

Phyllobacterium catacumbae sp. nov., a member of the order 'Rhizobiales' isolated from Roman catacombs

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Two strains were isolated from tuff, a volcanic rock that forms the walls of the Roman Catacombs of Saint Callixtus in Rome, Italy. A polyphasic approach using nutritional and physiological tests, reactions to antibiotics, fatty acid profiles, DNA base ratios, DNA–DNA reassociation and 16S rRNA gene sequence comparisons showed that the two isolates belong to a novel species within the genus *Phyllobacterium*. The species *Phyllobacterium catacumbae* sp. nov. is proposed. The type strain is CSC19^T (=CECT 5680^T=LMG 22520^T).

The genus *Phyllobacterium* was described by Knösel (1962), who defined two species, *Phyllobacterium myrsinacearum* and *Phyllobacterium rubiacearum*, isolated from leaf nodules of tropical ornamental plants (myrsine and madder, respectively). Both species were described and differentiated on the basis of phenotypic features. The genus name was not included in the Approved Lists (Skerman *et al.*, 1980) but was revived in the first edition of *Bergey's Manual of Systematic Bacteriology* by Knösel (1984a) and subsequently validly published (Knösel, 1984b). Based on fatty acid compositions and DNA–DNA hybridization, Mergaert *et al.* (2002) proposed that *P. rubiacearum* was a later heterotypic synonym of *P. myrsinacearum*.

This paper reports the isolation of two strains, CSC19^T (=LMG 22520^T=CECT 5680^T) and CSC32 (=LMG 22521=CECT 5681), from tuff, a volcanic rock that forms the walls of the Roman Catacombs of Saint Callixtus in Rome, Italy. A polyphasic approach showed that these isolates belong to a novel species within the genus *Phyllobacterium*.

Strains CSC19^T and CSC32 were isolated in B-4 medium composed (per litre) of 2.5 g calcium acetate, 4 g yeast extract and 15 g agar, pH 8 (Boquet *et al.*, 1973). Cultures were incubated at 28 °C. Morphological studies were carried out from cultures on tryptose soy agar (TSA; Oxoid). Wet slide suspensions of cultures grown in tryptose soy broth

(Oxoid) were observed by phase contrast microscopy. Acid production from a variety of substrates was tested using the API 50 CH B/E kit (bioMérieux) and assimilation tests were carried out using the API 20 NE kit (bioMérieux); API tests were performed according to the manufacturer's instructions. Nitrate reduction, catalase and hydrogen sulfide production, hydrolysis of Tweens 20 and 80, methyl red and Voges–Proskauer reactions were tested according to Lanyi (1987). Indole production (Smibert & Krieg, 1994), hydrolysis of starch (Cowan & Steel, 1965), 3-ketolactose (Bernaerts & De Ley, 1963) and DNase activities (Soto-Hernandez *et al.*, 1988) were also tested. Oxygen requirement for growth was studied using a GENbag Anaer incubation system (bioMérieux). Susceptibility to antibiotics was studied by placing antibiotic discs (Mast Diagnostics) on TSA plates inoculated with suspensions of the test strains. Oxidase activity was studied by monitoring the oxidation on dryslide oxidase (Becton Dickinson). For the Gram reaction, a 3% solution of potassium hydroxide was used (Haleblian *et al.*, 1981). Flagella were stained with flagella stain droppers (Becton Dickinson). N₂ fixation was evaluated as a growth response in minimal medium composed (per litre) of: 0.5 g CaCl₂·2H₂O, 0.5 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.2 g NaCl, 0.1 g FeCl₃, 10 ml trace element solution (Balch *et al.*, 1979) and 0.5 g sucrose, pH 6.8–7.0. Cultures were prepared in the presence and absence of ammonium (0.25 g NH₄Cl l⁻¹). A medium without ammonium was inoculated with the different strains and 1 ml was transferred to fresh medium both in the absence and presence of ammonium. Repeated transfers in ammonium-deficient medium revealed the inability of

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene sequences of *P. catacumbae* strains CSC32 and CSC19^T are respectively AY635999 and AY636000.

strains CSC19^T and CSC32, as well as the *P. myrsinacearum* type strain, to grow in the absence of ammonium. Morphological, physiological and biochemical traits are summarized in the species description and Table 1.

Cellular fatty acid profiles were analysed following standard methods described by Gonzalez *et al.* (2004). The predominant fatty acids (> 5%) of strains CSC19^T and CSC32 are shown in Table 2. The major fatty acid in *P. myrsinacearum* was 18:1 ω 7c (Mergaert *et al.*, 2002), as in strains CSC19^T and CSC32. Fatty acid 11-methyl 18:1 ω 7c was a minor constituent (2.3%) in *P. myrsinacearum*.

