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Streptomyces silvae sp. nov., isolated from forest soil

Ludovic Besauary^{1,*}, Lucas Martinet¹, Estelle Mühle², Dominique Clermont² and Caroline Rémond¹

Abstract

A bacterial strain, named For3^T, was isolated from forest soil sampled in Champenoux, France. Based on its 16S rRNA gene sequence, the strain was affiliated to the family *Streptomycetaceae* and, more specifically, to the genus *Streptomyces*. The strain had 99.93% 16S rRNA gene sequence similarity to its closest relative strains *Streptomyces pratensis* ATCC 33331^T, *Streptomyces anulatus* ATCC 27416^T, *Streptomyces setonii* NRRL ISP-5322^T and *Kitasatospora papulosa* NRRL B-16504^T. The phylogenomic tree using the genome BLAST distance phylogeny method showed that the closest relative strain was *Streptomyces atroolivaceus* NRRL ISP-5137^T and that For3^T represents a new branch among the *Streptomyces*. Genome relatedness indexes revealed that the average nucleotide identity and digital DNA–DNA hybridization values between For3^T and its closest phylogenomic relative (*S. atroolivaceus* NRRL ISP-5137^T) were 88.39 and 39.2%, respectively. The G+C content of the genome was 71.4 mol% and its size was 7.96 Mb with 7492 protein-coding genes. Strain For3^T harboured complete metabolic pathways absent in the closest relative strains such as cellulose biosynthesis, glycogen degradation I, glucosylglycerate biosynthesis I. Anteiso-C_{15:0}, iso-C_{15:0}, anteiso-C_{17:0} and MK-9(H4)/MK-9(H6) were the predominant cellular fatty acids and respiratory quinones, respectively. Phenotypic and genomic data supported the assignment of strain For3^T to a novel species *Streptomyces silvae* sp. nov., within the genus *Streptomyces*, for which the type strain is For3^T (=CIP 111908^T=LMG 32186^T).

Actinobacteria are Gram-positive bacteria characterized by a genome with a high G+C ratio. They are numerous and constitute a widely distributed group of soil microbes, representing 10–50% of the soil microflora community. They are important producers of diversified secondary metabolites with antifungal and antibacterial activities [1].

Among this phylum, *Streptomyces* is the most famous and well-described genus. *Streptomyces* members are Gram-stain-positive, aerobic and heterotrophic bacteria with aerial mycelia [2]. *Streptomyces* have a predominant presence of saturated iso and anteiso fatty acids as major cellular fatty acids [3]. The genus *Streptomyces*, which is very widespread in soils, is a major player in the degradation of organic matter and lignocellulose [4]. It has a large enzymatic arsenal encoding carbohydrate esterases, polysaccharide lyases, glycoside hydrolases and enzymes with auxiliary activities [5, 6] and it can produce approximately around 7600 bioactive compounds [7]. In this study, strain For3^T isolated from forest soil was subjected to a polyphasic taxonomy approach that relied on genomic, chemotaxonomic and physiological data.

Forest soil was sampled in Champenoux, France (48.44° N, 6.21° E) on 13 May 2019 in order to study the actinobacterial strains present in that soil. One gram of the soil sample was placed in 50 ml International *Streptomyces* Project (ISP) 2 [8] liquid medium at 30 °C for 1 week at pH 7 in order to isolate actinobacteria micro-organisms. After 1 week enrichment, dilution series up to 10⁻⁶ were made; 100 µl of each dilution was plated on ISP 2 agar medium and incubated for 10 days at 30 °C. Among all the isolates after 10 days, one isolate, named For3^T, was then purified by repeated streaking on the same ISP 2 medium and isolated for further study.

The almost-complete 16S rRNA gene sequence of strain For3^T was extracted from the genome sequence and represented a sequence of 1448 nucleotides long. It was deposited in GenBank/EMBL/DDBJ under the accession number MW479423.

Strain For3^T was most closely related to *Streptomyces pratensis* ATCC 33331^T, *Streptomyces anulatus* ATCC 27416^T, *Streptomyces setonii* NRRL ISP-5322^T and *Kitasatospora papulosa* NRRL B-16504^T [9] with 99.93% 16S rRNA gene sequence

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Keywords: forest soil; lignocellulose; *Streptomyces*.

Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization; ISP, International *Streptomyces* Project.

The accession numbers of 16S rRNA gene and whole genome sequences deposited at GenBank are MW479423 and JAERUC000000000, respectively. One supplementary table and four supplementary figures are available with the online version of this article.

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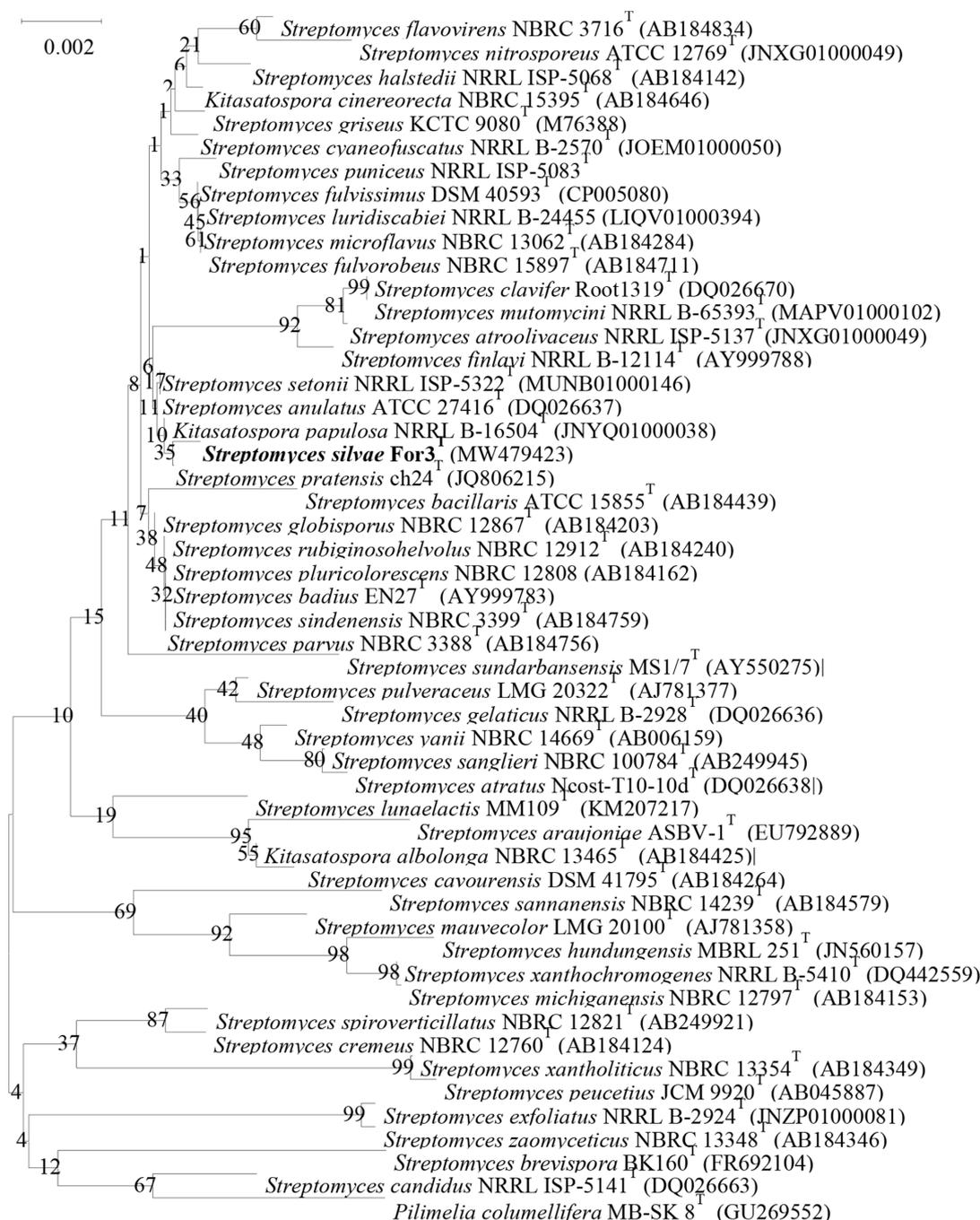


