Methylovirgula ligni gen. nov., sp. nov., an obligately acidophilic, facultatively methylotrophic bacterium with a highly divergent *mxaF* gene

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Two strains of Gram-negative, aerobic, non-pigmented, non-motile, rod-shaped bacteria were isolated from beechwood blocks during decay by the white-rot fungus *Hypholoma fasciculare* and were designated strains BW863[T] and BW872. They are capable of methylotrophic growth and assimilate carbon via the ribulose-bisphosphate pathway. In addition to methanol, the novel isolates utilized ethanol, pyruvate and malate. Strains BW863[T] and BW872 are obligately acidophilic, mesophilic organisms capable of growth at pH 3.1–6.5 (with an optimum at pH 4.5–5.0) and at 4–30 °C. Phospholipid fatty acid profiles of these bacteria contain unusually large amounts (about 90 %) of $C_{18:1}\omega 7c$, thereby resembling the profiles of *Methylobacterium* strains. The predominant quinone is Q-10. The DNA G+C content of the novel isolates is 61.8–62.8 mol%. On the basis of 16S rRNA gene sequence similarity, strains BW863[T] and BW872 are most closely related to the acidophilic methanotroph *Methylocapsa acidiphila* B2[T] (96.5–97 %). Comparative sequence analysis of *mxaF*, the gene encoding the large subunit of methanol dehydrogenase, placed the MxaF sequences of the two novel strains in a cluster that is distinct from all previously described MxaF sequences of cultivated methylotrophs. The identity between the MxaF sequences of the acidophilic isolates and those from known alpha-, beta- and gammaproteobacterial methylotrophs was respectively 69–75, 61–63 and 64–67 %. The data therefore suggest that strains BW863[T] and BW872 represent a novel genus and species of methylotrophic bacteria, for which the name *Methylovirgula ligni* gen. nov., sp. nov. is proposed. Strain BW863[T] (=DSM 19998[T] =NCIMB 14408[T]) is the type strain of *Methylovirgula ligni*.

Aerobic methylotrophic bacteria are a specialized group of micro-organisms that can utilize reduced carbon substrates with no carbon–carbon bonds as a sole source of carbon and energy (Lidstrom, 2006). Methanol is the major substrate for non-methanotrophic methylotrophs that inhabit terrestrial environments, where it is produced during the decomposition of fallen woody materials and plant debris (Ander & Eriksson, 1984; Warneke *et al.*, 1999). Most terrestrial environments in the Northern hemisphere, such as forest soils and wetlands of the temperate and boreal zones, are acidic (pH 3–5). Identification of active methylotroph populations in an acidic forest soil by stable-isotope probing (SIP) with ^{13}C-methanol suggested that a novel group of as-yet-uncultiv-

ated methylotrophs within the *Alphaproteobacteria* was a key player in this process (Radajewski *et al.*, 2000, 2002). In this paper, we describe two representatives of this previously uncultivated group of bacteria and propose a novel genus and species, *Methylovirgula ligni* gen. nov., sp. nov., for these obligately acidophilic methylotrophs.

