NOTE

***Brachybacterium fresconis* sp. nov. and *Brachybacterium sacelli* sp. nov., isolated from deteriorated parts of a medieval wall painting of the chapel of Castle Herberstein (Austria)**Jeroen Heyrman,¹ An Balcaen,¹ Paul De Vos,¹ Peter Schumann² and Jean Swings^{1,3}¹ Vakgroep BFM WE10V, Laboratorium voor Microbiologie, Universiteit Gent, KL Ledeganckstraat 35, B-9000 Gent, Belgium² DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1b, D-38124 Braunschweig, Germany³ BCCM/LMG Culture Collection, KL Ledeganckstraat 35, B-9000 Gent, Belgium

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From two samples of microbial biofilms, damaging the mural paintings at the Saint-Catherine chapel of Castle Herberstein (Austria), four and nine coryneform bacteria were isolated, respectively. A polyphasic taxonomic study of these isolates, including morphological, biochemical and chemotaxonomic characterization, REP-PCR fingerprinting, 16S rDNA sequence analysis, DNA base ratio and DNA–DNA hybridizations, allocated them to the genus *Brachybacterium*. The isolates of the two samples both represent new species, for which the names *Brachybacterium fresconis* sp. nov. and *Brachybacterium sacelli* sp. nov. are proposed. The respective type strains are LMG 20336^T (= DSM 14564^T) and LMG 20345^T (= DSM 14566^T).

Keywords: *Brachybacterium fresconis* sp. nov., *Brachybacterium sacelli* sp. nov., polyphasic taxonomy

It is now well recognized that wall paintings can be severely damaged by microbial growth (Ciferri, 1999). In studies on the bacterial community associated with such deterioration, members of the actinomycetes are frequently cultivated (Sorlini *et al.*, 1987; Weirich, 1989; Karpovich-Tate & Rebrikova, 1990; Altenburger *et al.*, 1996; Wieser *et al.*, 1999; Groth *et al.*, 1999b). During the course of classification of heterotrophic bacteria isolated from deteriorated parts of the wall paintings in the St Catherine chapel of the Castle of Herberstein (Austria), a group of 13 strains was allocated to the genus *Brachybacterium*. In this paper, we describe the characterization of this group of strains and describe two new species, *Brachybacterium fresconis* and *Brachybacterium sacelli*. Type strains were deposited in the BCCM/LMG (Collection of the Laboratorium voor Microbiologie, Rijksuniversiteit Gent, Ledeganckstraat 35, B-9000, Gent, Belgium)

as LMG 20336^T (= DSM 14564^T) and LMG 20345^T (= DSM 14566^T), respectively.

Four of the wall-painting strains (LMG 20333 and 20335–20337) originated from a paint layer sample of black discoloration located in the chancel vault of the chapel; nine strains (LMG 20338–20346) originated from black stripes in the upper area of the nave's north wall. The strains were isolated from trypticase soy agar (TSA; BBL) and R2A agar (Difco) with or without added salt; the isolation procedure used was described previously (Heyrman *et al.*, 1999). Isolated strains were further grown on marine agar (MA; Difco) at 28 °C. Reference strains were obtained from the DSMZ and sustained in the BCCM/LMG: *Brachybacterium alimentarium* DSM 10672^T (= LMG 19551^T), *Brachybacterium conglomeratum* DSM 10241^T (= LMG 19849^T), *Brachybacterium faecium* DSM 4810^T (= LMG 19847^T), *Brachybacterium neshterenkovii* DSM 9573^T (= LMG 19549^T), *Brachybacterium paraconglomeratum* DSM 46341^T (= LMG 19861^T), *Brachybacterium rhamnsum* DSM 10240^T (= LMG 19848^T) and *Brachybacterium tyrofermentans* DSM 10673^T (= LMG 19552^T). Reference strains were first cultured on PY-BHI medium (Takeuchi *et al.*, 1995) and further on MA at 28 °C.

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Abbreviation: UPGMA, unweighted pair group method with arithmetic averages.

The EMBL accession numbers for the 16S rRNA gene sequences of *Brachybacterium fresconis* LMG 20336^T and *Brachybacterium sacelli* LMG 20345^T are AJ415378 and AJ415384, respectively.

Fig. 1. Grouping of normalized REP-PCR patterns of 13 *Brachybacterium* isolates from damaged mural paintings in a dendrogram based on a UPGMA clustering of Pearson correlation similarity coefficients.

