Arcanobacterium bialowiezense sp. nov. and *Arcanobacterium bonasi* sp. nov., isolated from the prepuce of European bison bulls (*Bison bonasus*) suffering from balanoposthitis, and emended description of the genus *Arcanobacterium* Collins *et al.* 1983

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A taxonomic study was performed on 13 bacterial strains isolated from preputial swabs of European bison (*Bison bonasus*) bulls suffering from balanoposthitis. The isolates were Gram-positive, non-motile, facultatively anaerobic, diphtheroid-shaped cells. Based on biochemical profiles and BOX-PCR-generated genomic fingerprints, the isolates were grouped into two clusters represented by four and nine strains, respectively. Strains 1(W3/01)^T and 2(W106/04)^T, selected as representatives of the two clusters, shared 97.2% 16S rRNA gene sequence similarity. The highest gene sequence similarities found (95.5–96.4%) were to *Arcanobacterium pyogenes* DSM 20630^T and *Arcanobacterium bernardiae* DSM 9152^T, demonstrating that the novel strains are members of the genus *Arcanobacterium*, but are not members of a recognized species. The polar lipid profiles of the two novel strains displayed the major characteristics also found in *A. pyogenes* DSM 20630^T and *Arcanobacterium haemolyticum* DSM 20595^T. Detection of a quinone system with MK-10(H₄) as the predominant compound confirmed phylogenetic relatedness of the novel strains to *A. pyogenes* and separated them from the type species of the genus, *A. haemolyticum*, which contains MK-9(H₄) as the predominant quinone. Results from DNA–DNA hybridizations clearly demonstrated that strains 1(W3/01)^T and 2(W106/04)^T represent separate species. Based on these data, two novel species of the genus *Arcanobacterium* are described, for which the names *Arcanobacterium bialowiezense* sp. nov. [type strain 1(W3/01)^T=DSM 17162^T=NCTC 13354^T] and *Arcanobacterium bonasi* sp. nov. [type strain 2(W106/04)^T=DSM 17163^T=NCTC 13355^T] are proposed.

Since 1980, a chronic disease of the external genital organs of male European bison (*Bison bonasus*) has been observed on the Polish side of the Białowieża Forest (Kita *et al.*, 1994).

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 1(W3/01)^T and 2(W106/04)^T are AJ879696 and AJ879697, respectively.

The polar lipid profile of strain 1(W3/01)^T is available as a supplementary figure in IJSEM Online.

The advanced stage of the disease is characterized by oedema of the skin surrounding the preputial orifice and accumulation of thick exudate and necrotic tissue within the preputial cavity (Jakob *et al.*, 2000).

In the course of a study concerning the aetiological agent of the balanoposthitis, several bacterial strains were isolated. Based on preliminary classification, these isolates were identified as members of the genera *Arcanobacterium*, *Corynebacterium*, *Bacillus*, *Fusobacterium*, *Peptostreptococcus*, *Prevotella*, *Staphylococcus* and *Streptococcus* and the family

Enterobacteriaceae (A. Lehen, unpublished results). Thirteen strains isolated from the prepuces of 13 European bison bulls suffering from balanoposthitis [strains 1(W3/01)^T, 1(W10/02), 1(W89/03), 1(W91/04), 2(W106/04)^T, 2(W4/01), 2(W34/02), 2(W76/03), 2(W78/03), 2(W87/03), 2(W101/04), 2(W103/04) and 2(W105/04)] were found to be morphologically similar to *Arcanobacterium pyogenes*, but could not be identified by standard diagnostic tests. Although these strains were isolated exclusively from bulls with balanoposthitis, their pathological significance is still unknown. However, the phylogenetic relationship to *A. pyogenes* underlines their possible role in the aetiology of balanoposthitis. In order to clarify the taxonomic status of this set of isolates, the strains were characterized by a combination of genomic, physiological and chemotaxonomic methods.

Genomic analyses

Based on almost identical genomic fingerprints generated after BOX-PCR according to the method described by Louws *et al.* (1994), the *Arcanobacterium* isolates were grouped into two clusters and each cluster was assumed to represent a distinct species. Strains 1(W3/01)^T and 2(W106/04)^T were selected as representative strains from each of the clusters. Cluster I, represented by strain 1(W3/01)^T, exhibited four predominant bands, with sizes of approximately 450, 550, 750 and 1500 bp. Cluster II, represented by strain 2(W106/04)^T, exhibited three predominant bands, with sizes of approximately 550, 850 and 1100 bp. *A. pyogenes* DSM 20630^T exhibited a distinct BOX fingerprint (Fig. 1).

