International Journal of Systematic and Evolutionary Microbiology (2009), **59**, 3105–3110 DOI 10.1099/ijs.0.008664-0

Hoyosella altamirensis gen. nov., sp. nov., a new member of the order *Actinomycetales* isolated from a cave biofilm

Valme Jurado,[1] Reiner M. Kroppenstedt,[2] Cesáreo Saiz-Jimenez,[1] Hans-Peter Klenk,[2] Delphine Mouniée,[3,4] Leonila Laiz,[1] Andrée Couble,[3,4] Gabriele Pötter,[2] Patrick Boiron[3,4] and Verónica Rodríguez-Nava[3,4]

Correspondence
Verónica Rodríguez-Nava
veronica.rodriguez@sante.univ-lyon1.fr

[1]Instituto de Recursos Naturales y Agrobiología, CSIC, Apartado 1052, E-41080 Sevilla, Spain

[2]Deutsche Sammlung von Mikroorganismen und Zellkulturen, D-38124 Braunschweig, Germany

[3]Université de Lyon and UMR CNRS 5557, Center for Microbial Ecology, Opportunistic Pathogens Bacterial and Environment Research Group, French Observatory for Nocardiosis, Faculté de Pharmacie, Université Lyon I, F-69622 Villeurbanne, France

[4]Ecole Nationale Vétérinaire de Lyon, F-69280 Marcy l'Etoile, France

A novel actinomycete, strain OFN S31^T, was isolated from a complex biofilm in the Altamira Cave, Spain. A polyphasic study was carried out to clarify the taxonomic position of this strain. Phylogenetic analysis with 16S rRNA gene sequences of representatives of the genera *Corynebacterium*, *Dietzia*, *Gordonia*, *Millisia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Segniliparus*, *Skermania*, *Tsukamurella* and *Williamsia* indicated that strain OFN S31^T formed a distinct taxon in the 16S rRNA gene tree that was more closely associated with the *Mycobacterium* clade. The type strain of *Mycobacterium fallax* was the closest relative of strain OFN S31^T (95.6 % similarity). The cell wall contained *meso*-diaminopimelic acid, arabinose and galactose, which are characteristic components of cell-wall chemotype IV of actinomycetes. The sugars of the peptidoglycan were acetylated. The polar lipid pattern was composed of phosphatidylinositol, phosphatidylglycerol, phosphatidylethanolamine and diphosphatidylglycerol. Strain OFN S31^T is characterized by the absence of mycelium and mycolic acids. Strain OFN S31^T had MK-8 as the major menaquinone. The DNA G+C content was 49.3 mol%, the lowest found among all taxa included in the suborder *Corynebacterineae*. Based on morphological, chemotaxonomic, phenotypic and genetic characteristics, strain OFN S31^T is considered to represent a novel species of a new genus, for which the name *Hoyosella altamirensis* gen. nov., sp. nov. is proposed. The type strain of *Hoyosella altamirensis* is strain OFN S31^T (=CIP 109864^T =DSM 45258^T).

A number of novel bacterial taxa have been isolated from caves and described in recent years. The new genera and species described belong mainly to the suborders *Corynebacterineae*, *Pseudonocardineae* and *Micrococcineae* (Lee *et al.*, 2000, 2001; Lee, 2006a, b; Seo *et al.*, 2007; Jurado *et al.*, 2005a, b; Groth *et al.*, 1999, 2002; Margesin *et al.*, 2004; Schumann *et al.*, 2004). White colonies are widespread in Altamira Cave, Cantabria, Spain, from the entrance as far as the Polychromes Hall, in a transect of less than 100 m. Analysis of this microbial community has revealed that the colonies are formed by a consortium of more than 30 different species, as detected by denaturing gradient gel electrophoresis, many of them uncultured and unidentified (Schabereiter-Gurtner *et al.*, 2002; Gonzalez *et al.*, 2006). Recently, some novel species have been described from these white colonies, such as *Aurantimonas altamirensis* (Jurado *et al.*, 2006) and *Nocardia altamirensis* (Jurado *et al.*, 2008). In this work, a polyphasic approach was used to determine the taxonomic position of bacterial strain OFN S31^T. This strain was collected as a member of the complex microbial community that produces the white colonies, isolated or in masses of hundreds on the cave ceiling and walls.