Total genomic DNA was purified for 16S rDNA sequencing and REP-PCR using a slight modification of the method of Pitcher *et al.* (1989), as described by Heyndrickx *et al.* (1996). For determination of the G+C content and DNA–DNA hybridization, approximately 1 g biomass was harvested from agar plates and prepared further using a combination of the protocols of Marmur (1961) and Pitcher *et al.* (1989), as described by Logan *et al.* (2000).

REP-PCR genomic fingerprinting was performed with the REP1 and REP2 primers (Versalovic *et al.*, 1994) using the PCR conditions as described by Rademaker & de Bruijn (1997). For each strain, 6 µl PCR product mixed with 2 µl loading buffer (Rademaker & de Bruijn, 1997) was electrophoresed in a 1.5% (w/v) agarose gel and TAE buffer [1.21 g Tris 2-amino-2(hydroxymethyl)-1,3 propanediol l⁻¹, 0.2 ml 0.5 M EDTA l⁻¹, pH 8] for 15 h at a constant 55 V and 4 °C. The first and every sixth lane were loaded with 6 µl of the molecular ruler [45.5% (v/v) 100 bp ruler, 36.5% (v/v) 500 bp ruler and 18% (v/v) loading buffer]. After staining with ethidium bromide (0.5 µg ml⁻¹) and visualization, the patterns were digitalized and a Pearson correlation of the resulting band patterns was performed using BioNumerics 2.0 Software (Applied Maths).

16S rDNA sequence analysis was performed as described previously by Heyrman & Swings (2001). A phylogenetic tree was constructed based on global alignment and the neighbour-joining method, including all described members of the genus *Brachybacterium*, and *Dermabacter hominis* as an outgroup. A similarity matrix was constructed using pairwise alignment of the sequences and the unweighted pair group method with arithmetic averages (UPGMA) algorithm.

The G+C content of DNA was determined by HPLC as described by Mesbah *et al.* (1989) using a Waters

Symmetry Shield RP₈ column thermostatically controlled at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ with 1.5% acetonitrile (pH 4.0). Non-methylated λ phage DNA was used as the calibration reference.

DNA–DNA hybridization was performed using a modification of the microplate method (Ezaki *et al.*, 1989), as described by Willems *et al.* (2001).

Analysis of the fatty acid content of the cell wall was performed as described previously (Heyrman *et al.*, 1999), starting from cells grown on TSA for 48 h. Purified cell wall preparations were obtained by the method of Schleifer & Kandler (1972). The amino acids and peptides in peptidoglycan hydrolysates were analysed as described by Groth *et al.* (1999a). The glycolate content of the peptidoglycan was examined by the colorimetric method of Uchida *et al.* (1999). Menaquinones were extracted as described by Collins *et al.* (1977) and were analysed by HPLC (Groth *et al.*, 1997). Polar lipids extracted by the method of Minnikin *et al.* (1979) were identified by two-dimensional TLC and spraying with specific reagents (Collins & Jones, 1980). Absence of mycolic acids was demonstrated by TLC (Minnikin *et al.*, 1975).

Cell morphology and Gram staining was examined respectively by phase-contrast and normal light microscopy. Growth under anaerobic conditions (on MA) and catalase production were recorded. Oxidase activity was determined after 48 h growth on MA and using the reagents described by Fallor & Schleifer (1981). Tolerance to different NaCl concentrations and pH 5 was measured spectrophotometrically at OD₅₅₀ in marine broth after 48 h incubation at 28 °C. Results were scored positive if the change in OD₅₅₀ was greater than 0.300. Hydrolysis of starch was determined by the method of Smibert & Krieg (1994). Hydrolysis of gelatin was tested by streaking the isolates on agar medium with 1.2% (w/v) gelatin

Fig. 2. Phylogenetic positions based on 16S rDNA sequences of representative mural painting isolates among the type strains of *Brachybacterium*. *Dermabacter hominis* was included as outgroup. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points.

added, incubating for 5 days and visualizing hydrolysis with gelatin-precipitating reagent [15% (w/v) HgCl₂ in 10% (v/v) HCl]. API 20NE tests were performed according to the recommendations of the manufacturer (bioMérieux). Acid production from carbohydrates was studied as described by Takeuchi *et al.* (1995).

The mural painting strains were first screened by REP-PCR fingerprinting to group the isolates, since it has been shown that in general REP fingerprinting is in close agreement with DNA–DNA relatedness studies (Nick *et al.*, 1999; Rademaker *et al.*, 2000). The 13 isolates showed four REP types that can be subdivided into three major groups at less than 10% similarity (Pearson coefficient). In group 3, two subgroups (3a and 3b) can be delineated at 50% similarity (Fig. 1). The clustering corresponds well with the origin of the strains, with REP clusters 1 and 2 containing strains isolated from the chapels chancel vault and REP clusters 3a and 3b from the nave's north wall.