Bacterial DNA was extracted following the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR using the conserved primers 27F (5'-AGAGTTTGATCCTGGCTCAG) and 1522R (5'-AAGG-AGGTGATCCAGCCGCA). PCR thermal conditions were as follows: 95 °C for 1 min; 35 cycles of 95 °C for 15 s, 55 °C for 15 s, 72 °C for 2 min; and a final extension cycle at 72 °C for 10 min. Forward and reverse strands of the amplified DNA fragment were sequenced in an ABI 3700 sequencer

Table 1. Phenotypic characteristics of strains CSC19^T, CSC32 and *P. myrsinacearum*

Taxa: 1, *P. myrsinacearum*; 2, strain CSC19^T; 3, strain CSC32. All three taxa bear polar, subpolar or lateral flagella, stain negative in the Gram reaction and do not grow in the presence of 2% NaCl. *P. catacumbae* strains CSC19^T and CSC32 are negative for hydrolysis of β -galactoside, gelatin, 3-ketolactose, starch and Tweens 20 and 80. They are positive for the hydrolysis of arginine, β -glucoside and urea. They are positive for the production of hydrogen sulfide. Catalase and oxidase tests give positive results. Voges-Proskauer, methyl red and indole tests give negative results. DNase activity is negative. They are susceptible to doxycycline (30 μ g), framycetin (50 μ g), novobiocin (30 μ g) and tetracycline (30 μ g) and resistant to streptomycin (10 μ g). -, Negative; +, positive; (+) weakly positive.

Characteristic	1	2	3
Nitrate reduction	+*	-	-
Acid production (API 50 CH B/E) from:			
D-Adonitol	+*	-	-
Dulcitol	+*	-	-
Erythritol	-	+	+
Glycerol	+*	-	-
Lactose	-	+	(+)
Methyl α -D-glucopyranoside	+	-	-
N-Acetylglucosamine	-	+	+
L-Sorbose	+	-	-
D-Tagatose	+	-	-
Xylitol	+	-	-
Assimilation (API 20 NE) of:			
Trisodium citrate	+	-	-

*Data from Mergaert *et al.* (2002).

Table 2. Comparison of the major fatty acids found in *P. myrsinacearum* and strains CSC19^T and CSC32

Taxa: 1, *P. myrsinacearum*; 2, strain CSC19^T; 3, strain CSC32. Values represent percentages of total fatty acid content and standard deviations are showed in parentheses. Data for *P. myrsinacearum* were taken from Mergaert *et al.* (2002). ND, Not detected.

Fatty acid	1	2	3
16:0	6.0-9.1	16.0 (0.3)	16.9 (0.8)
16:0 3-OH	5.4-5.7	0.5 (0.0)	0.5 (0.0)
17:0 cyclo	ND	1.1 (0.1)	1.3 (0.1)
17:0	ND	1.1 (0.2)	1.8 (0.0)
18:1 ω 7c	61.0-64.9	47.9 (2.7)	44.2 (1.8)
18:0	ND-3.3	2.4 (0.1)	2.1 (0.2)
11-methyl 18:1 ω 7c	ND-2.3	10.6 (0.7)	11.2 (1.0)
19:0 cyclo ω 8c	4.8-6.2	13.7 (1.3)	15.0 (1.9)
18:1 2-OH	4.5-5.2	ND	ND
20:0	3.7-4.4	ND	ND

(Applied Biosystems). A homology search was performed using the BLAST algorithm (Altschul *et al.*, 1990) at NCBI (National Centre for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/>). Sequence alignment was performed using the software suite ARB (Ludwig *et al.*, 2004). The alignment was manually edited considering the expected sequence secondary structure. An unrooted phylogenetic tree was constructed by maximum-likelihood using the program fastDNAmL (Felsenstein, 1981) embedded in ARB. The obtained tree topology was reconstructed by quartet-puzzling using the program Treepuzzle (Strimmer & von Haeseler, 1996) also available in the ARB package. The quartet-puzzling tree represented a consensus tree showing well-supported branching. It was based on 1000 puzzling trials. The reliability value of each internal branch indicates as a percentage how often the corresponding cluster was found. Comparisons of 16S rRNA gene sequences from strains CSC19^T and CSC32 revealed similarities of 97.4 and 97.2% with *P. myrsinacearum* strains DSM 5892^T and DSM 5893, respectively. A phylogenetic tree showing the relationships between *P. myrsinacearum*, isolates CSC19^T and CSC32 and members of closely related genera is shown in Fig. 1.

The G+C content of genomic DNA was determined following the fluorimetric method described by Gonzalez & Saiz-Jimenez (2002) using the thermal denaturation temperature. The DNA G+C contents of strains CSC19^T and CSC32 were 55.9 \pm 0.4 and 53.8 \pm 0.6 mol%, respectively. These values are slightly lower than the proposed for the emended species *P. myrsinacearum*, 60.3-61.3 mol% (Mergaert *et al.*, 2002).

