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

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A novel actinomycete, strain OFN S31T, 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 S31T 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 S31T (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 S31T is characterized by the absence of mycelium and mycolic acids. Strain OFN S31T 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 S31T 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 S31T (5CIP 109864T =DSM 45258T).

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 S31T. 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 S31T 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;...
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 S31T was 2–4 % NaCl. This differs from the phylogenetically and chemotaxonomically closest strains (Table 1). Strain OFN S31T 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 S31T from its closest relatives

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

Phenotypic properties were determined for strain OFN S31T and for the following type strains: Mycobacterium fallax DSM 44179T, Corynebacterium amycolatum DSM 6922T, Corynebacterium kroppenstedtii DSM 44385T and Turicella otitidis DSM 8821T. 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 aрыlsulfatase production, we used techniques described by Boiron et al. (1993), Goodfellow & Lechevalier (1989) and Goodfellow (1992, 1998). Testosterone is hydrolysed by OFN S31T but casein, hypoxanthine, uric acid and xanthine are not. Other physiological results are summarized in Table 1, revealing several differences between strain OFN S31T 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 10⁵ 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 S31T was susceptible to ampicillin.
(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), ceftazidine (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 was amplified by using primers SQ1 and SQ6 as described previously by Rodriguez-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 was distinct from all cultured members of the suborder Corynebacterineae. The closest relative of strain OFN S31 was Mycobacterium fallax ATCC 35219, 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. DQ539A1 revealed 99.8 % similarity to the 16S rRNA gene sequence of strain OFN S31. This sequence belongs to a strain isolated from an oil-polluted soil, suggesting that strains of the species represented by strain OFN S31 are present in different environments.

The 16S rRNA gene sequence of strain OFN S31 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. The similarity values are too low to assign OFN S31 to any of the described taxa of the suborder Corynebacterineae. According to the phylogenetic tree shown in Fig. 2, strain OFN S31 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 showed the same pattern of signature nucleotides as that published by Stackebrandt et al. (1997) for the families belonging to the suborder Corynebacterineae (Corynebacterineae, Dietziaceae, Gordoniaceae, Mycobacteriaceae, Nocardiaceae and Tsukamurellaceae) in their hierarchical classification system of the class Actinobacteria based on phylogenetic analyses of the 16S rRNA gene.

![Phylogenetic tree derived from 16S rRNA gene sequences showing the relationships between strain OFN S31 and species belonging to the suborder Corynebacterineae.](http://ijs.sgmjournals.org)
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_v9 (20 %) and C_17:1_v9 (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*

<table>
<thead>
<tr>
<th>Marker</th>
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<th>10</th>
<th>11</th>
<th>12</th>
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</thead>
<tbody>
<tr>
<td>Acyl type*</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Major menaquinone(s)</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>(H4, v-cycl.)</td>
<td>8</td>
<td>(H4, v-cycl.)</td>
<td>8</td>
<td>(H2)</td>
<td>9</td>
<td>(H2)</td>
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<td>(H2)</td>
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<tr>
<td>Presence of Phosphatidyl ethanolamine</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Tuberculostearic acid</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>49.3</td>
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</table>

*Present in *C. ammonigenes*, *C. bovis*, *C. minutissimum*, *C. urealyticum* and *C. variabilis* (Kämpfer & Kroppenstedt, 1996).

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

pattern of OFN S31T 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 S31T 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 present, together with phosphatidylglycerol, diphosphatidylglycerol and diphosphatidylmycolate. The DNA G+C content of the type strain of the type species is 49.3 mol%. Phyleogenetically, 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. Colonies are flat and white. Slightly acid–alcohol-fast. 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 S31T (= CIP 109864T = DSM 45258T), was isolated from a white colony in Altamira Cave, Spain.

Acknowledgements

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