Two strains of Gram-negative bacteria were isolated from beechwood blocks incubated on the surface of an acidic (pH 3.3–3.6) forest soil during decay by the white-rot fungus *Hypholoma fasciculare* (Folman *et al.*, 2008), and were designated strains BW863[T] and BW872. The media used for isolation of strains BW863[T] and BW872 were water yeast agar of pH 5 (medium W5) and 1/10-strength trypticase soy broth agar of pH 5 (medium T5), respectively. Medium W5 had the following composition (per litre of demineralized water): NaCl, 1 g; yeast extract (Difco Technical grade), 0.1 g; 2-[*N*-morpholino]ethanesulfonic acid (MES; Sigma) as a buffering compound, 1.95 g; and agar (Baker's Agar Technical), 20 g. Medium T5 contained (per litre): NaCl, 1 g; trypticase soy broth (Oxoid), 3 g; MES, 1.95 g; and agar, 20 g. Details of the isolation procedure were published previously (Folman *et al.*, 2008). Partial sequencing of the 16S rRNA genes from these novel isolates showed that they are affiliated with the *Alphaproteobacteria* and are most closely related (97 % sequence similarity) to the acidophilic, obligately methanotrophic bacterium *Methylocapsa acidiphila* B2[T]. This unexpected finding suggested that the isolates may be capable of C_1 metabolism and that the media used to isolate these bacteria may not support their optimal growth. The latter became evident with further attempts to maintain strains BW863[T] and BW872 on media W5 and T5, since only a trace of growth could be observed after 3–4 weeks of incubation. Replacement of W5 and T5 with slightly modified medium M2 (designated medium MM2), which is used routinely for cultivation of *Methylocapsa acidiphila* (Dedysh *et al.*, 2002), resulted in good development of strains BW863[T] and BW872 when methanol was provided as a growth substrate. Thus, further maintenance and cultivation experiments with these bacteria were performed using medium MM2 of the following composition (per litre distilled water): KH_2PO_4, 100 mg; $(NH_4)_2SO_4$, 200 mg; $MgSO_4$, 50 mg; $CaCl_2 . 2H_2O$, 20 mg; yeast extract, 10 mg; methanol, 0.5 % (v/v); pH 4.8–5.2. For growth in liquid media, 500 ml screw-capped serum bottles were used with a headspace/liquid space ratio of 4 : 1. After inoculation, methanol was added aseptically to the cultures and the bottles were capped with silicone rubber septa to prevent loss of methanol by evaporation. Bottles were incubated on a rotary shaker (120 r.p.m.) at 24 °C.

Morphological observations and cell-size measurements were made with a Zeiss Axioplan 2 microscope and Axiovision 4.2 software (Zeiss). For the preparation of ultrathin sections, cells of exponentially growing cultures were collected by centrifugation and pre-fixed with 1.5 % (w/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 1 h at 4 °C and then fixed in 1 % (w/v) OsO_4 in the same buffer for 4 h at 20 °C. After dehydration in an ethanol series, samples were embedded in Epon 812 epoxy resin. Thin sections were cut on an LKB-2128 Ultrotome, stained with 3 % (w/v) uranyl acetate in 70 % (v/v) ethanol and then post-stained with lead citrate (Reynolds, 1963) at 20 °C for 4–5 min. Specimen samples were examined with a JEM-100B transmission electron microscope at 80 kV accelerating voltage.

Physiological tests were performed in liquid medium MM2 with methanol as the sole growth substrate. Growth of isolates was monitored by nephelometry at 600 nm for 3 weeks under a variety of growth conditions, including temperatures of 4–37 °C, pH 2.9–8.0 and NaCl concentrations of 0.01–2 % (w/v). Variations in pH were achieved by mixing 0.1 M solutions of H_2SO_4 and NaOH. The range of potential growth substrates was examined using 0.05 % (w/v) concentrations of the following carbon sources: formate, formamide, formaldehyde, methylamine, dimethylamine, trimethylamine, glucose, arabinose, xylose, lactose, maltose, rhamnose, raffinose, sucrose, sorbose, fructose, acetate, malate, oxalate, pyruvate, succinate, citrate, mannitol, *myo*-inositol and ethanol. The ability to grow with methane as the sole growth substrate was tested with 10–20 % (v/v) methane in the headspace of experimental bottles. Nitrogen sources were tested by replacing $(NH_4)_2SO_4$ in MM2 with 0.05 % (w/v) KNO_3, $NaNO_2$, urea, glycine, alanine or yeast extract.