Strain OFN S31^T was isolated on starch-casein agar at 28 °C but also grew on brain heart infusion (BHI; Difco), Bennett's agar (Jones, 1949) and trypticase soy agar (TSA;

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain OFN S31^T is FJ179485.

The cellular fatty acid profile of strain OFN S31^T is available as supplementary material with the online version of this paper.

MacFaddin, 1985). The growth temperature was tested in the range 10–46 °C. The strain grew on Bennett's agar at 20–37 °C, with optimum growth at 28 °C. The colonies were circular, smooth and cream in colour, with a diameter of 1–2 mm after 3 weeks on Bennett's medium at 28 °C. Tolerance of NaCl was studied on Bennett's agar supplemented with 0–10 % (w/v) NaCl. Growth occurred with 6 % NaCl, although the optimum NaCl concentration for the growth of strain OFN S31^T was 2–4 % NaCl. This differs from the phylogenetically and chemotaxonomically closest strains (Table 1). Strain OFN S31^T was slightly acid–alcohol-fast in a modified Ziehl–Neelsen test (1 % acid decoloration) (Boiron *et al.*, 1993). For determination of cell morphology, samples were fixed in 2.5 % (w/v) glutaraldehyde (0.1 M cacodylate buffer, pH 7.2–7.4). After 1 h of fixation, the cells were washed three times in cacodylate buffer and post-fixed for 1 h in 1 % (w/v) osmium tetroxide, dehydrated in a graded ethanol series and substituted with acetone. Finally, the samples were sputter-coated with gold in a sputter coater (Edwards Scancoat Six) and observed using a scanning electron microscope (SEM; Philips XL-30). Colony morphology of 3- and 14-day-old cultures was studied using a stereo microscope. Cells were spherical (0.7–1.3 μm in diameter) and occurred singly, in pairs, in tetrads or in small clumps (Fig. 1). Cells were not motile and were non-spore-forming.

Table 1. Physiological characteristics that can be used to differentiate strain OFN S31^T from its closest relatives

Strains: 1, strain OFN S31^T; 2, *M. fallax* DSM 44179^T; 3, *C. amycolatum* DSM 6922^T; 4, *C. kroppenstedtii* DSM 44385^T; 5, *T. otitidis* DSM 8821^T. Data were obtained in this study. All strains were negative for acid production from xylose, D-mannitol, lactose and glycogen, hydrolysis of gelatin and activities of arylsulfatase, β-glucuronidase and β-galactosidase and positive for catalase activity. −, Negative; +, positive; V, variable; ND, not determined.

Characteristic	1	2	3	4	5
Growth in the presence of NaCl at:					
5 % (w/v)	+	−	+	+	+
7 % (w/v)	−	−	+	+	ND
Growth on carbon sources (at 1.0 % w/v)					
L-Rhamnose	+	−	+	+	−
myo-Inositol	+	−	−	−	−
D-Galactose	−	V	−	−	−
D-Sorbitol	+	V	+	+	ND
Maltose	+	+	+	V	V
Sucrose	+	+	V	−	V
Glucose	+	+	+	+	V
Acid production from:					
D-Glucose	−	−	+	+	−
D-Ribose	−	−	+	−	−
Maltose	−	−	+	+	−
Sucrose	−	−	+	−	−
Nitrate reduction	−	+	−	−	−
Decomposition or hydrolysis of:					
Aesculin	+	−	−	+	−
Urea	+	−	+	−	−
Enzyme activities					
Alkaline phosphatase	+	−	+	+	+
α-Glucosidase	+	−	−	−	−
N-Acetyl-β-glucosaminidase	+	−	−	−	−
Pyrazinamidase	−	−	−	+	+
Pyrrolidonyl arylamidase	−	−	+	−	−