Fig. 1. Phylogenetic tree of type strains closely related to strain For3^T (in bold) based on 16S rRNA gene sequences. The evolutionary history was inferred by MEGA 7.0 [34] using the neighbour-joining method [35]. There were a total of 1315 positions in the final dataset. Bar represents 0.002 substitutions per nucleotide position. Strain For3^T is highlighted in bold.

similarity. High levels of 16S rRNA gene sequence similarity were previously reported within the genus *Streptomyces* [10, 11], demonstrating that it is difficult to differentiate *Streptomyces* species by 16S rRNA gene sequences.

Phylogenetic analysis based on the 16S rRNA gene sequences of strain For3^T and its closest species (determined by BLASTn) was performed after alignment of sequences using

the ClustalW program [12]. Neighbour-joining (Fig. 1) and maximum-likelihood phylogenetic trees were built using MEGA7 software (Fig. S1, available in the online version of this article) [13]. The stability of the grouping was estimated by bootstrap analysis (1000 replicates). The results showed that strain For3^T represents a new branch in both neighbour-joining and phylogenetic trees. Strain For3^T fell within the

Table 1. Genome size, DNA G+C content, digital DNA–DNA relatedness and average nucleotide identity of strain For3^T and closely related type strains of the genus *Streptomyces* (with a 16S rRNA similarity above 98.65%)

Strain	Size (bp)	DNA G+C content (mol%)	DNA–DNA relatedness (%)	Average nucleotide identity (%)
<i>Streptomyces</i> sp. For3 ^T	8072519	71.4		
<i>Streptomyces atroolivaceus</i> NRRL ISP-5137 ^T	8216207	70.7	39.20	88.39
<i>Streptomyces mutomycini</i> NRRL B-65393 ^T	7070041	70.6	39.20	88.85
<i>Streptomyces pratensis</i> ATCC 33331 ^T	7656104	71.0	37.2	87.90
<i>Kitasatospora papulosa</i> NRRL B-16504 ^T	7568795	71.0	37.10	88.05
<i>Streptomyces clavifer</i> Root1319 ^T	6744287	71.3	32.60	86.22
<i>Streptomyces halstedii</i> NRRL ISP-5068 ^T	7740838	71.9	30.40	84.66
<i>Streptomyces nitrosporeus</i> ATCC 12769 ^T	7581562	72.2	29.30	84.30
<i>Streptomyces setonii</i> NRRL ISP-5322 ^T	7819863	71.7	27.40	82.05
<i>Streptomyces brevispora</i> BK160 ^T	7540224	70.3	27.40	82.18
<i>Streptomyces atratus</i> Ncost-T10-10d ^T	9361102	69.6	27.00	80.94
<i>Streptomyces anulatus</i> ATCC 27416 ^T	9057348	71.4	27.00	81.46
<i>Streptomyces peucetius</i> JCM 9920 ^T	8179821	72.5	27.00	81.93
<i>Streptomyces cavourensis</i> DSM 41795 ^T	7670394	72.1	27.00	82.19
<i>Streptomyces bacilliaris</i> ATCC 15855 ^T	7888441	72.0	27.00	82.22
<i>Streptomyces griseus</i> KCTC 9080 ^T	8630192	72.2	26.80	81.78
<i>Streptomyces cyaneofuscatus</i> NRRL B-2570 ^T	7899007	71.6	26.80	82.11
<i>Streptomyces globisporus</i> NBRC 12867 ^T	8120903	71.4	26.70	81.82
<i>Streptomyces parvus</i> NBRC 3388 ^T	8340747	71.7	26.60	81.52
<i>Streptomyces badius</i> EN27 ^T	7550241	71.6	26.60	82.02
<i>Streptomyces lunaelactis</i> MM109 ^T	8570191	69.8	23.90	78.73
<i>Streptomyces michiganensis</i> NBRC 12797 ^T	8656216	71.2	23.60	77.88
<i>Streptomyces xantholiticus</i> NBRC 13354 ^T	8023114	70.6	23.10	78.27
<i>Streptomyces exfoliatus</i> NRRL B-2924 ^T	7874957	71.9	22.80	78.16

genus *Streptomyces* and was closely related to *S. pratensis* ATCC 33331^T, *S. anulatus* ATCC 27416^T, *S. setonii* NRRL ISP-5322^T and *K. papulosa* NRRL B-16504^T with whom it formed a subgroup.