Cell biomass for cellular fatty acid and isoprenoid quinone analyses and for DNA extraction was obtained from batch cultures grown in liquid medium MM2 with methanol at 24 °C for 7 days. Isoprenoid quinones were extracted according to Collins (1985) and analysed using an LCQ Advantage Max tandem-type mass spectrometer and a Finnigan Mat 8430 ionization mass spectrometer. Fatty acid analysis was performed as described previously (Dedysh *et al.*, 2007). Polar lipids were extracted from cell biomass with chloroform/methanol (1 : 2, v/v) by stirring for 1 h in an ice bath, followed by centrifugation at 5000 *g* for 20 min; during this time, the two phases were separated. The procedure of extraction was performed twice and the upper phases were collected and combined. Two millilitres of chloroform and 2 ml distilled water were added to the supernatant and the resulting mixture was subjected to careful shaking for 15 min in an ice bath. The mixture was then centrifuged and, during this time, three phases were separated. The lower phase with polar lipids was withdrawn, evaporated at 30 °C and dissolved in 200 µl chloroform. Polar lipids were separated by two-dimensional TLC (Kieselgel 60, 10 × 10 cm; Merck) using chloroform/methanol/7 M sodium hydroxide (60 : 30 : 4 by vol.) in the first direction followed by chloroform/methanol/acetic acid/water (85 : 12.5 : 12.5 : 3 by vol.) in the second direction. Plates were sprayed with various specific reagents for detection of different phospholipids (Kates, 1972). Phospholipid standards (Sigma) were used for identification of phospholipids during comparative analysis.

The DNA base composition was determined by thermal denaturation using a Unicam SP1800 spectrophotometer at a heating rate of 0.5 °C min^{-1}. The G+C content was calculated according to Owen et al. (1969). PCR-mediated amplification of the 16S rRNA gene was performed using primers 9f and 1492r and reaction conditions described by Weisburg et al. (1991). Partial fragments of the mxaF gene (encoding the large subunit of methanol dehydrogenase), the nifH gene (encoding dinitrogenase reductase) and the cbbL gene [encoding the large subunit of form I ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)] were amplified using primers and reaction conditions described, respectively, by McDonald & Murrell (1997), Dedysh et al. (2004b) and Spiridonova et al. (2004). The PCR-amplified gene fragments were purified using the Wizard PCR Preps DNA Purification System (Promega) and sequenced on an ABI Prism 377 DNA sequencer (PE Applied Biosystems). Phylogenetic analysis was carried out using the ARB program package (Ludwig et al., 2004).

Small (0.5–1 mm), opaque, light-cream, circular colonies with entire edges and a smooth surface developed on agar medium MM2 after incubation for 2 weeks at 24 °C. Liquid cultures displayed white turbidity; a surface pellicle was not formed. Cells of strains BW863^T and BW872 were Gram-negative, non-motile, straight or slightly curved, thin rods, 0.3–0.65 μm wide and 1.2–2.5 μm long. They reproduced by binary fission and occurred singly or were arranged in rosettes and misshapen cell clusters (Fig. 1a). The formation of rosettes was most pronounced in old cultures (2 weeks or more). No capsule formation and no resting cell forms were observed. Overall, the cell morphology of the novel isolates was strikingly different from that of Methylocapsa acidiphila B2^T (Supplementary Fig. S1, available in IJSEM Online).

Thin sections were prepared from both plate and liquid cultures of strains BW863^T and BW872 (Fig. 1b–d). Cells grown on agar medium possessed 5-nm-thick pilus-like structures that covered both cell poles (Fig. 1b). The nature and function of these structures remain unknown; remarkably, they were not observed in cells grown in liquid cultures. Regardless of the growth conditions used, a well-developed system of intracytoplasmic membranes (ICM) typical of Methylocapsa acidiphila B2^T and other obligate methanotrophs was absent from cells of strains BW863^T and BW872. However, more precise examination of cross-sections (Fig. 1c) revealed that a few loosely arranged, rudimentary ICM were visible in some cells. Similar to members of the genus Methylocella, intracellular granules of poly-β-hydroxybutyrate were formed at each cell pole in strains BW863^T and BW872 (Fig. 1d).