Phenotypic properties were determined for strain OFN S31^T and for the following type strains: *Mycobacterium fallax* DSM 44179^T, *Corynebacterium amycolatum* DSM 6922^T, *Corynebacterium kroppenstedtii* DSM 44385^T and *Turicella otitidis* DSM 8821^T. All physiological tests were performed at 28 °C. Oxidase activity was determined by monitoring the oxidation of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine on filter paper (Steel, 1961). Catalase production was shown by the production of bubbles after a suspension of the cells was mixed with a drop of a 3 % hydrogen peroxide solution on a slide. To determine decomposition of casein, hypoxanthine, xanthine, uric acid and testosterone, the utilization of substrates as carbon sources and arylsulfatase production, we used techniques described by Boiron *et al.* (1993), Goodfellow & Lechevalier (1989) and Goodfellow (1992, 1998). Testosterone is hydrolysed by OFN S31^T but casein, hypoxanthine, uric acid and xanthine are not. Other physiological results are summarized in Table 1, revealing several differences between strain OFN S31^T and the closest strains.

Antibiotic susceptibility patterns were determined by using the disc-diffusion method on Mueller–Hinton medium according to the criteria of the Comité de l'Antibiogramme de la Société Française de Microbiologie (Cavallo *et al.*, 2008). Mueller–Hinton agar plates were inoculated with a final inoculum of approximately 10^5 c.f.u. ml^{-1} and then antibiotic discs were applied. Diameters of zones of growth inhibition (mm) were recorded after 72 h of incubation at 28 °C. Strain OFN S31^T was susceptible to ampicillin

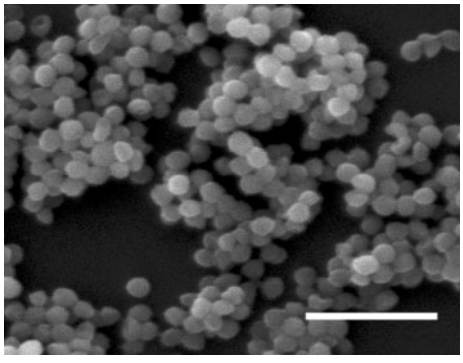

Fig. 1. Scanning electron micrograph of cells of strain OFN S31^T. Bar, 5 μm.

(10 μg), amoxicillin (25 μg), amoxicillin/clavulanic acid (20/10 μg), imipenem (10 μg), gentamicin (10 μg), amikacin (30 μg), linezolid (30 μg), sulfamethoxazole/trimethoprim (1.25/23.75 μg), ciprofloxacin (5 μg), rifampicin (30 μg), cefalotin (30 μg) and ticarcillin (75 μg) and was resistant to trimethoprim (5 μg), ceftazidime (30 μg), cefamandole (30 μg) and clindamycin (15 IU).

A fragment [around 1333 nt; positions 46–1400 according to the *Escherichia coli* numbering (Brosius *et al.*, 1978)] of the 16S rRNA gene of strain OFN S31^T was amplified by using primers SQ1 and SQ6 as described previously by Rodríguez-Nava *et al.* (2004). The PCR products were purified by using a Microspin gel extraction kit (Omega; Bio-tek) and sequenced by using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) in an Applied Biosystems model 373A DNA sequencer.

The sequence determined was aligned with those of phylogenetically close reference strains obtained from the GenBank (16S rRNA) or Bioinformatic Bacterial Identification (BIBI) database (Devulder *et al.*, 2003) by using the program CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.*, 2007) and PHYLO_WIN (Galtier *et al.*, 1996) with three treeing algorithms, the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) methods. 16S rRNA gene sequence analysis revealed that strain OFN S31^T was distinct from all cultured members of the suborder *Corynebacterineae*. The closest relative of strain OFN S31^T was *Mycobacterium fallax* ATCC 35219^T, with only 95.6 % sequence similarity.

A sequence deposited in the GenBank database under the accession number EF564379 (Y.-N. Wang and X.-L. Wu, unpublished) as *Mycobacterium* sp. DQS39A1 revealed 99.8 % similarity to the 16S rRNA gene sequence of strain OFN S31^T. This sequence belongs to a strain isolated from an oil-polluted soil, suggesting that strains of the species represented by strain OFN S31^T are present in different environments.