Representatives of the different REP types were chosen and analysed for their 16S rDNA sequence together with the type strains of *B. rhamnosum* and *B. paraconglomeratum*, since the sequences of the latter were not available from the EMBL database. The sequences were compared with those of all described *Brachybacterium* species and *Dermabacter hominis* was included as outgroup (Fig. 2). The seven mural painting isolates studied fall into two groups (bootstrap value of 94), one containing representatives of REP groups 1 and 2, and the other those of REP group 3 (a and b). The similarity matrix of the sequences revealed high internal pairwise similarity values within each group (99.8% or more). Between the two 16S rDNA groups,

a mean sequence similarity of 98.7% was calculated. 16S group 1 showed high sequence similarity towards *B. alimentarium* (mean of 98.2%), *B. faecium* (98.0%) and *B. nesterenkovii* (97.6%). 16S group 2 had the highest sequence similarities with *B. alimentarium* (mean of 98.3%), *B. faecium* (98.1%), *B. nesterenkovii* (98.1%) and *B. tyrofermentans* (97.9%). For both groups, mean sequence similarities with the other *Brachybacterium* species were below 97.5%.

Total DNA–DNA relatedness values were determined between a representative of the three main REP types (LMG 20333, 20336^T and 20345^T, respectively) and the closest related *Brachybacterium* type species as determined by 16S rDNA sequencing (sequence similarities above 97.5%). The mean percentage of DNA–DNA binding was 80% between the representatives of REP clusters 1 and 2; 56% between those of 1 and 3 and 46% between those of 2 and 3. The overall DNA relatedness values of the isolates and the closest related type strains of *Brachybacterium* species were all below 50%, thus well below the species level (70%; Wayne *et al.*, 1987). According to these genomic analyses, the novel isolates clearly belong to two distinct groups that can be allocated to two new species within *Brachybacterium*. A table of DNA–DNA relatedness data is available as supplementary data at <http://ijs.sgmjournals.org> – Table A.

Chemotaxonomic characterizations of the mural painting isolates were compared with those given in the literature for validated *Brachybacterium* species (Collins *et al.*, 1988; Gvozdyak *et al.*, 1992; Takeuchi *et al.*, 1995; Schubert *et al.*, 1996).

The mean values of fatty acids of the strains of both taxa are given below as a percentage of the total fatty

acid content $\pm$ SD (fatty acids representing less than 1% of the total amount are omitted). Taxon 1 (REP groups 1 and 2, 16S group 1): anteiso- $C_{15:0}$, $75.5 \pm 2.4\%$; iso- $C_{16:0}$, $6.9 \pm 0.7\%$; $C_{16:0}$, $1.6 \pm 0.5\%$; anteiso- $C_{17:0}$, $8.5 \pm 0.9\%$; $C_{18:1}$, $4.3 \pm 0.3\%$. Taxon 2 (REP group 3, 16S group 2): iso- $C_{15:0}$, $1.8 \pm 0.4\%$; anteiso- $C_{15:0}$, $73.7 \pm 2.7\%$; iso- $C_{16:0}$, $5.5 \pm 0.8\%$; iso/anteiso- $C_{17:1}$, $1.8 \pm 0.4\%$; anteiso- $C_{17:0}$, $8.6 \pm 1.2\%$; $C_{18:1}$, $4.5 \pm 0.3\%$. It can be concluded that both taxa have highly similar fatty acid profiles with anteiso- $C_{15:0}$, accounting for approximately 75% of the total fatty acid content. However, some discrimination can be obtained on the basis of the fatty acids that occur in low amounts, such as iso- $C_{15:0}$, $C_{16:0}$ and iso/anteiso- $C_{17:1}$. The dominance of anteiso methyl-branched fatty acids (anteiso- $C_{15:0}$ and to a lesser extent anteiso- $C_{17:0}$) in the profile of the new isolates is also found in all other species of *Brachybacterium*. A table of fatty acid profile data is available as supplementary data at <http://ijs.sgmjournals.org> – Table B.

The menaquinone profiles of the new isolates are only partially in agreement with those found for other *Brachybacterium* species. For taxon 1 (REP groups 1 and 2, 16S group 1), the principal menaquinone is MK-7 (mean $63.3 \pm 12.5\%$) with MK-8 present at a considerable level ($25.8 \pm 8.6\%$). In addition, minor amounts of the partially saturated menaquinone MK-7(H_2) are also present ($5.8 \pm 3.0\%$). The latter character has not been mentioned in the description of all other *Brachybacterium* species. In taxon 2 (REP group 3, 16S group 2), MK-7 occurs as the major menaquinone ($61.0 \pm 12.3\%$) in combination with substantial to approximately equal amounts of MK-8 ($34.4 \pm 9.7\%$). The profiles of four isolates, contain minor amounts of MK-9 ($1.2 \pm 0.4\%$) and two of these isolates also contain minor amounts of MK-7(H_2) (both 3%).