Despite their close phylogenetic relationship to the methanotrophic bacterium Methylocapsa acidiphila B2^T, the novel isolates were unable to grow on methane. All attempts to amplify a pmoA gene fragment (encoding the
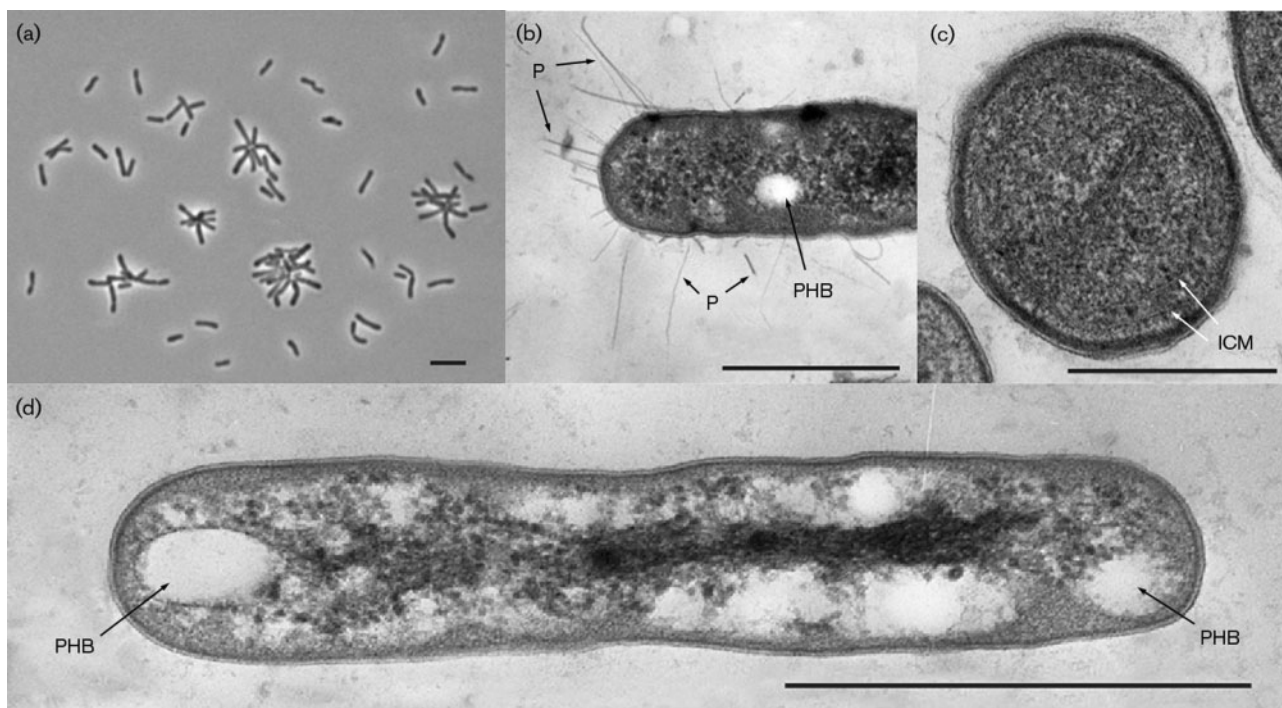
Fig. 1. (a) Phase-contrast micrograph of cells of strain BW863^T grown in liquid mineral medium with methanol for 10 days. (b–d) Electron micrographs of ultrathin sections of cells of strain BW863^T grown on solid mineral medium with methanol. PHB, Poly-β-hydroxybutyrate; P, pilus-like structures; ICM, intracytoplasmic membranes. Bars, 5 μm (a) and 0.5 μm (b–d).

large subunit of the particulate methane monooxygenase) from DNA of strains BW863[T] and BW872 using any of the primer sets A189/A682 (Holmes *et al.*, 1995), A189/Mb661r (Costello & Lidstrom, 1999), A189/Mcap630r and A189/Forest675r (Kolb *et al.*, 2003) for this gene were unsuccessful.