16S rRNA gene amplification was performed according to Wyss *et al.* (1996). Amplified PCR products were sequenced bidirectionally using an ABI 3100 Genetic Analyzer (Applied Biosystems). The resulting 16S rRNA gene sequences of strains 1(W3/01)^T and 2(W106/04)^T consisted of 1481 [positions 36–1517, *Escherichia coli* numbering (Brosius *et al.*, 1978)] and 1444 nucleotides (36–1480, *E. coli* numbering), respectively. In a FASTA search (Pearson & Lipman, 1988), the two isolates shared 97.2% gene sequence similarity. Isolates 1(W3/01)^T and 2(W106/04)^T exhibited highest sequence similarities to *A. pyogenes* DSM 20630^T (96.1 and 96.4%, respectively) and *Arcanobacterium bernardiae* DSM 9152^T (95.5 and 95.8%, respectively). Sequence similarities with other species of *Arcanobacterium* were in the range 93.3–94.7%. These results indicate that strains 1(W3/01)^T and 2(W106/04)^T are affiliated to the

genus *Arcanobacterium*, but are not members of an established species. In order to display the phylogenetic relationships of the two representative strains with established species of the genus *Arcanobacterium*, a dendrogram was constructed after multiple alignment with publicly available 16S rRNA gene sequences using MEGA version 2.1 (Kumar *et al.*, 2001). The results clearly demonstrated that the two isolates are closely related and that they are located on the *Arcanobacterium* lineage (Fig. 2), with *A. pyogenes* DSM 20630^T and *A. bernardiae* DSM 9152^T as the closest related species.

DNA–DNA hybridizations (Ziemke *et al.*, 1998; Kämpfer *et al.*, 2003) between strains 1(W3/01)^T and 2(W106/04)^T revealed a DNA–DNA relatedness of only 9.4% (reciprocal 13.2%). This low value unambiguously demonstrated that strains 1(W3/01)^T and 2(W106/04)^T are representatives of two separate species.

Biochemical characteristics

An investigation of *A. bernardiae* DSM 9152^T, *Arcanobacterium haemolyticum* DSM 20595^T, *Arcanobacterium hippocoleae* DSM 15539^T, *Arcanobacterium pluranimalium* DSM 13483^T, *Arcanobacterium phocae* DSM 10002^T, *A. pyogenes* DSM 20630^T and strains 1(W3/01)^T and 2(W106/04)^T using commercially available API Coryne, API CH50 and API ZYM tests (bioMérieux) revealed metabolic profiles that were distinct for each strain (Table 1). The other isolates which, based on BOX band patterns, had been grouped with strain 1(W3/01)^T [strains 1(W10/02), 1(W89/03) and 1(W91/04)] or strain 2(W106/04)^T [strains 2(W4/01), 2(W34/02), 2(W76/03), 2(W78/03), 2(W87/03), 2(W101/04), 2(W103/04) and 2(W105/04)] showed profiles which were almost indistinguishable from the selected representative of each cluster. The two similarity groups could be distinguished from one another by their biochemical profiles, confirming that each group represents a distinct species. Gram-staining (Quinn *et al.*, 2000) of cells of the two novel isolates revealed Gram-positive behaviour. Isolates 1(W3/01)^T and 2(W106/04)^T were catalase- and oxidase-negative, showed no serolysis on Löffler serum agar and no growth on Gassner or MacConkey agar. The optimum growth temperature on sheep blood agar was 37 °C. No growth occurred at 42 °C. The best growth occurred under aerobic conditions. Growth in a CO₂-enriched (CO₂Gen) or anaerobic atmosphere (AnaeroGen) was slowed. Other characteristics are listed in the species descriptions and in Table 1.

Fig. 1. BOX-PCR-generated fingerprints of the *Arcanobacterium* isolates. Lanes: M, 100 bp standard marker; 1, 1(W3/01)^T; 2, 1(W10/02); 3, 1(W89/04); 4, 1(W91/04); 5, *Arcanobacterium pyogenes* DSM 20630^T; 6, 2(W106/04)^T; 7, 2(W4/01); 8, 2(W34/02); 9, 2(W76/03); 10, 2(W78/03); 11, 2(W87/03); 12, 2(W101/04); 13, 2(W103/04); 14, 2(W105/04).