The 16S rRNA gene sequence of strain OFN S31^T showed highest similarity to those of type strains of the suborder *Corynebacterineae*. The highest value of 95.6 % (58 differences) was found to *M. fallax* ATCC 35219^T. The similarity values are too low to assign OFN S31^T to any of the described taxa of the suborder *Corynebacterineae*. According to the phylogenetic tree shown in Fig. 2, strain OFN S31^T formed a distinct subclade close to the genus *Mycobacterium*, indicating that this isolate is not closely related at the 16S rRNA gene sequence level to any previously described taxa, so could represent a new genus.

The 16S rRNA gene sequence of strain OFN S31^T showed the same pattern of signature nucleotides as that published by Stackebrandt *et al.* (1997) for the families belonging to the suborder *Corynebacterineae* (*Corynebacteriae*, *Dietziaceae*, *Gordoniaceae*, *Mycobacteriaceae*, *Nocardiaceae* and *Tsukamurellaceae*) in their hierarchic classification system of the class *Actinobacteria* based on phylogenetic analyses of the 16S rRNA gene.

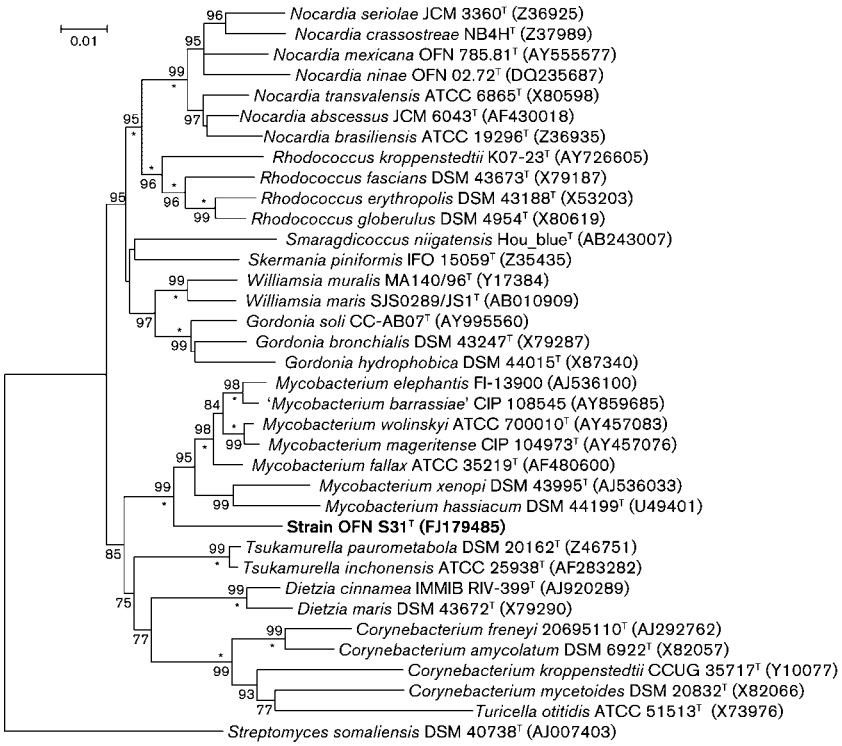

Fig. 2. Phylogenetic tree derived from 16S rRNA gene sequences showing the relationships between strain OFN S31^T and species belonging to the suborder *Corynebacterineae*. The tree was constructed by using the neighbour-joining method and was based on a comparison of 1333 nucleotides. Bootstrap values are expressed as percentages of 1000 replications. Asterisks indicate branches of the tree that were also recovered using maximum-likelihood and maximum-parsimony treeing algorithms. Bar, 0.01 substitutions per nucleotide position.

Hypervariable regions were identified in the 16S rRNA gene sequence between strain OFN S31^T and the species of the genus *Mycobacterium*, the genus that is most closely related genotypically. These hypervariable regions covered positions 73–160, 210–315, 469–858 and 1015–1340 according to the *E. coli* sequence numbering (Brosius *et al.*, 1978).