The major carbon and energy source for strains BW863[T] and BW872 was methanol. It was utilized over a wide range of concentrations (0.01–2 %), with an optimum between 0.5 and 1 % (v/v). None of the other C_1 compounds tested supported growth of these strains. In addition to methanol, the novel isolates were able to utilize ethanol, malate and pyruvate, but they did not grow on a range of sugars or other multicarbon compounds tested. Thus, strains BW863[T] and BW872 should be regarded as restricted facultatively methylotrophic bacteria. Both isolates had an absolute requirement for the presence of growth factors in the cultivation medium (20–50 mg yeast extract l^{-1}). They utilized ammonium salts, nitrates and yeast extract as nitrogen sources. Strains BW863[T] and BW872 were capable of slow growth in liquid nitrogen-free medium under microaerobic conditions (1.0–2.0 % O_2 in flask headspace). The sequences of *nifH* gene fragments from these

methylotrophs displayed highest similarity (86–88 %) to the corresponding gene fragments from acidophilic methanotrophs of the genus *Methylocella* and members of the genus *Bradyrhizobium*.

The isolates were obligately acidophilic and grew in the pH range 3.1–6.5, with an optimum at pH 4.5–5.0 (Supplementary Fig. S2). The temperature range for growth was 4–30 °C (optimum at 20–24 °C). No growth occurred at 37 °C. Growth of strains BW863[T] and BW872 was inhibited in the presence of NaCl in the medium at concentrations above 0.7 % (w/v).

Phospholipid fatty acid (PLFA) profiles of the novel methylotrophic isolates contained unusually large amounts of 11-*cis*-octadecenoic fatty acid ($C_{18:1}\omega 7c$), which comprised 87–92 % of the total PLFAs (Table 1), and were remarkably similar to the profiles described for representatives of the genus *Methylobacterium* (Kato *et al.*, 2005, 2008). The latter, however, contain larger amounts of $C_{18:0}$ and do not possess $C_{19:0}$ cyclo, which is one of the characteristic PLFAs in strains BW863[T] and BW872. The predominance of $C_{18:1}\omega 7c$ in the PLFA profiles is also a feature specific to acidophilic methanotrophs of the genera

Table 1. PLFA compositions of strains BW863[T] and BW872 in comparison with phylogenetically related acidophilic methanotrophs

Values are percentages of total PLFAs; components representing <0.1 % of the total are not included. Major PLFAs are shown in bold. Data for reference species were taken from Dedysh *et al.* (2002) (*Methylocapsa acidiphila*) and from Dedysh *et al.* (2000, 2004a) and Dunfield *et al.* (2003) (*Methylocella* species).

Fatty acid	BW863[T]	BW872	*Methylocapsa acidiphila*	*Methylocella*
$C_{14:0}$	0	0	0	0–4.1
iso-$C_{15:0}$	0	0	0.1	0.2–1.2
$C_{15:0}$	0	0	0	0–0.1
$C_{16:0}$	1.80	1.96	**7.3**	**3.0–7.7**
10-Methyl $C_{16:0}$	0	0.37	0	0
$C_{16:1}\omega 7c$	0.33	0.36	4.7	**4.7–11.3**
$C_{16:1}\omega 7t$	0	0	0	0–5.8
iso-$C_{17:1}\omega 9c$	0	0	0	0–0.3
anteiso-$C_{17:0}$	0	0.21	0	0–0.3
$C_{17:0}$	0.53	0.64	0.1	0–0.1
iso-$C_{17:0}$	0.23	0	0.6	0–2.5
br-$C_{17:0}$	0.23	0.25	0	0
$C_{17:0}$ cyclo	0	0.18	0	0–6.5
$C_{17:1}\omega 8c$	0	0	0	0–0.3
$C_{17:1}\omega 7c$	0.16	0.19	1.0	0
iso-$C_{18:0}$	0	0	0	0–0.5
$C_{18:0}$	0.83	0.91	**7.6**	0.4–1.2
$C_{18:1}\omega 9c$	0	0.41	0	0
$C_{18:1}\omega 8c$	0	0	0	0
$C_{18:1}\omega 7c$	**87.07**	**92.76**	**78.3**	**59.2–82.2**
$C_{18:1}\omega 5c$	0	0	0	0–0.1
$C_{19:0}$ cyclo	2.57	1.39	0	0
$C_{19:1}\omega 8c$ cyclo	0	0	0	**0–13.6**
$C_{19:0}$	0	0	0	0–0.6
$C_{20:0}$	0.11	0.13	0	0