Fig. 2. Neighbour-joining dendrogram based on 16S rRNA gene sequences showing the phylogenetic relationships of *Arcanobacterium* species and related genera. The dendrogram was constructed after multiple alignment of data using MEGA version 2.1 (Kumar *et al.*, 2001). Bootstrap values based on 500 replications are given at branch points. Bar, 0.01 substitutions per nucleotide position.

Chemotaxonomic characteristics

Biomass for fatty acid, quinone and polar lipid analysis was collected from blood agar and washed twice with 0.9 % NaCl solution. Fatty acid analysis was conducted as described by Kämpfer & Kroppenstedt (1996). The fatty acid profiles of the representatives of each BOX similarity group, strains 1(W3/01)^T, 1(W10/02), 2(W106/04)^T and 2(W34/02), as well as *A. haemolyticum* DSM 20595^T and *A. pyogenes* DSM 20630^T, consisted predominantly of unbranched acids (> 98 %). The six strains shared the presence of the major components C_{16:0}, C_{18:1}ω9c and C_{18:0} which was in good agreement with the profiles of *A. pyogenes* and *A. haemolyticum* as reported by Bernard *et al.* (1991). The fatty acid profiles distinguished the representatives of the two BOX similarity groups by revealing significant differences in the relative amounts of C_{14:0}, C_{18:1}ω9c, C_{18:1}ω6c, C_{16:0} and/or C_{18:0} (Table 2) and also distinguished them from the two reference strains.

Polar lipids were analysed as described by Ventosa *et al.* (1993). The polar lipid profiles of strains 1(W3/01)^T (see Supplementary Fig. S1 in IJSEM Online) and 2(W106/04)^T were rather complex. Both strains contained diphosphatidylglycerol, phosphatidylglycerol, three unknown phosphoglycerolipids, an unknown aminolipid (AL2), two unknown phospholipids and an unknown aminophospholipid as predominant compounds, but they could be distinguished by the presence of two hydrophilic lipids in strain 1(W3/01)^T; an unknown aminoglycerolipid and an unknown aminophosphoglycerolipid. Similar, but not identical, profiles were detected for *A. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T (results not shown).

Analyses of quinones were performed as described previously (Altenburger *et al.*, 1996). Strain 1(W3/01)^T exhibited a quinone system with menaquinone MK-10(H₄) as the predominant compound (98 %) and MK-9(H₄) (2 %).

A similar quinone system, with MK-10(H₄) (91 %) and MK-9(H₄) (9 %), was detected in strain 2(W106/04)^T. This result was not in agreement with the previously determined quinone systems of *A. haemolyticum*, which was reported to contain MK-9(H₄) as the predominant compound (Collins *et al.*, 1982). Hence, we analysed the quinone system of *A. haemolyticum* DSM 20595^T and *A. pyogenes* DSM 20630^T. *A. haemolyticum* DSM 20595^T was found to possess a quinone system with MK-9(H₄) as the predominant compound (85 %) and minor amounts of MK-8(H₄) (15 %). In contrast, *A. pyogenes* DSM 20630^T, which is the closest phylogenetic relative of strains 1(W3/01)^T and 2(W106/04)^T, also contained a quinone system with the major compounds MK-10(H₄) (85 %) and MK-9(H₄) (15 %). The results from the quinone analyses reflect the phylogenetic relatedness of strains 1(W3/01)^T and 2(W106/04)^T to *A. pyogenes* DSM 20630^T and clearly distinguish them from the type species of the genus, *A. haemolyticum*.

Taxonomic considerations

16S rRNA gene sequence analyses and the chemotaxonomic characteristics of the isolates suggest the assignment of strains 1(W3/01)^T and 2(W106/04)^T to the genus *Arcanobacterium*. Unique physiological profiles and genomic fingerprints and characteristic fatty acid and polar lipid profiles demonstrate that strains 1(W3/01)^T and 2(W106/04)^T each represent a separate species within the genus.