The isomer of diaminopimelic acid was analysed by TLC of whole-organism hydrolysates as described by Boiron *et al.* (1993). Whole-cell sugar analyses were performed following the methods described previously (Staneck & Roberts, 1974). The acyl type of the peptidoglycan was determined by a modification of the colorimetric method of Uchida & Aida (1977). In contrast to the original procedure, our whole-cell hydrolysate was neutralized by passing it through an ion-exchange column (Analytichem Bond Elut SCX; Varian).

Standard procedures for the analyses of fatty acids by gas chromatography were adopted with the Microbial Identification System (MIDI, Inc.) for automated GC analyses (Kroppenstedt, 1985). Mycolic acids and isoprenoid quinones were separated by HPLC (Minnikin *et al.*, 1975; Kroppenstedt, 1982) and polar lipids were extracted and analysed by TLC using the integrated method described by Minnikin *et al.* (1984).

Analysis of whole-cell hydrolysates revealed *meso*-diaminopimelic, arabinose and galactose, consistent with an arabinogalactan polymer wall (chemotype IV) characteristic of members of the suborder *Corynebacterineae*. The sugars of the peptidoglycan are acetylated. This type of peptidoglycan is found only in members of three other genera of this suborder, namely *Corynebacterium*, *Dietzia* and *Turicella*. While phylogenetic data suggested that strain OFN S31^T was related to the genus *Mycobacterium*, the fact that the strain lacks mycolic acids indicates that it is related to *C. amycolatum*, *C. kroppenstedtii* and *T. otitidis* (Liebl, 2006) (Table 2).

The fatty acid pattern was composed mainly of $C_{16:0}$ (26 %), $C_{18:1}\omega9$ (20 %) and $C_{17:1}\omega9$ (13 %) (Supplementary Table S1, available in IJSEM Online). $C_{18:0}$ was present in smaller amounts for strain OFN S31^T compared with *M. fallax*, *C. amycolatum*, *C. kroppenstedtii* and *T. otitidis*. Another difference was the presence of tuberculostearic acid in strain OFN S31^T and its absence from *C. amycolatum*.

The menaquinone pattern revealed that MK-8 (96.7 %) was the principal menaquinone and MK-9 was present at 3.3 %. This feature distinguished strain OFN S31^T from *M. fallax*, *C. amycolatum* and *T. otitidis* and related it to *C. kroppenstedtii*, in which MK-8 was the major menaquinone.

The polar lipid pattern was composed of phosphatidylinositol, phosphatidylglycerol, phosphatidylethanolamine and diphosphatidylglycerol. The occurrence of the diagnostic phosphatidylethanolamine in the phospholipid

Table 2. Chemotaxonomic markers of OFN S31^T and other genera of the suborder *Corynebacterineae*

Taxa: 1, OFN S31^T; 2, *Nocardia*; 3, *Skermania*; 4, *Rhodococcus*; 5, *Gordonia*; 6, *Williamsia*; 7, *Mycobacterium*; 8, *Tsukamurella*; 9, *Dietzia*; 10, *Corynebacterium*; 11, *Turicella*; 12, *Smaragdicoccus*. All taxa contain saturated and unsaturated fatty acids. V, Variable; ND, no data available. Data for reference taxa were taken from Adachi *et al.* (2007).

Marker	1	2	3	4	5	6	7	8	9	10	11	12
Acyl type*	A	G	G	G	G	G	G	G	A	A	ND	G
Major menaquinone(s)	8	8(H_4,ω-cycl.)	8(H_4,ω-cycl.)	8(H_2)	9(H_2)	9(H_2)	9(H_2)	9	8(H_2)	8(H_2)†	10, 11	8(H_4,ω-cycl.), 8(H_4,ω-dicycl.)
Presence of:												
Phosphatidylethanolamine	+	+	+	+	+	+	+	+	–	V‡	ND	+
Tuberculostearic acid	+	+	+	+	+	+	+	+	+	V§	+	–
Mycolic acids (no. of carbons)	None	50–62	58–64	34–54	54–66	50–56	70–90	64–78	34–38	22–36‖	None	43–49
DNA G+C content (mol%)	49.3	64–72	68	63–73	63–69	64–65	70–72	67–68	73	51–67	65–72	64

*A, Acetyl; G, glycolyl.
†*C. amycolatum* synthesizes MK-9.
‡Present in *C. bovis* and *C. urealyticum* (Kämpfer *et al.*, 1999).
§Present in *C. ammoniagenes*, *C. bovis*, *C. minutissimum*, *C. urealyticum* and *C. variabilis* (Kämpfer & Kroppenstedt, 1996).
‖*C. amycolatum* and *C. kroppenstedtii* lack mycolic acids.

pattern of OFN S31^T separates this taxon from species of the genera *Corynebacterium*, *Dietzia* and *Turicella*, which lack this phospholipid.

