strain is T6^T (PFGE type E) (=LMG 21773^T=NCIMB 13939^T). Isolated from a pig caecum (contents and epithelium) in Fermoy, Ireland. Previously termed '*Bifidobacterium aerophilum*'.

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