In general, the quinone system is a rather conserved characteristic. The finding of MK-10(H₄) in one lineage within the genus *Arcanobacterium*, represented by our isolates and *A. pyogenes*, is rather exciting because the representative of the second lineage, *A. haemolyticum*, is characterized by the MK-9(H₄) quinone system. Hence, both quinone system and phylogeny indicate a subdivision within the genus *Arcanobacterium*, and a restriction of the genus to the species *A. haemolyticum*, *A. phocae*, *A. pluranimalium* and

Table 1. Biochemical characteristics of *Arcanobacterium* species

Species/strains: 1, *A. bernardiae* DSM 9152^T; 2, *A. haemolyticum* DSM 20595^T; 3, *A. hippocoleae* DSM 15539^T; 4, *A. pluranimalium* DSM 13483^T; 5, *A. phocae* DSM 10002^T; 6, *A. pyogenes* DSM 20630^T; 7, *A. bialowiezense* sp. nov. (*n*=4); 8, *A. bonasi* sp. nov. (*n*=9). +, Positive; w, weakly positive; -, negative; v(+), type strain shows positive reaction; v(-), type strain shows negative reaction. All strains are positive for D-glucose; all strains are negative for reduction of nitrate, urease, lipase (C14), valine arylamidase, cystine arylamidase, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α -mannosidase.

Characteristic	1	2	3	4	5	6	7	8
N-Acetyl- β -glucosaminidase	-	+	+	w	-	w	-	-
Adonitol	-	-	-	-	-	-	v(-)	w
Alkaline phosphatase	-	+	+	-	+	-	-	-
D-Arabitol	-	w	-	-	-	-	v(-)	+
L-Arabitol	-	-	-	-	-	-	v(-)	+
Catalase	-	-	-	+	-	-	-	-
Erythritol	-	-	-	-	-	-	-	w
Esterase (C4)	-	-	-	-	-	-	+	+
Esterase lipase (C8)	-	-	-	-	-	-	+	+
D-Fructose	-	-	-	-	-	-	+	+
α -Galactosidase	-	-	-	-	+	-	-	-
β -Galactosidase	-	+	+	-	+	+	-	-
Gelatin hydrolysis	-	-	-	+	-	+	-	-
α -Glucosidase	+	+	+	-	-	+	+	-
β -Glucosidase	-	-	-	w	w	-	-	-
β -Glucuronidase	-	w	+	+	-	+	+	+
Glycerol	-	-	-	-	-	-	v(+)	w
Glycogen	w	-	-	-	+	w	-	-
D-Lactose	-	+	+	-	w	+	-	w
Leucine arylamidase	-	-	-	-	+	+	+	w
D-Maltose	+	+	-	w	w	+	v(-)	w
D-Mannitol	-	-	-	-	w	-	-	-
Pyrazinamidase	+	+	-	-	-	-	+	-
Pyrrolidonyl arylamidase	w	w	-	-	w	+	-	+
D-Ribose	+	+	-	+	+	+	-	-
Sucrose	-	w	-	-	-	w	-	-
Trypsin	-	-	-	-	-	-	v(+)	+
D-Xylose	-	-	-	-	w	+	-	-

A. hippocoleae might be desirable. This would lead to *A. pyogenes*, *A. bernardiae* and the two novel species described in this study being reclassified as a novel genus. In a reclassified genus *Arcanobacterium*, the presence of a MK-9(H₄) quinone system might be a genus-specific trait, while MK-10(H₄) might characterize the neighbouring genus. However, the suitability of the quinone system as the basis for such differentiation would have to be substantiated by examination of all species of *Arcanobacterium*.

Since the presence of MK-9(H₄) is listed as a characteristic trait of the genus *Arcanobacterium* (Collins *et al.*, 1982), the description of the genus has to be emended to include the presence of MK-10(H₄).

Emended description of the genus *Arcanobacterium* Collins *et al.* 1983

In contrast to the original description of the genus, some species do not contain a quinone system with menaquinone MK-9(H₄) as the major compound; MK-10(H₄) can also predominate. Other characteristics of the genus are as given by Collins *et al.* (1982).

Description of *Arcanobacterium bialowiezense* sp. nov.

Arcanobacterium bialowiezense (bi.a.lo.wi.e.zen'se. N.L. neut. adj. *bialowiezense* pertaining to Bialowieza, Poland, where the type strain was isolated).