Genomic DNA was extracted using PureLink Genomic DNA Mini Kit (Invitrogen) according to manufacturer's instructions. DNA was quantified using the ND-1000 spectrophotometer (NanoDrop Technologies). DNA quality was controlled after electrophoresis on a 0.8% (w/v) agarose gel made in Tris–acetate–EDTA buffer. Genomic DNA was sequenced using a NovaSeq system (Illumina) and was performed by Novogene (Cambridge, UK). The obtained genome sequence was annotated by using the RAST server (Rapid Annotation using Subsystem Technology; <https://rast.nmpdr.org/>) and deposited at DDBJ/ENA/GenBank under the accession number JAERUC000000000. Comparisons between the For3^T genome and those of its closest relatives

available in GenBank are presented in Table 1. The genome size of strain For3^T was 7.96 Mbp, slightly smaller than the average genome sizes (8–9 Mbp) reported for the genus *Streptomyces* [14]. The DNA G+C content of strain For3^T was 71.4 mol%, is near the average of 70 mol% reported for the genus *Streptomyces* [15].

The genome BLAST distance was calculated using the annotation platform system provided by DSMZ (<https://tygs.dsmz.de/>). The phylogenomic tree obtained (Fig. 2) showed that strain For3^T is individualized in a new branch. Based on that phylogenomic comparison, its closest strain is *S. atroolivaceus* NRRL ISP-5137^T with an average branch support of 84.9%. Moreover, based on the genomes available, an automated multi-locus species tree was realized using the web server at <https://automlst.ziemertlab.com/> [16]. Based on 89 house-keeping core genes (listed in the Table S1), the phylogenomic tree obtained showed that our strain is closely related to *S.*