Further analyses were performed on the three mural painting strains of which the DNA–DNA relatedness was determined. The amino acids present in all cell wall hydrolysates of these strains were *meso*-diaminopimelic acid (*m*-Dpm), alanine, glycine and larger amounts of glutamic acid. The peptides D-Ala $\rightarrow$ D-Glu $\rightarrow$ D-Glu and D-Glu $\rightarrow$ Gly were found in the partial hydrolysate (data not shown). From these results the peptidoglycan type A4 γ *m*-Dpm $\leftarrow$ D-Glu $\leftarrow$ D-Glu with glycine bound to the α -carboxyl group of D-Glu at position 2 of the peptide subunit (type A31.2, *DSMZ Catalogue of Strains*, 7th edn, 2001) was concluded. The three isolates studied share this peptidoglycan type with *B. faecium*, *B. rhamnosum*, *B. conglomeratum* and *B. paraconglomeratum*. The peptidoglycan structure differentiates the isolates from *B. nesterenkovi* that lacks glycine, and from *B. alimentarium* and *B. tyrofermentans* that both contain aspartic acid. The strains do not contain mycolic acids and the muramic acid residues of the peptidoglycan are *N*-glycolylated, which further supports the affiliation to *Brachybacterium*. Strains LMG 20336^T and LMG 20345^T correspond in displaying phosphatidyl-

glycerol, diphosphatidylglycerol and one unidentified glycolipid.

Further testing showed that both novel taxa tolerate high levels of NaCl in their growth medium, with optimal growth from 2.5 to 10% NaCl and weak growth at 15%. This is not unexpected since halotolerant and halophilic bacteria belonging to different genera have been reported to occur on deteriorated monuments (Saiz-Jimenez & Laiz, 2000). Also, several other *Brachybacterium* species have a high level of tolerance to salt (Takeuchi *et al.*, 1995; Schubert *et al.*, 1996). The two novel taxa can be easily distinguished from each other on the basis of gelatinase and urease activity. Other discriminative characteristics between all present species of *Brachybacterium* are given in Table 1.

Description of *Brachybacterium fresconis* sp. nov.

Brachybacterium fresconis [fres.co'nis. N.L. gen. n. *fresconis*, pertaining or belonging to fresco(es)].

Cells vary in shape from coccoid forms (single or in agglomerates) in the stationary phase, to short rods in the exponential phase. Cells are non-motile, do not form endospores and stain Gram-positive. Colonies on MA after 24 h are small (< 1 mm), cream-coloured, smooth, glistening, circular in outline and low convex. No growth in an anaerobic chamber on MA. The temperature range for growth is 5–40 °C, with optimal growth between 20 and 30 °C. The NaCl concentration range for growth is 0–15% (w/v). Grows at pH 5. Catalase and urease are produced, oxidase is not produced. Aesculin and gelatin are hydrolysed, starch is not hydrolysed. Nitrate is reduced to nitrite. Indole is not produced. Arginine dihydrolase-negative and β -galactosidase-positive. Assimilation of arabinose, glucose, maltose and mannose is positive; caprate, citrate and malate are not assimilated. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid and is of the type A4 γ (*m*-Dpm $\leftarrow$ D-Glu $\leftarrow$ D-Glu) with glycine bound to the α -carboxyl group of D-glutamic acid at position 2 of the peptide subunit. Muramic acid residues are glycolylated. Phosphatidylglycerol, diphosphatidylglycerol and one unidentified glycolipid were found as polar lipids. Mycolic acids are absent. The principal menaquinone is MK-7, MK-8 is present in substantial amounts and MK-7(H_2) in minor amounts. The main fatty acid is anteiso- $C_{15:0}$. The type strain produced acid from D-fructose, D-glucose, DL-lactose, maltose, D-mannose and sucrose, but not from D-arabinose, L-arabinose, D-galactose, D-melezitose and D-sorbitol. The G+C content is 70.4 mol% for the type strain. The type strain was deposited in the BBCM/LMG as LMG 20336^T (= DSM 14564^T). The 16S rRNA sequence of this strain is deposited at EMBL under accession number AJ415378.

Description of *Brachybacterium sacelli* sp. nov.

Brachybacterium sacelli (sa.cel'li. L. gen. n. *sacelli*, of the chapel).