Cells are short pleomorphic rods that stain Gram-positive. After 48 h of growth under aerobic conditions on sheep blood agar, colonies are translucent, convex, approx. 0.5 mm in diameter and surrounded by a narrow zone of β -haemolysis. Cells are non-motile (motility agar), facultatively anaerobic and are catalase- and oxidase-negative. Best growth occurs at 37 °C; no growth at 42 °C. No growth is observed on Gassner or MacConkey agar. Other physiological and biochemical characteristics are summarized in Table 1. The quinone system contains MK-10(H₄) as the predominant compound. Diphosphatidylglycerol is predominant in the polar lipid profile. Phosphatidylglycerol, three unknown phosphoglycerolipids, two unknown aminolipids, two unknown phospholipids, an unknown aminophospholipid, an unknown aminoglycerolipid and an unknown aminophosphoglycerolipid are present in moderate to minor amounts. The fatty acid profile consists predominantly of unbranched acids with the major compounds C_{16:0}, C_{18:1}ω9c, C_{18:0} and C_{14:0}. Complete fatty acid profiles are listed in Table 2.

The type strain, strain 1(W3/01)^T (=DSM 17162^T=NCTC 13354^T), was isolated from the prepuce of a European bison.

Description of *Arcanobacterium bonasi* sp. nov.

Arcanobacterium bonasi [bo.na'si. L. gen. n. *bonasi* of the European bison (*Bison bonasus*) from which the type strain was isolated].

Cells are short pleomorph rods that stain Gram-positive. After 48 h of growth under aerobic conditions on sheep blood agar, colonies are translucent, convex, approx. 0.5 mm in diameter and surrounded by a narrow zone of β -haemolysis. Cells are non-motile (motility agar), facultatively anaerobic and catalase- and oxidase-negative. Best growth occurs at 37 °C; no growth at 42 °C. No growth is observed on Gassner or MacConkey agar. Other physiological and biochemical characteristics are summarized in Table 1. The quinone system contains MK-10(H₄) as the predominant compound. Diphosphatidylglycerol is predominant in the polar lipid profile. Phosphatidylglycerol, three unknown phosphoglycerolipids, an unknown aminolipid, two unknown phospholipids and an unknown

Table 2. Fatty acid content (%) of *Arcanobacterium* strains

Summed feature 3 contains C_{16:1}ω7c/C₁₅ iso 2-OH; summed feature 5 contains C_{18:2}ω6,9c/C_{18:0} ante.

Fatty acid	<i>A. bialowiezense</i> sp. nov.		<i>A. bonasi</i> sp. nov.		<i>A. haemolyticum</i> DSM 20595 ^T	<i>A. pyogenes</i> DSM 20630 ^T
	1(W3/01) ^T	1(W10/02)	2(W106/04) ^T	W(34/02)		
C _{9:0}	0.1				0.3	
C _{10:0}	1.5	1.7			0.3	0.8
C _{12:0}	1.3	0.9				1.4
C _{14:0}	7.8	7.5	1.6	1.8		8.5
C _{15:0}	1.3	2.0	0.7	0.7		
C _{16:1} ω9c	1.7	1.8		0.4		3.7
Summed feature 3	1.7	1.9	2.7	3.0	2.4	0.8
C _{16:0}	30.6	33.4	24.4	25.2	22.5	35.3
C _{17:1} ω8c		0.6		0.7		
Summed feature 5	3.5	2.6	5.5	5.7	10.7	0.8
C _{18:1} ω9c	20.9	24.7	30.4	32.4	37.0	33.2
C _{18:1} ω6c	5.9	3.9	9.6	6.8		1.3
iso C _{17:1} ω9c						0.5
C _{17:0} iso				0.6		
C _{17:0} anteiso		0.4	0.7	0.8		
C _{17:0}	1.2	1.5	1.3	1.4		
C _{18:0}	20.6	16.4	22.0	19.8	24.7	12.3
C _{19:1} iso I						0.8
C _{19:0} iso						0.8
C _{20:4} ω6,9,12,15c	2.0	0.6	1.3	0.9	2.1	

aminophospholipid are present in moderate to minor amounts. The fatty acid profile consists predominantly of unbranched acids with the major compounds C_{16:0}, C_{18:1}ω9c and C_{18:0}. Complete fatty acid profiles are listed in Table 2.

The type strain, strain 2(W106/04)^T (=DSM 17163^T = NCTC 13355^T), was isolated from the prepuce of a European bison.

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