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Streptomyces coriariae sp. nov., a novel streptomycete isolated from actinorhizal nodules of *Coriaria intermedia*

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Abstract

An actinobacterial strain, CMB-FB, was isolated from surface-sterilized root nodules of a *Coriaria intermedia* plant growing along Halsema Highway in the province of Benguet (Luzon, Philippines). The 16S rRNA gene sequence of CMB-FB showed high sequence similarity to those of the type strains of *Streptomyces rishiriensis* (99.4%), *Streptomyces humidus* (99.1%), *Streptomyces cacaoi* subsp. *asoensis* (99.0%), and *Streptomyces phaeofaciens* (98.6%). The major menaquinones of CMB-FB were composed of MK-9(H₄), MK-9(H₈) and MK-9(H₁₀), and there was a minor contribution of MK-9(H₁₀). The polar lipid profile consisted of phosphatidylethanolamine, unidentified aminolipids and phospholipids, a glycopospholipid and four unidentified lipids. The diagnostic diamino acid of the peptidoglycan was meso-diaminopimelic acid. The major fatty acids were iso-C_{16:0}, anteiso-C_{15:0} and anteiso-C_{17:0}. The results of physiological analysis indicated that CMB-FB was mesophilic. The results of phylogenetic, genome-genome distance calculation and average nucleotide identity analysis indicated that the isolated strain represents the type strain of a novel species. On the basis of these results, strain CMB-FB (=DSM 112754^T=LMG 32457^T) is proposed as the type strain of the novel species *Streptomyces coriariae* sp. nov.

INTRODUCTION

The genus *Streptomyces*, first described in 1943 [1], is the largest genus of the class *Actinomycetia* [2] encompassing Gram-positive aerobic bacteria with DNA G+C contents (mol%) of 69–78 % [3]. They are recognized for a varied secondary metabolism that leads to the production of diverse bioactive compounds [4]. Most streptomycetes are soil bacteria, and several interact with plants, as rhizosphere bacteria [5], endophytes [6, 7] or pathogens [8, 9].

Gram-negative rhizobia and actinobacteria of the genus *Frankia* can engage in symbiosis with root-nodule-forming host plants of the orders Fabales, Fagales, Cucurbitales and Rosales [10]. Aside from their microsymbionts, root nodules can host non-nitrogen-fixing bacterial endophytes, such as members of the genus *Streptomyces* [11, 12]. Plant members of the actinorhizal genus *Coriaria* (Coriariaceae, Cucurbitales) are endemic to disjunct areas across the globe. During investigations of nodules from a cluster of *Coriaria intermedia* bushes from the Philippines, metagenome analysis revealed the presence of non-*Frankia* actinobacterial strains at significant levels: among the bacterial sequences obtained from a group of nodules, only 75% were from members of the genus *Frankia*. One strain of a member of the genus *Streptomyces* was isolated from surface-sterilized nodules collected nearby; its 16S rRNA gene sequence showed high similarity to those of *Streptomyces rishiriensis* NRRL B-3239^T, *Streptomyces humidus*

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Abbreviations: ANI, average nucleotide identity; BUSCO, benchmarking universal single-copy orthologs; dDDH, digital DNA–DNA hybridization; GBDP, genome BLAST distance phylogeny.

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The GenBank accession number for the 16S rDNA of *Streptomyces* sp. CMB-FB is OU572639 The genome sequence accession numbers for NCBI GenBank are CAJYZZ010000001–CAJYZZ010000211; the Bioproject number is PRJEB46697; the EBI accession number is <https://www.ebi.ac.uk/ena/browser/view/CAJYZZ010000000.1>

Two supplementary figures and five supplementary tables are available with the online version of this article.

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JCM 4386^T and *Streptomyces cacaoi* subsp. *asoensis* JCM 4185^T. This strain turned out to represent 0.5% of the bacterial sequences from the nodule sample used for DNA isolation.