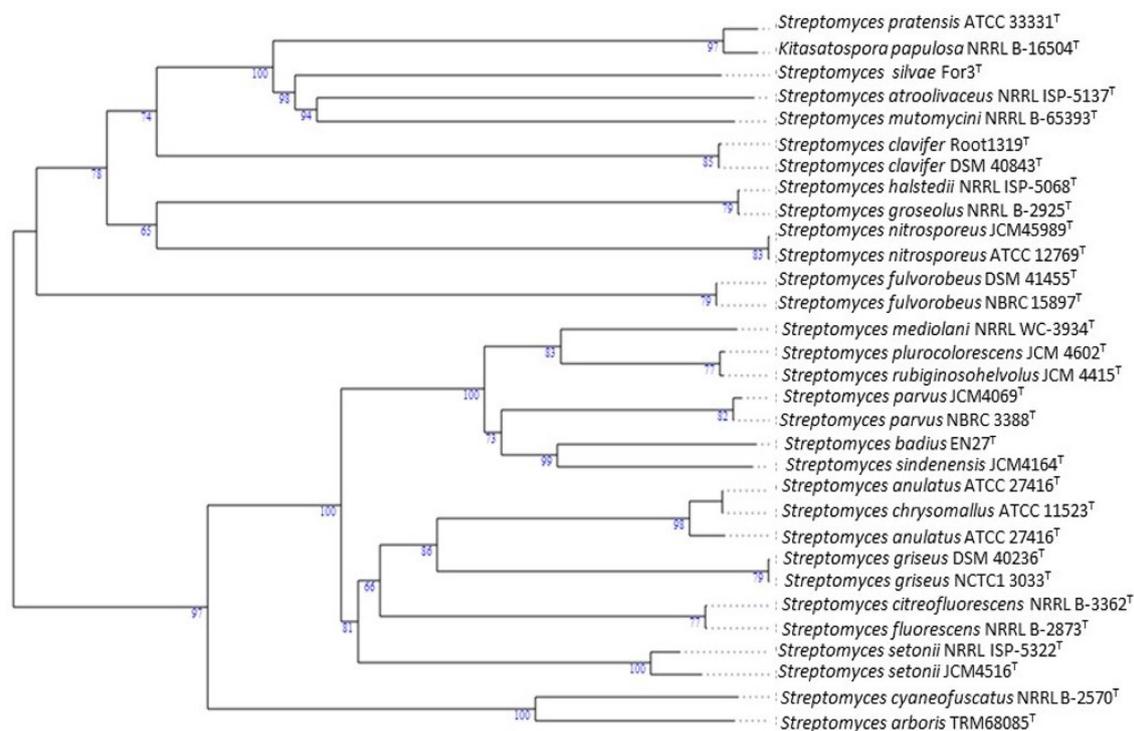


Fig. 2. Tree inferred with FastME 2.1.6.1 [36] from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d_g . The tree was rooted at the midpoint [37].

atroolivaceus NRRL ISP-5137^T (Fig. S2). The pairwise average nucleotide identity (ANI) values were determined by using an ANI calculator (<http://jspecies.ribohost.com/jspeciesws/#home>) [17]. The ANI values between For3^T and its closest relatives *S. mutomycini* NRRL B-65393^T, *S. atroolivaceus* NRRL ISP-5137^T (the closest phylogenomic strain based on AutoMLST and TYGS), *S. pratensis* ATCC 33331^T and *K. papulosa* NRRL B-16504^T were 88.85, 88.39, 87.9 and 88.05% respectively, well below the cut-off value of 95–96% proposed for species delineation [18] (Table 1).

Digital DNA–DNA hybridization (dDDH) or DNA–DNA relatedness values were calculated between strain For3^T and the type strains of closest species using the Genome-to-Genome Distance Calculator [19]. The dDDH values between strain For3^T and *S. atroolivaceus* NRRL ISP-5137^T and *S. mutomycini* NRRL B-65393^T reached 39.2% (the highest value obtained), lower than the 70% species cut-off value [19, 20] (Table 1). For *S. pratensis* ATCC 33331^T and *K. papulosa* NRRL B-16504^T, the ANI values were 37.2 and 37.1% respectively. The TETRA method [21] was applied and supported this result with a TETRA index of 0.994, below the species delimitation cut-off (>0.999) (data not shown).

Genomic comparisons between strain For3^T and its closest relative strain *S. atroolivaceus* NRRL ISP-5137^T [22] were performed. Strain For3^T harboured several genes coding enzymes such as 3-hydroxyacyl-[acyl-carrier-protein] dehydratase, 4-carboxy-4-hydroxy-2-oxoadipate aldolase, 4-oxalomesaconate hydratase, clavaldehyde

dehydrogenase, creatinine amidohydrolase and threonyl-tRNA synthetase-related protein, unlike the other analysed genomes. Those enzymes could not be related to any specific metabolic pathway. Strain For3^T had 61 genes that were not detected in *S. pratensis* ATCC 33331^T such as genes encoding for 3-carboxy-cis-muconate cycloisomerase and protocatechuate-3,4-dioxygenase. Strain For3^T had 91 genes that were not detected in *K. papulosa* NRRL B-16504^T such as several genes in the CRISPR system encoding for CRISPR-associated helicase Cas2, Cas3, Cse3 and Cse4. Strain For3^T had 98 genes that were not detected in *S. mutomycini* NRRL B-65393^T such as several genes in the arginine and ornithine degradation metabolic pathway such as arginine decarboxylase, ornithine decarboxylase and isochorismatase.