Thus, this polyphasic taxonomic study clearly showed that isolate OFN S31^T could be distinguished readily from representatives of all phylogenetically related genera; therefore, it is concluded that this strain should be assigned to a new genus and species, for which we propose the name *Hoyosella altamirensis* gen. nov., sp. nov.

Description of *Hoyosella* gen. nov.

Hoyosella (Ho.yo.sel′la. N.L. fem. dim. n. *Hoyosella* named in honour of Dr Manuel Hoyos, a pioneer in research towards the protection of the Altamira Cave paintings).

Cells are non-motile, non-spore-forming and spherical and occur singly, in pairs, in tetrads or in small clumps. Gram-stain-positive, aerobic, catalase-positive and oxidase-negative. Colonies are circular, smooth and cream coloured. The sugars of the murein are acetylated. Mycolic acids are absent. The fatty acid pattern is composed mainly of $C_{16:0}$, $C_{18:1}\omega9$ and $C_{17:1}\omega9$. The major menaquinone is MK-8. The diagnostic phospholipid phosphatidylethanolamine is found, together with phosphatidylglycerol, diphosphatidylglycerol and diphosphatidylinositol. The DNA G+C content of the type strain of the type species is 49.3 mol%. Phylogenetically, the genus is a member of the suborder *Corynebacterineae*. The type species is *Hoyosella altamirensis*.

Description of *Hoyosella altamirensis* sp. nov.

Hoyosella altamirensis [al.ta.mi.ren′sis. N.L. fem. adj. *altamirensis* referring to Altamira Cave (Cantabria, Spain), where the type strain was isolated].

Displays the following properties in addition to those given for the genus. Cells are 0.7–1.2 μm in diameter. Colonies are about 1 mm in diameter. Slightly acid–alcohol-fast. Grows at 20–37 °C (optimum 28 °C) and in the presence of up to 6 % NaCl (optimal growth with 2–4 % NaCl). Good growth on BHI and TSA media. Phenotypic characteristics are reported in Table 1. Shows high susceptibilities to some antibiotics tested, for example penicillins, aminosides and some cephalosporins; resistance is observed to trimethoprim, ceftazidime, cefamandole and clindamycin.

The type strain, OFN S31^T (=CIP 109864^T =DSM 45258^T), was isolated from a white colony in Altamira Cave, Spain.

Acknowledgements

We thank Peter Schumann for performing the analyses of G+C content and J. P. Flandrois for technical contributions. This work was supported by the Centre National de la Recherche Scientifique (CNRS). The authors acknowledge funding from the Spanish Ministry of Culture for the microbiological study of Altamira Cave and from the Spanish Ministry of Education and Science, project CGL2006-07424. The facilities provided by the Museum of Altamira are acknowledged.

References

Adachi, K., Katsuta, A., Matsuda, S., Peng, X., Misawa, N., Shizuri, Y., Kroppenstedt, R. M., Yokota, A. & Kasai, H. (2007). *Smaragdicoccus niigatensis* gen. nov., sp. nov., a novel member of the suborder *Corynebacterineae*. *Int J Syst Evol Microbiol* **57**, 297–301.

Boiron, P., Provost, F. & Dupont, B. (1993). Technical protocols. In *Méthodes de Laboratoire pour le Diagnostic de la Nocardiose*, pp. 107–126. Edited by Institut Pasteur. Paris: Institut Pasteur.

Brosius, J., Palmer, M. L., Poindexter, J. K. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci U S A* **75**, 4801–4805.