Isolation and ecology

Nodules, seeds and leaves of *Coriaria intermedia* were collected from a group of bushes along Halsema Highway on the slopes of the Cordillera Central range in the province of Benguet (Luzon, Philippines), at an altitude of 2027–2032 m above sea level. Collection took place at Baguio-La Trinidad-Bontoc Road, Buguias, Benguet (Philippines), coordinates 16°46′42.0″N (16.778330)–16°46′46.7″N (16.779634) and 120°48′32.0″E (120.808875)–120°48′33.8″E (120.809397). The plant species was verified on the basis of *matK* and *rbcL* sequences [13] and vouchers are deposited in the Herbarium at the Department of Botany, University of the Philippines, leg. C.M. Bandong s.n. (Reg. No. 21414) and in the herbarium of the Swedish Museum of Natural History, leg. K. Pawlowski s.n. (S; Reg. No. S19-5452). DNA was isolated from 100 mg of nodules collected in the field and the metagenome was sequenced and assembled according to the methods described by Nguyen *et al.* [14]; the raw data are available at ENA (Project PRJEB53824). The high contribution of the DNA of a strain of a member of the genus *Streptomyces* in the metagenome – 12% of the bacterial DNA in the metagenome based on metaCV [15], while bacterial DNA represented 32% of the whole metagenome – indicated that, like members of the genus *Frankia*, members of the genus *Streptomyces* were stably internally accommodated in infected nodule cortical cells.

The seeds of *C. intermedia* were germinated in the greenhouse, and after 12 weeks, young plantlets were infected using the remaining crushed nodules which had been collected in the field in order to propagate the collected inoculum. In order to isolate the strain of the member of the genus *Streptomyces*, root nodules induced on *C. intermedia* plants in the greenhouse were collected and rinsed with sterile water to remove any soil. Nodules were surface sterilized using 70% (v/v) ethanol for 1 min, followed by 10% NaClO (v/v) for 5 min and six washes in autoclaved milliQ H₂O. To confirm the effectiveness of the surface sterilization, nodules were rolled over yeast malt agar plates (YMA; Sigma-Aldrich), and the plates were incubated at 28 °C overnight. Nodules were crushed with a sterile mortar and pestle, and the suspension was plated on YMA plates, which were incubated at 28 °C. Colonies that appeared after 12 h were considered contaminants from the nodule periderm and were sliced from the plate using a scalpel. After 3 days, several distinct colonies were observed and transferred to individual YMA plates, which were incubated for 3 days. Gram staining was performed on the different isolates according to the instructions of the manufacturer (Gold Biotechnology). Only Gram-staining-positive colonies showing the typical hyphal morphology of member of the genus *Streptomyces* were retained. Some colonies turned out to represent a mix of bacteria. Colonies were therefore cultivated in liquid YM medium to perform serial dilutions, which were plated on YMA. Ultimately, seven strains with different colony phenotypes were obtained and used to inoculate liquid cultures (YM medium) to be used for DNA extraction according to the cetyltrimethylammonium bromide (CTAB) method of Wilson [16] modified by Ribeiro *et al.* [17] but without achromopeptidase.

The metagenome assembly from the root nodules was used to identify genes present in the incomplete metagenome of members of the genus *Streptomyces*, but not in that of members of the genus *Frankia*. One such gene was *arnB*, encoding UDP-4-amino-4-deoxy-L-arabinose-oxoglutarate-aminotransferase. To identify which of the seven strains represented the strain of the member of the genus *Streptomyces* previously detected in metagenomes, specific primers were designed for the gene encoding *arnB* (*arnB_S_Fw* 5'-CATCGTGTACGCCTGTCTA-3', *arnB_S_Rv* 5'-CATACGGCTACCTCGCACAG-3'). Only one colony tested positive. The strain was maintained on YMA and on International Streptomyces Project 2 (ISP2) medium [18] at 28 °C.

For full genome sequencing of the strain, genomic DNA (gDNA) was isolated from cells grown in liquid ISP2 medium using the geneJET genomic DNA purification kit (ThermoFisher). Library preparation was performed with 500 ng gDNA using the Nextera Flex Library Prep Kit (Illumina) according to the manufacturer's instructions. Libraries were quality controlled with a High Sensitivity DNA Kit on a Bioanalyzer (Agilent) and quantified on a Qubit 2.0 Fluorometer (ThermoFisher Scientific with ds HS Assay Kit). Genome sequencing was performed in the Genomics Service Unit (LMU Biocenter, Munich, Germany) on a MiSeq (Illumina) with v3 chemistry (2×300 bp paired-end sequencing). Assembly and annotation were performed as previously described [14, 19] with small modifications. In brief, *de novo* assembly was performed by applying gsAssembler 2.8. (Roche) with default settings. Completeness, contamination and strain heterogeneity were estimated with benchmarking universal single-copy orthologs (BUSCO) (v3.0.2 [20]), using the bacterial-specific single-copy marker genes database (odb9). For the annotation of the genome, Prokka [21] and GenDB [22] were used.