Growth tests at various temperatures, and at different pH and NaCl concentrations, were performed using ISP 2 agar as culture medium. Growth of strain For3^T occurred at 15–30 °C (optimum, 25 °C), at pH 6.0–9.0 (pH 7.0) and in the presence up to 5% w/v NaCl (0% w/v NaCl). Compared to *S. pratensis* ATCC 33331^T [23], which is the closest strain with physiologic data available, strain For3^T could be distinguished only by a more restricted temperature range growth while the rest of the physiological parameters were the same. Indeed, the growth range of *S. pratensis* ATCC 33331^T was between 15 and 37 °C. *K. papulosa* NRRL B-16504^T grew at 15–30 °C (optimum, 25 °C), at pH 6.0–9.0 (pH 7.0) and in the presence up to 5% w/v NaCl (0% w/v NaCl), which are the same values obtained for

For3^T. The optimum physiological parameters (pH, temperature) were also close compared to the others strains, especially *S. atroolivaceus* NRRL ISP-5137^T and *S. mutomycini* NRRL B-65393^T. Strain For3^T was aerobic, Gram-stain-positive and catalase-positive. Colonies were greenish-white with rough surfaces when the strain was cultivated on ISP 2 medium. Green diffusible pigment was produced on that medium.

The assimilation of carbon sources by strain For3^T was tested using Biolog MicroPlates [24]. Compared to a closest phylogenomic strain *S. atroolivaceus* NRRL ISP-5137^T [25, 26], strain For3^T was unable to degrade D-glucose and sucrose. Strain For3^T was able to degrade D-fructose, D-mannitol, raffinose and rhamnose, like *S. atroolivaceus* NRRL ISP-5137^T. Compared to *K. papulosa* NRRL B-16504^T, strain For3^T was unable to degrade D-glucose; however, strain For3^T was able to utilize D-fructose, D-mannitol, raffinose and rhamnose, whereas *K. papulosa* NRRL B-16504^T was not. Compared to *S. pratensis* ATCC 33331^T, both strains were able to degrade fructose, D-mannitol, raffinose and rhamnose; For3^T did not degrade D-glucose in contrast to *S. pratensis* ATCC 33331^T.

For chemotaxonomic affiliation: (1) the fatty acid methyl esters were obtained from 40 mg fresh scraped colonies from Petri dishes by saponification, methylation and extraction using minor modifications of the method of [27] and [28]; (2) the respiratory quinones were first extracted from 100 mg freeze-dried cell material using methanol-hexane extraction and then separated by using the hexane method described in [29, 30]; (3) the polar lipids were extracted from 200 mg freeze-dried cell material using a chloroform-methanol-0.3% aqueous NaCl mixture; polar lipids were separated by two dimensional silica gel thin-layer chromatography. The first direction was developed in chloroform-methanol-water and the second in chloroform-methanol-acetic acid-water. All analyses relating to fatty acids (www.dsmz.de/services/services-microorganisms/identification/analysis-of-cellular-fatty-acids.html), respiratory quinones (www.dsmz.de/services/services-microorganisms/identification/analysis-of-respiratory-quinones.html) and polar lipids (www.dsmz.de/services/microorganisms/biochemical-analysis/polar-lipids) were carried out by DSMZ (Braunschweig, Germany). The detection of diamino and mycolic acids in the strain were performed according to standard procedures [31, 32].

For3^T contained MK-9(H6) (60.2%), MK-9(H4) (29.4%), MK-9(H2) (3.2%) and MK-9(H8) (7.2%) as major respiratory quinones. For3^T had a respiratory quinone content very similar to those of *K. papulosa* NRRL B-16504^T and *S. lunaelactis* MM109^T with the presence of MK-9(H2), MK-9(H4), MK-9(H6) and MK-9(H8). Only *S. lunaelactis* MM109^T had several quinones (MK-9 and MK-10). However, differences in the relative abundance of MK-9(H2), MK-9(H4), MK-9(H6) (always the most abundant) and MK-9(H8) were detected. *S. pratensis* ATCC 33331^T had only MK-9(H6) and MK-9(H8).

The dominant fatty acids of strain For3^T were anteiso-C_{15:0} (32.97%), iso-C_{15:0} (15.37%) and anteiso-C_{17:0} (13.83%). This cellular fatty acid profile was consistent with those of members of the genus *Streptomyces* such as *S. pratensis* ATCC 33331^T, *S. lunaelactis* MM109^T and *S. brevispora* BK160^T.