Cavallo, J. D., Chardon, H., Chidiac, C., Courvalin, P., Dabernat, H., Drugeon, H., Dubreuil, L., Goldstein, F., Guery, B. & other authors (2008). *Recommandations du CASFM*. Paris: Comité de l'antibiogramme de la Société Française de Microbiologie. http://www.sfm.asso.fr/

Devulder, G., Pierre, G., Baty, F. & Flandrois, J. P. (2003). BIBI, a bioinformatics bacterial identification tool. *J Clin Microbiol* **41**, 1785–1787.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.

Galtier, N., Gouy, M. & Gautier, C. (1996). SeaView and PHYLO_WIN, two graphic tools for sequence alignment and molecular phylogeny. *Comput Appl Biosci* **12**, 543–548.

Gonzalez, J. M., Portillo, M. C. & Saiz-Jimenez, C. (2006). Metabolically active Crenarchaeota in Altamira Cave. *Naturwissenschaften* **93**, 42–45.

Goodfellow, M. (1992). The family *Nocardiaceae*. In *The Prokaryotes*, 2nd edn, pp. 1188–1213. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York, Springer.

Goodfellow, M. (1998). The genus *Nocardia* Trevisan 1889. In *Topley & Wilson's Microbiology & Microbial Infections*, 9th edn, pp. 464–489. Edited by A. Balows & B. I. Duerden. London: Edward Arnold.

Goodfellow, M. & Lechevalier, M. P. (1989). Genus *Nocardia* Trevisan 1889, 9^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 4, pp. 2350–2361. Edited by S. T. Williams, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

Groth, I., Schumann, P., Schuetze, B., Augsten, K., Kramer, I. & Stackebrandt, E. (1999). *Beutenbergia cavernae* gen. nov., sp. nov., an L-lysine-containing actinomycete isolated from a cave. *Int J Syst Bacteriol* **49**, 1733–1740.

Groth, I., Schumann, P., Schuetze, B., Augsten, K. & Stackebrandt, E. (2002). *Knoellia sinensis* gen. nov., sp. nov. and *Knoellia subterranea* sp. nov., two novel actinobacteria isolated from a cave. *Int J Syst Evol Microbiol* **52**, 77–84.

Jones, K. L. (1949). Fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelia is a fluctuating characteristic. *J Bacteriol* **57**, 141–145.

Jurado, V., Groth, I., Gonzalez, J. M., Laiz, L. & Saiz-Jimenez, C. (2005a). *Agromyces salentinus* sp. nov. and *Agromyces neolithicus* sp. nov. *Int J Syst Evol Microbiol* **55**, 153–157.

Jurado, V., Groth, I., Gonzalez, J. M., Laiz, L. & Saiz-Jimenez, C. (2005b). *Agromyces subbeticus* sp. nov., isolated from a cave in southern Spain. *Int J Syst Evol Microbiol* **55**, 1897–1901.

Jurado, V., Gonzalez, J. M., Laiz, L. & Saiz-Jimenez, C. (2006). *Aurantimonas altamirensis* sp. nov., a member of the order *Rhizobiales* isolated from Altamira Cave. *Int J Syst Evol Microbiol* **56**, 2583–2585.

Jurado, V., Boiron, P., Kroppenstedt, R. M., Laurent, F., Couble, A., Laiz, L., Klenk, H.-P., Gonzalez, J. M., Saiz-Jimenez, C. & other authors (2008). *Nocardia altamirensis* sp. nov., isolated from Altamira Cave, Cantabria, Spain. *Int J Syst Evol Microbiol* **58**, 2210–2214.

Kämpfer, P. & Kroppenstedt, R. M. (1996). Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.

Kämpfer, P., Andersson, M. A., Rainey, F. A., Kroppenstedt, R. M. & Salkinoja-Salonen, M. (1999). *Williamsia muralis* gen. nov., sp. nov., isolated from the indoor environment of a children's day care centre. *Int J Syst Bacteriol* **49**, 681–687.

Kluge, A. G. & Farris, J. S. (1969). Quantitative phyletics and the evolution of anurans. *Syst Zool* **18**, 1–32.

Kroppenstedt, R. M. (1982). Separation of bacterial menaquinones by HPLC using reverse phase (RP18) and a silver loaded ion exchanger as stationary phases. *J Liq Chromatogr* **5**, 2359–2367.