The assembled draft genome of strain CMB-FB was 9378825 bp long, composed of 216 contigs with an N50 of 78852 bp and a DNA G+C content of 71.23 mol%; the results of BUSCO analysis indicated that it was 93.9% complete with 139 complete and single-copy BUSCOs and seven missing BUSCOs (Table S1, available in the online version of this article). Annotation revealed 8316 CDS, 1 tmRNA, 90 tRNA genes and five rRNA operons. In total 0.87% of the original metagenome data could be mapped on the CMB-FB genome, indicating that the isolated strain was either closely related to the dominant strain of members of the genus *Streptomyces* in the nodules, or represented one of the dominant strains.

Phylogeny

The genome sequence data were uploaded to the Type (Strain) Genome Server (TYGS) at <https://tygs.dsmz.de> for a whole genome-based taxonomic analysis [23]. The results were provided by the TYGS platform on 2021-07-07. Determination of the most closely related type strain genomes was performed in two complementary ways:

First, the CMB-FB genome was compared against all type strain genomes available in the TYGS database via the MASH algorithm [24]; the ten type strains with the smallest MASH distances were chosen for further analysis. Second, an additional set of ten closely related type strains was determined on the basis of the 16S rRNA gene sequences. The 16S rRNA sequence of CMB-FB (GenBank accession number OU572639), was compared by means of BLAST [25] against the 16S rRNA gene sequence of each of the 14917 type strains available currently in the TYGS database. This was used as a proxy to find the best 50 matching type strains (according to the bitscore) for each genome and to subsequently calculate precise distances using the genome BLAST distance phylogeny (GBDP) approach under the algorithm 'coverage' and distance formula d_5 [26]. These distances were used to determine the ten type strain genomes most closely related to CMB-FB.

For the phylogenomic inference, all pairwise comparisons among the set of genomes were conducted using GBDP and accurate intergenomic distances inferred using the algorithm 'trimming' and distance formula d_5 . In total, 100 distance replicates were calculated for each comparison. Digital DNA–DNA hybridization (dDDH) values and confidence intervals were calculated using the recommended settings of the GGDC 2.1 [26]. The resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FASTME 2.1.6.1 including subtree pruning and regrafting postprocessing [27]. Branch support was inferred from 100 pseudo-bootstrap replicates each. The trees were rooted at the midpoint [28] and visualized with PhyD3 [29].

The type-based species clustering using a 70% dDDH radius around each of the 14 type strains was performed as described by Meier-Kolthoff and Göker [23]. The resulting groups are shown in Table S2a. Subspecies clustering was performed using a 79% dDDH threshold as previously introduced [30].

The 16S rRNA gene sequence of the isolated strain, which had been amplified and sequenced, showed high similarity to *Streptomyces rishiriensis* NRRL B-3239^T (NR_044141.1; 99.4%), followed by *Streptomyces humidus* NRRL B-3172^T = JCM 4386^T (DQ442508; 99.1%), *Streptomyces cacaoi* subsp. *asoensis* JCM 4185^T (CP049838.1; 99.1%) and *Streptomyces phaeofaciens* JCM 4814^T (NR_041126.1; 98.6%). Whole-genome phylogeny inferred with FastME 2.1.6.1 [31] from GBDP distances calculated from genome sequences showed a slightly different picture with *S. cacaoi* subsp. *asoensis* JCM 4185^T as the closest relative (Fig. 1a). Genome-to-Genome-Distance-Calculation (GGDC) was performed on the TYGS server using digital DNA–DNA hybridization (dDDH) and formula 2 (Table S2b). Values were 37.5% for *S. cacaoi* subsp. *asoensis*, 34.4% for *S. rishiriensis*, 34.0% for *S. humidus* and 31.1% for *S. phaeofaciens*, indicating that strain CMB-FB represents a different species. When calculating average nucleotide identity (ANI, Table S3) using the orthoANiU algorithm [30, 32], ANI between CMB-FB and *S. cacaoi* subsp. *asoensis* was lower than 95%. The 16S rRNA-based phylogenies inferred from GBDP distances using FastME 2.1.6.1 [31] are shown in Fig. 1b; the 16S rRNA-based phylogeny inferred using maximum likelihood and maximum-parsimony are shown as well (Fig. 1c and d). A 16S-based phylogeny inferred using maximum-parsimony based on ggdc.dsmz.de is shown in Fig. S1. In summary, on the basis of the genome sequence, CMB-FB represents a novel species of the genus *Streptomyces*.