Strain For3^T had lipid, glycolipid, aminolipid, phosphatidylethanolamine and diphosphatidylglycerol as polar lipids (Fig. S3). Only this strain had such diversity in term of polar lipid profile: indeed, for the others strains, *S. lunaelactis* MM109^T had lipid, phosphatidylethanolamine and diphosphatidylglycerol, whereas *K. papulosa* NRRL B-16504^T had only phosphatidylethanolamine. Concerning the detection of diamino acid in the whole-organism hydrolysate, LL-diaminopimelic acid (A2pm) was found, confirming that the strain belongs to the genus *Streptomyces* [33]. Strain For3^T lacked mycolic acid.

In conclusion, the respiratory quinone, fatty acid and polar lipid profiles, as well as 16S rRNA gene sequencing results, showed unambiguously that strain For3^T is affiliated to species of the genus *Streptomyces*. On the basis of the phenotypic differences observed and the results of genomic studies, strain For3^T can be clearly distinguished from the most closely related species of the genus *Streptomyces*. Therefore, we propose a novel species within the genus *Streptomyces*, with the name *Streptomyces silvae* sp. nov. and with strain For3^T as type strain.

DESCRIPTION OF *STREPTOMYCES SILVAE* SP. NOV.

Streptomyces silvae (sil'vae. L. gen. n. *silvae* of a forest).

Aerobic, Gram-stain positive, catalase-positive actinobacterium (0.5×1.5 μm) (Fig. S4). Green diffusible pigment is formed on ISP 2 medium and the strain differentiates into spiral chains of spores with rough surfaces on sporulation medium. Growth occurs at 15–30 °C (optimum, 25 °C), at pH 6.0–9.0 (pH 7.0) and in the presence up to 5% w/v NaCl (0% w/v NaCl). Able to degrade D-arabitol, D-aspartic acid, cellobiose, dextrin, D-fructose, D-fructose-6-PO₄, D-fucose, D-galactose, D-gluconic acid, D-glucose-6-PO₄, D-glucuronic-acid, D-lactic acid, methyl ester, D-malic acid, maltose, D-mannitol, D-mannose, melibiose, raffinose, D-salicin, D-sorbitol, formic acid, gentiobiose, glucuronamide, L-alanine, L-arginine, L-aspartic acid, L-fucose, L-galactonic acid, lactone, L-glutamic acid, lithium, chloride, L-lactic acid, L-rhamnose, L-serine, mucic acid, myo-inositol, N-acetyl neuraminic acid, N-acetyl-D-galactosamine, pectin, p-hydroxy-phenylacetic acid, potassium tellurite, rifamycin SV, sodium bromate, sodium butyrate, stachyose, α-hydroxy-butyric acid and α-keto-butyric acid. Strain For3^T is not able to degrade 3-methyl-glucose, acetic acid, acetoacetic acid, aztreonam, bromo-succinic acid, citric acid, D-galacturonic acid, D-saccharic acid, D-serine, trehalose, turanose, fusidic acid, gelatin, glycerol, glycyl-L-proline, guanidine HCl, inosine, L-histidine, lincomycin, L-malic acid, L-pyroglytamic acid,

methyl pyruvate, minocycline, *N*-acetyl- β -D-glucosamine, *N*-acetyl- β -D-mannosamine, nalidixic acid, niaproof 4, propionic acid, quinic acid, sucrose, troleandomycin, Tween 40, vancomycin, α -D-glucose, lactose, α -keto-glutaric acid, β -hydroxy-D, L-butyric acid, methyl β -D-glucoside and γ -amino-butyric acid. Lipid, glycolipid, aminolipid, phosphatidylethanolamine and diphosphatidylglycerol are present as polar lipids. Anteiso-C_{15:0}, iso-C_{15:0} and anteiso-C_{17:0} are the most abundant fatty acids. MK-9(H4) and MK-9(H6) are the predominant quinones.

The type strain, For3^T (=CIP 111908^T=LMG 32186^T), was isolated from a forest soil sample collected near Champenoux, France.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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