Table 1. Distinguishing characteristics between the two novel taxa and species in the genus *Brachybacterium*

B. fr. [*B. fresconis* (LMG 20333, 20335–20337)] and *B. s.* [*B. sacelli* (LMG 20338–20346)] data from this study given as a percentage (percentages of 89 and 11 are given as positive or negative, respectively, in the species description) except for acid production and G + C content that apply only to the respective type strains LMG 20336^T and LMG 20345^T; *B. fa.* (*B. faecium*), data from Collins *et al.* (1988) and Takeuchi *et al.* (1995); *B. n.* (*B. nesterenkovi*), data from Gvozdyak *et al.* (1992) and Takeuchi *et al.* (1995); *B. c.* (*B. conglomeratum*), *B. p.*, (*B. paraconglomeratum*) and *B. r.* (*B. rhamnosum*), data from Takeuchi *et al.* (1995); *B. a.* (*B. alimentarium*) and *B. t.* (*B. tyrofermentans*), data from Schubert *et al.* (1996). +, Positive; +w, weakly positive; v, variable; –, negative; ND, not determined.

Characteristic	<i>B. fr.</i> (n = 4)	<i>B. s.</i> (n = 9)	<i>B. fa.</i>	<i>B. n.</i>	<i>B. c.</i>	<i>B. p.</i>	<i>B. r.</i>	<i>B. a.</i>	<i>B. t.</i>
Growth in the presence of:									
7.5 % NaCl	100	100	+	+w	+	+	+	+	+
10 % NaCl	100	100	+w	–	+w	+w	+w	+	+
15 % NaCl	100w	100w	+w	–	+w	+w	–	+w	+
Growth at pH 5.0	100	100	–	+	v	–	+	ND	ND
Hydrolysis of gelatin	100	0	–	–	–	–	+	+	+
Urease production	100	0	–	–	+	+	+	–	–
Assimilation of:									
Mannose	100	100	–	–	ND	ND	ND	+	+
Mannitol	75	100	–	–	ND	ND	ND	+	+
N-Acetylglucosamine	25	100	–	–	ND	ND	ND	+	+
Gluconate	75	100	–	–	ND	ND	ND	+	+
Acid production from:									
D-Fructose	+	+	ND	v	v	+	+	–	–
DL-Lactose	+	+	ND	+	v	+w	–	–	–
Maltose	+	+	+	+	v	+	+	–	–
D-Mannose	+	+	+	–	v	+	+	ND	ND
Sucrose	+	+	v	v	v	+	+	–	–
G + C content of the type strain (mol %)	70.4	70.3	69.4	70.0	70.6	68.6	71.5	73.0	73.0

Cells vary in shape from coccoid forms (single or in agglomerates) in the stationary phase, to short rods in the exponential phase. Cells are non-motile, do not form endospores and stain Gram-positive. Colonies on MA after 24 h are small (< 1 mm), cream-coloured, smooth, glistening, circular in outline and low convex. No growth in an anaerobic chamber on MA. The temperature range for growth is 5–40 °C, with optimal growth between 20 and 30 °C. The NaCl concentration range for growth is 0–15% (w/v). Grows at pH 5. Catalase is produced, oxidase and urease are not produced. Aesculin is hydrolysed, gelatin and starch are not hydrolysed. Nitrate is reduced to nitrite. Indole is not produced. Arginine dihydrolase-negative and β -galactosidase-positive. Strains assimilate gluconate, maltose, mannose, mannitol and N-acetylglucosamine, but not adipate, arabinose, caprate, citrate, glucose and malate. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid and is of the type A4 γ (*m*-Dpm $\leftarrow$ D-Glu $\leftarrow$ D-Glu) with glycine bound to the α -carboxyl group of D-glutamic acid at position 2 of the peptide subunit. Muramic acid residues are glycosylated. Phosphatidylglycerol, diphosphatidylglycerol and one unidentified glycolipid were found as polar lipids. Mycolic acids are absent. The principal menaquinone is MK-7 in combination with either MK-8 in lower but still considerable amounts, or in approximately equal amounts. MK-7(H₂) or MK-9 are present

in minor amounts in some strains. The main fatty acid is anteiso-C_{15:0}. The type strain produced acid from D-fructose, D-glucose, DL-lactose, maltose, D-mannose and sucrose, but not from D-arabinose, L-arabinose, D-galactose, D-melezitose and D-sorbitol. The G + C content is 70.3 mol% for the type strain. The type strain was deposited in the BCCM/LMG as LMG 20345^T (= DSM 14566^T). The 16S rRNA sequence of this strain is deposited at EMBL under accession number AJ415384.

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