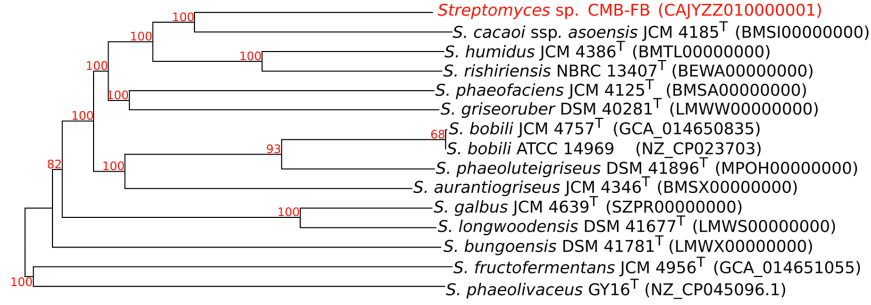
Genome features

The secondary metabolism of CMB-FB was assessed using antiSMASH v. 6.0 [33]; the results can be found in Table S4. The data indicate that the strain is able to produce several antibiotics and siderophores, including germicidin, scabichelin, albaflavenone, alkylresorcinol and, like all other strains of members of the genus *Streptomyces* examined, also geosmin.

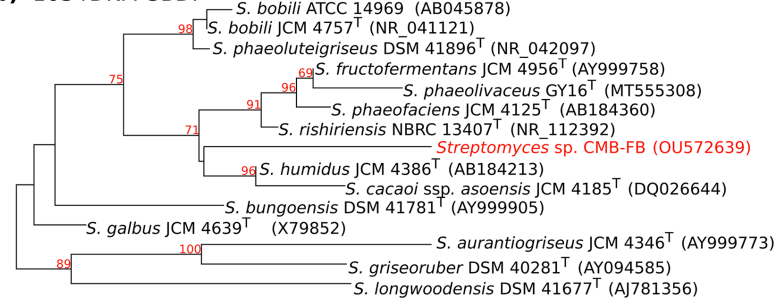
CMB-FB has several features of plant-growth-promoting rhizobacteria. Its genome contains the operons for the production of some siderophores (Table S4) and it seems to have the potential to degrade the ethylene precursor 1-aminocyclopropne-1-carboxylate (ACC), since it contains an ACC deaminase gene *acdS* (GenBank accession number OU548656; the encoded protein shows 86.4% amino acid identity with the ACC deaminase from *Streptomyces venezuelae* [34]). The strain also seems to have the ability to produce indole acetic acid (auxin): it contains an *iaaM* gene encoding a tryptophan 2-monooxygenase that catalyses the conversion of tryptophan to indole-3-acetamide (IAM; GenBank accession number OU548758), and the *iaaH* gene encoding an amidase for the conversion of IAM to IAA was found directly upstream (GenBank accession number OU548759). Furthermore, it has the potential to synthesize melanin on the basis of the presence of the *melC* gene (GenBank accession number OU596102), which has been shown to be a trait associated with improved plant colonization [35]. The production of melanin is further supported by the antiSMASH data (Table S4).