Kroppenstedt, R. M. (1985). Fatty acid and menaquinone analysis of actinomycetes and related organisms. In *Chemical Methods in Bacterial Systematics* (Society for Applied Bacteriology Technical Series vol. 20), pp. 173–199. Edited by M. Goodfellow & D. E. Minnikin. New York: Academic Press.

Lee, S. D. (2006a). *Amycolatopsis jejuensis* sp. nov. and *Amycolatopsis halotolerans* sp. nov., novel actinomycetes isolated from a natural cave. *Int J Syst Evol Microbiol* **56**, 549–553.

Lee, S. D. (2006b). *Nocardia jejuensis* sp. nov., a novel actinomycete isolated from a natural cave on Jeju Island, Republic of Korea. *Int J Syst Evol Microbiol* **56**, 559–562.

Lee, S. D., Kim, E. S., Roe, J. H., Kim, J., Kang, S. O. & Hah, Y. C. (2000). *Saccharothrix violacea* sp. nov., isolated from a gold mine cave, and *Saccharothrix albidocapillata* comb. nov. *Int J Syst Evol Microbiol* **50**, 1315–1323.

Lee, S. D., Kim, E. S., Min, K. L., Lee, W. Y., Kang, S. O. & Hah, Y. C. (2001). *Pseudonocardia kongjuensis* sp. nov., isolated from a gold mine cave. *Int J Syst Evol Microbiol* **51**, 1505–1510.

Liebl, W. (2006). *Corynebacterium* – nonmedical. In *The Prokaryotes. A Handbook on the Biology of Bacteria*, 3rd edn, vol. 3, pp. 796–818. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. New York: Springer.

MacFaddin, J. F. (1985). *Media for Isolation–Cultivation–Identification–Maintenance of Medical Bacteria*, vol. 1. Baltimore: Williams & Wilkins.

Margesin, R., Schumann, P., Spröer, C. & Gounot, A. M. (2004). *Arthrobacter psychrophenolicus* sp. nov., isolated from an alpine ice cave. *Int J Syst Evol Microbiol* **54**, 2067–2072.

Minnikin, D. E., Alshamaony, L. & Goodfellow, M. (1975). Differentiation of *Mycobacterium*, *Nocardia*, and related taxa by thin-layer chromatographic analyses of whole-cell methanolysates. *J Gen Microbiol* **88**, 200–204.

Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A. & Parlett, J. H. (1984). An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* **2**, 233–241.

Rodríguez-Nava, V., Couble, A., Molinard, C., Sandoval, H., Boiron, P. & Laurent, F. (2004). *Nocardia mexicana* sp. nov., a new pathogen isolated from human mycetomas. *J Clin Microbiol* **42**, 4530–4535.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Schabereiter-Gurtner, C., Saiz-Jimenez, C., Piñar, G., Lubitz, W. & Rölleke, S. (2002). Altamira cave Paleolithic paintings harbor partly unknown bacterial communities. *FEMS Microbiol Lett* **211**, 7–11.

Schumann, P., Cui, X., Stackebrandt, E., Kroppenstedt, R. M., Xu, L. & Jiang, C. (2004). *Jonesia quinghaiensis* sp. nov., a new member of the suborder *Micrococcineae*. *Int J Syst Evol Microbiol* **54**, 2181–2184.

Seo, J. P., Yun, Y.-W. & Lee, S. D. (2007). *Nocardia speluncae* sp. nov., isolated from a cave. *Int J Syst Evol Microbiol* **57**, 2932–2935.

Stackebrandt, E., Rainey, F. A. & Ward-Rainey, N. L. (1997). Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *Int J Syst Bacteriol* **47**, 479–491.

Staneck, J. L. & Roberts, G. D. (1974). Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* **28**, 226–231.

Steel, K. J. (1961). The oxidase reaction as a taxonomic tool. *J Gen Microbiol* **25**, 297–306.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Uchida, K. & Aida, K. (1977). Acyl type of bacterial cell wall: its simple identification by a colorimetric method. *J Gen Appl Microbiol* **23**, 249–260.