The degree of DNA-DNA relatedness between the two isolates and *P. myrsinacearum* was determined by two independent methods: the DNA-DNA hybridization method described by Ziemke *et al.* (1998) and by measuring

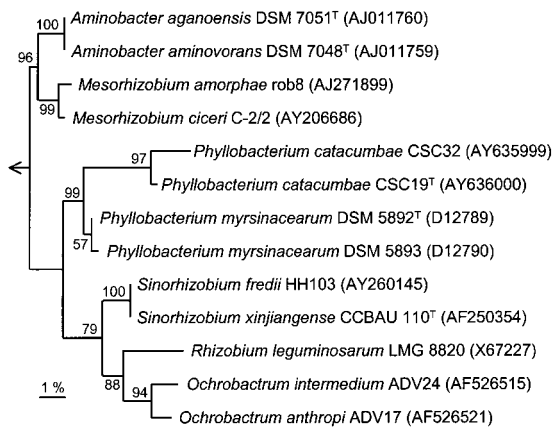


Fig. 1. Phylogenetic tree showing the relationships between *P. myrsinacearum*, strains CSC19^T and CSC32 proposed as *P. catacumbae*, and members of closely related genera. Reliability values are given at branching points as percentages from 1000 trials. Strain name and accession number for the strains represented in the tree are shown in parentheses. *Escherichia coli* O157:H7 (GenBank accession no. AY513502) was used as the outgroup.

the divergence between the thermal denaturation midpoint of homoduplex DNA and heteroduplex DNA (ΔT_m) as described by Gonzalez & Saiz-Jimenez (2005). Strains CSC19^T and CSC32 showed 100% DNA–DNA hybridization and no significant ΔT_m was observed. However, the hybridization between CSC19^T and *P. myrsinacearum* DSM 5892^T was 43.4% and the two strains showed a ΔT_m of >9 °C (equivalent to <45% hybridization as proposed by Rosselló-Mora & Amann, 2001). Altogether, these results indicate that both strains show sufficient genomic coherence and sufficient hybridization differences from their closest relatives to be considered as a single species (Rosselló-Mora & Amann, 2001; Stackebrandt *et al.*, 2002).

The two strains under study derived from the same hypogean environment, the Roman Catacombs of Saint Callixtus. A polyphasic approach, considering both genotypic and phenotypic characteristics, suggests a divergent classification of strains CSC19^T and CSC32 within the genus *Phyllobacterium* (Lambert *et al.*, 1990). On this basis, we propose a novel species, *Phyllobacterium catacumbae* sp. nov. comprising two strains, CSC19^T and CSC32.

Members of the genus *Phyllobacterium* were described as the second most frequently occurring bacteria on root surfaces during a large-scale assessment of the rhizobacterial communities of young sugar beet plants. Extensive analyses of the microflora from the rhizoplane of other crop plants did not reveal the presence of strains of *Phyllobacterium* (Lambert *et al.*, 1990). This paper describes a novel species, *P. catacumbae*, inhabiting the walls of Roman catacombs. This novel species occupies a completely different niche and poses some questions about the relationship of members of the genus *Phyllobacterium* with plants.

Description of *Phyllobacterium catacumbae* sp. nov.

Phyllobacterium catacumbae (ca.ta.cum'bae. L. gen. n. *catacumbae* of catacombs).

Gram-negative, aerobic cells, motile by a polar tuft of flagella. Colonies are circular, smooth and beige with a colony diameter of less than 1 mm after 2 days of incubation; after 6 days, colonies reach a maximum diameter of 4 mm. Cells are short rods, 0.4–1.9 µm long and 0.4–0.7 µm wide, in exponential growth. Optimum temperature for growth is 28 °C. Phenotypic characteristics including antibiotic susceptibility are shown in Table 1. In addition, acid is produced from DL-arabinose, D-cellobiose, aesculin, D-fructose, DL-fucose, D-galactose, D-glucose, inositol, D-lyxose, D-maltose, D-mannitol, D-mannose, D-melibiose, N-acetylglucosamine, L-rhamnose, D-ribose, D-sucrose, D-sorbitol, D-trehalose, D-turanose and DL-xylose. Glucose, arabinose, mannose, mannitol, N-acetylglucosamine, maltose, potassium gluconate and malate are assimilated. Predominant fatty acids are 18:1 ω 7c, 16:0, 19:0 cyclo ω 8c and 11-methyl 18:1 ω 7c. The DNA G + C content is 55.9 ± 0.4 mol%.

The type strain is CSC19^T (= CECT 5680^T = LMG 22520^T). The organism was isolated from the tuff walls of the Roman catacombs of Saint Callixtus, Rome, Italy.

Emended description of the genus *Phyllobacterium* (ex Knösel 1962) Knösel 1984

The description is as given by Knösel (1984a) with the following modifications. Acid production from adonitol, dulcitol and glycerol is variable. Members of the genus *Phyllobacterium* are not restricted to tropical leaf nodules but can also be present in hypogean environments. The DNA G + C content ranges from 53.8 to 61.3 mol%. The type species is *Phyllobacterium myrsinacearum*. The genus includes two species, *P. myrsinacearum* (heterotypic synonym *P. rubiacearum*) and *P. catacumbae*.

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