CMB-FB was isolated from surface-sterilized nodules and thus represents a putative endophyte. For legumes, many data are available on Gram-negative non-rhizobial strains found in nodules; fewer studies have reported on Gram-positive ones: Deng *et al.* [11] reported on members of the genera *Mycobacterium*, *Nocardia* and *Streptomyces* while Alonso-Vega *et al.* [36] described intracellular members of the genus *Micromonospora* in legume nodules. For actinorhizal nodules, mostly

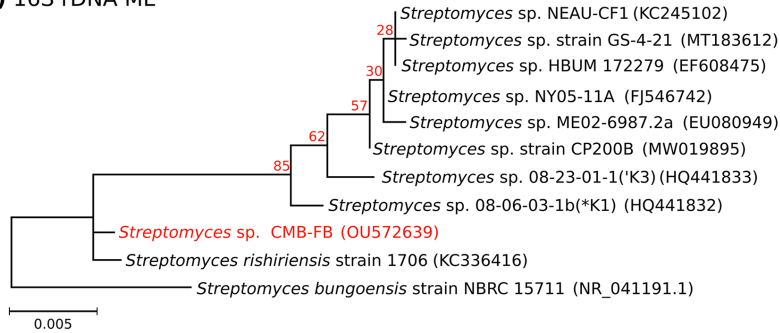
(a) whole genome



(b) 16S rDNA GBDP



(c) 16S rDNA ML



(d) 16S rDNA MP

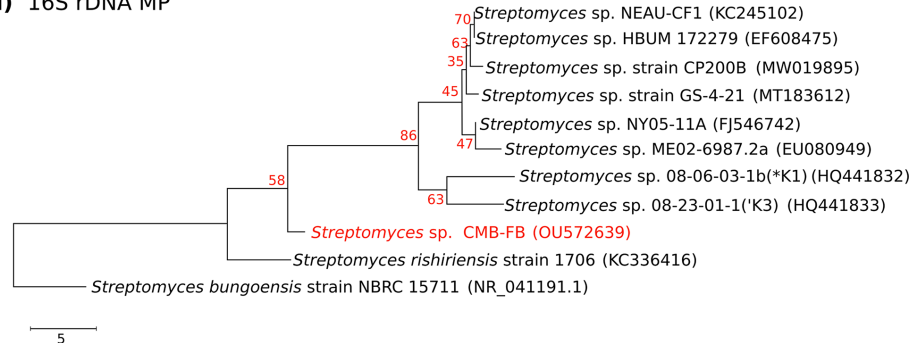


Fig. 1. Phylogenies. (a) Tree inferred with FastME 2.1.6.1 [31] from GBDP distances calculated from type strain genome sequences. The branch lengths are scaled in terms of GBDP distance formula d_g . The numbers above branches are GBDP pseudo-bootstrap support values >60% from 100 replications, with an average branch support of 95.3%. The tree was rooted at the midpoint. (b) Tree inferred with FastME 2.1.6.1 [31] from GBDP distances calculated from type strain 16S rRNA gene sequences. The branch lengths are scaled in terms of GBDP distance formula d_g . The numbers above branches are GBDP pseudo-bootstrap support values >60% from 100 replications, with an average branch support of 73.6%. The tree was rooted at the midpoint. (c) 16S rDNA tree inferred using maximum-likelihood method and Tamura–Nei model [53] for the 16S rRNA genes of CMB-FB and the nine most similar 16S rRNA genes irrespective of type strains, rooted with *Streptomyces bungoensis* NBRC 15711^T. Evolutionary analyses were conducted in MEGA X [54]. (d) Maximum-parsimony (MP) tree for the 16S rRNA genes of CMB-FB and the nine most similar 16S rRNA genes irrespective of type strains, rooted with *S. bungoensis* NBRC 15711^T. Tree #1 out of 10 most parsimonious trees (length=63) is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [54]. Evolutionary analyses were conducted in MEGA X [54]. The scale bars denote number of substitutions per site.

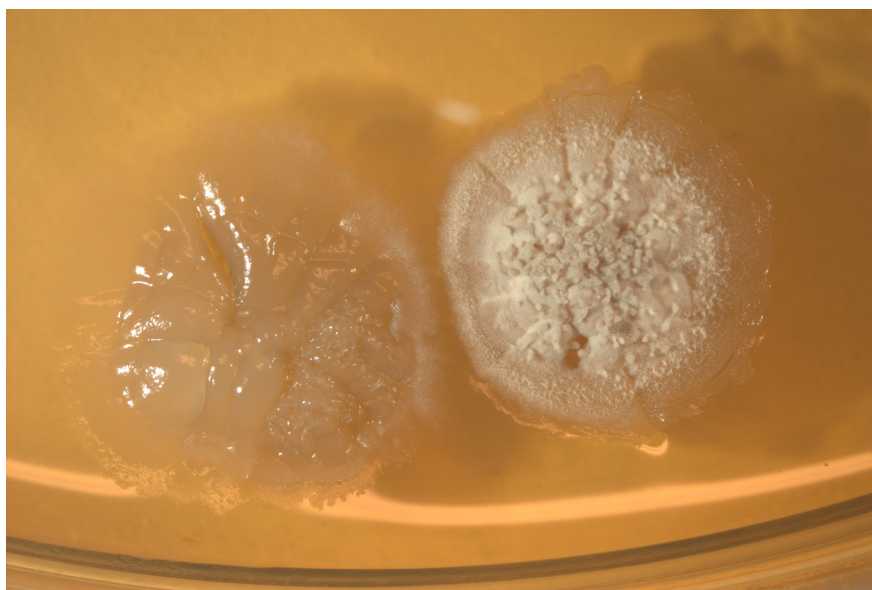


Fig. 2. Growth phenotype of CMB-FB on YMA plates. The colony on the left shows the light brown phenotype. After prolonged incubation on YMA, colonies start to sporulate, resulting in white spores as seen on the colony on the right.

members of the genera *Micromonospora*, *Nocardia* and *Streptomyces* have been reported as non-*Frankia* nodule endophytes [37]; in particular, strains of members of the genus *Micromonospora* have been found in actinorhizal nodules of several different host plants [38]. However, thus far the only members of the actinobacteria isolated from actinorhizal nodules and sequenced are two strains of members of the genus *Micromonospora* [39, 40], two strains of members of the genus *Nocardia* [41, 42] and one strain of a member of the genus *Streptomyces* [43]. Many strains of members of the genus *Streptomyces* have been reported as endophytes [44] or plant pathogens [45] of non-nodulating plant species. This study is the first, to our knowledge, to characterize a strain of a member of the genus *Streptomyces* from actinorhizal nodules. Further analyses would be needed to identify the role of this strain in the plant–microbe community.

Morphology

CMB-FB grew as light brown mycelium on YMA or ISP2 medium, forming white spores after prolonged incubation (Fig. 2). On Qmod agar, the strain sporulated readily. The morphology of CMB-FB on ISP2 and Qmod agar was examined using scanning electron microscopy (SEM). Samples were fixed with 1.5% glutaraldehyde (EM grade) and subsequently critical point dried using a critical point dryer (Bal-tec). Samples were sputter coated with 5 nm gold–palladium before observation with a model 7900F (Jeol) operating at 5kV. CMB-FB grew well on ISP2. The mycelia are smooth but increasing decorations can be observed towards the extremities. The morphology of the aerial hyphae was never straight, but always curly (Fig. 3a). Hyphae were sometimes connected by polymeric material (Fig. 3b). Interestingly, the strain did not seem to be able to grow into the ISP2 agar, as no substrate hyphae were observed entering the agar, thus limiting nutrient collection to the surface of the agar plates (Fig. 3c). On Qmod agar, aerial hyphae differentiated into chains of spores with heavily decorated surfaces (Fig. 3d). The spores were cylindrical and approximately 0.6–0.9 μm in length.

Physiology and chemotaxonomy

To determine the optimal pH, bacteria were plated on Qmod [46], ranging from pH 5 to pH 10, at increments of 1, and incubated at 28 °C in triplicate. Optimal growth, based on speed of growth, was found between pH 6 and pH 8. Carbon source utilization was examined on streptomyces minimal medium [47] supplied with 55 mM of either arabinose, fructose, galactose, glucose, maltose, mannitol, *myo*-inositol, sucrose, rhamnose or xylose. Cultures were incubated at 28 °C in triplicate. The strain did not grow on arabinose, fructose or xylose. It grew well on maltose, mannitol, *myo*-inositol, sucrose and rhamnose, but only slowly on galactose and glucose. Since this strain had been isolated from nodules induced by members of the genus *Frankia*, growth in basic alkaline propionate (BAP) liquid medium [48], normally used for members of the genus *Frankia*, was also examined. BAP cultures turned out to work best for biomass production, leading to neither biofilm formation nor sporulation. This means that the strain grew well on propionate as the sole carbon source. Salt tolerance was determined on minimal medium containing mannitol, on which it grew best, in triplicate, with added NaCl ranging from 1–10%, at 1% intervals. It was found to grow in media containing up to 4% NaCl. Optimal growth temperature was determined on

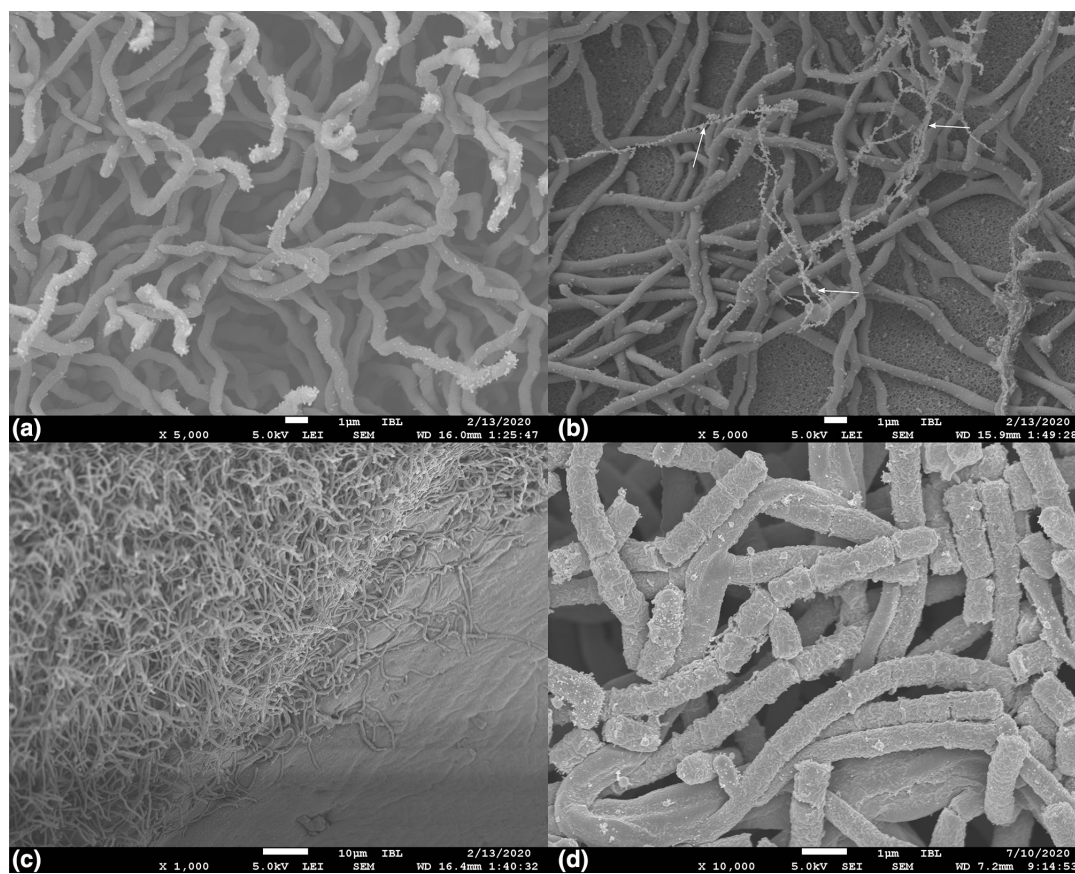


Fig. 3. Morphology of CMB-FB. (a) CMB-FB formed mycelia on ISP2. (b) On ISP2, hyphae were sometimes connected by polymeric material (arrows). (c) A view from the border of the colony shows that the hyphae did not extend into the agar. (d) Aerial hyphae differentiated into chains of spores with heavily decorated surfaces.

minimal medium containing mannitol in triplicate at 8, 15, 20, 28, 32 and 37 °C. The strain grew at temperatures between 20 and 37 °C, with the best temperature range being between 28 and 32 °C. Growth was monitored every day for the course of 4 (carbon source and salt tolerance) to 6 days (temperature range).

Analysis of cellular fatty acids, whole-cell sugars, and cell wall characteristics was carried out by the Identification Service, German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany, using bacteria harvested from cultures grown in BAP in the exponential phase. Galactose, glucose and ribose were detected as whole-cell sugars. The diagnostic diamino acid of the cell wall peptidoglycan was LL-diaminopimelic acid; thus CMB-FB contains a type I peptidoglycan [49]. The fatty acid composition is given in Table 1 (minor components are listed in Table S5). The main respiratory quinones were MK-9(H₈) (56.4%), MK-9(H₆) (28.5%), MK-9(H₄) (13.9%) and MK-9(H₁₀) (1.2%). The polar lipid profile consisted of phosphatidylethanolamine, unidentified aminolipids and phospholipids, a glycolipid and four unidentified lipids (Fig. S2).

Altogether, the spore morphology clearly distinguishes CMB-FB from the closely related species *S. rishiriensis* and *S. phaeofaciens*, which produce cylindrical spores with smooth surfaces in spiral chains [50]. The morphology of the strains of

Table 1. Fatty acid composition of CMB-FB

Fatty Acid	Percentage in CMB-FB
anteiso-C _{15:0}	13.4
iso-C _{16:0}	25.7
anteiso-C _{17:0}	10.8

S. cacaui subsp. *asoensis* and *S. humidus* has never been characterized, since interest was focused on their secondary metabolites [51, 52]; however, the antiSMASH results indicate that CMB-FB can produce neither polyoxins nor glebomycin (Table S4). Thus, while a comparison based on chemotaxonomic features is not possible, morphological features and secondary metabolism support the results of the genome analysis that CMB-FB represents a novel species.

PROTOLOGUE

Description of *Streptomyces coriariae* sp. nov

Streptomyces coriariae sp. nov. (co.ri.a'ri.ae. N.L. gen. n. coriariae, of the plant genus *Coriaria*); referring to the origin of the strain from nodules of a *Coriaria intermedia* bush growing in the Philippine province of Benguet.

Aerobic, Gram-staining-positive, mesophilic, filamentous actinobacterium that forms curly aerial hyphae, but does not seem to form agar penetrating substrate hyphae on ISP2 medium. The surfaces of aerial hyphae are smooth, but become increasingly decorated towards the extremities. Aerial hyphae differentiate into chains of white cylindrical spores, 0.6–0.9 µm in length, with heavily decorated surfaces. Its major menaquinones are composed of MK-9(H₄), MK-9(H₆) and MK-9(H₈) and there is a minor contribution of MK-9(H₁₀). The diagnostic diamino acid of its peptidoglycan is meso-diaminopimelic acid. Its polar lipid profile contains phosphatidylethanolamine, unidentified aminolipids and phospholipids, a glycopospholipid and four unidentified lipids. The major fatty acids are iso-C_{16:0}, anteiso-C_{15:0} and anteiso-C_{17:0}. Grows well on ISP2, YMA, streptomyces minimal medium and Qmod agar plates. No pigment production was detected on the media examined. In liquid culture, the strain sporulates readily in ISP2 and YM, while it shows a flakey growth phenotype in Qmod medium and growth in liquid streptomyces minimal medium leads to biofilm formation; the best biomass production is observed in BAP medium. Grows at 20–37 °C (optimally at 28–32 °C), pH 6.0–8.0 and in the presence of 0–4% (w/v) NaCl. Good growth is observed on streptomyces minimal medium agar plates with the following carbon sources: maltose, sucrose, rhamnose, mannitol or *myo*-inositol.

The type strain is CMB-FB^T (= DSM 112754^T= LMG 32457^T), which was isolated from root nodules of *Coriaria intermedia* collected on Mount Pulag (Luzon, Philippines). The DNA G+C content of the type strain is 71.21% and the genome size is 9.324 Mb. The ENA/Genbank/DDBJ accession numbers for the draft genome sequence are CAJYZZ010000001–CAJYZZ010000211. The accession number of the 16S sequence is OU572639.

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Author contributions

Conceptualization, F.B., C.M.B., J.S. and K.P.; investigation, F.B., C.M.B., J.W., and A.B.; formal analysis, D.W.; supervision, K.P., J.K. and J.S.; writing – original draft, F.B. and K.P.; writing – review and editing, all authors; funding acquisition, K.P.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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