Methylocapsa and *Methylocella* (Dedysh *et al.*, 2000, 2002, 2004a; Dunfield *et al.*, 2003). However, the PLFA profiles of acidophilic methanotrophs also include other major components, such as $C_{16:0}$, $C_{16:1}\omega 7c$, $C_{18:0}$ and $C_{19:1}\omega 8c$ cyclo, while these PLFAs were either present in minor amounts or absent from cells of strains BW863[T] and BW872. Polar lipid profiles of strains BW863[T] and BW872 were nearly identical and consisted of phosphatidylethanolamine (about 35%), phosphatidylcholine (30%) and phosphatidylglycerol (30%). By contrast, the major phospholipids in *Methylocapsa acidiphila* and *Methylocella* species were phosphatidylglycerol and phosphatidylmethylethanolamine, respectively (Dedysh *et al.*, 2000; Fang *et al.*, 2000).

In order to establish the routes of primary C_1 metabolism employed by the novel methylotrophs, enzyme activities were determined in extracts of cells grown on methanol. Since the two strains displayed nearly identical enzyme profiles, results are shown only for strain BW863[T] (Table 2). This bacterium possessed a pyrroloquinoline quinone-containing methanol dehydrogenase, which required alkaline pH and NH_4^+ ions for activity *in vitro* with phenazine methosulfate (PMS) as an artificial electron acceptor. Two formaldehyde-oxidizing enzymes were present, NAD-dependent formaldehyde dehydrogenase and the PMS-linked form. Also, the NAD-dependent and PMS-linked forms of formate dehydrogenase were detected. Thus, strain BW863[T] appears to possess the complete set of enzymes involved in methanol oxidation to CO_2 through formaldehyde and formate that provides metabolic energy for methylotrophic growth. The presence of the two enzymes unique to the ribulose bisphosphate (RuBP) pathway for C_1 assimilation, phosphoribulokinase

and RuBisCO, suggested that the carbon derived from the oxidation of methanol was assimilated at the level of CO_2 via the RuBP pathway. Detection and partial sequence analysis of *cbbL*, the gene encoding form-I RuBisCO, provided genotypic evidence for autotrophic metabolism in strains BW863[T] and BW872. The two strains displayed identical *cbbL* gene sequences. The highest nucleotide sequence identity to the *cbbL* gene fragments of the novel methylotrophic isolates was shown by strains of *Beijerinckia mobilis* (86%), *Telmatospirillum sibiriense* (86%) and *Methylocapsa acidiphila* (85%). Neither the serine pathway nor the ribulose monophosphate pathway of C_1 assimilation was operative as a result of the absence of the appropriate specific enzymes, i.e. serine-glyoxylate aminotransferase, malyl-CoA-lyase, glycerate kinase and hexulosephosphate synthase. Isocitrate dehydrogenase was active with NADP.

A phylogenetic tree constructed on the basis of 16S rRNA gene sequences (Fig. 2) indicated that strains BW863[T] and BW872 belong to the family *Beijerinckiaceae* of the class *Alphaproteobacteria*. The closest taxonomically described phylogenetic neighbour of these isolates was the acidophilic, obligately methanotrophic bacterium *Methylocapsa acidiphila* B2[T] (3–3.5% sequence difference). However, 16S rRNA gene sequences from the two novel strains and *Methylocapsa acidiphila* B2[T] did not group together if environmental sequences OP1, OP3 and OP8, which were retrieved by means of the SIP technique from different acidic soils amended with ^{13}C-methanol (Radajewski *et al.*, 2000, 2002), were included in comparative sequence analysis (Supplementary Fig. S3). In this case, strains BW863[T] and BW872 grouped together with these environmental sequences, and the bootstrap value for the common branch point of this cluster was quite high (90%). Other phylogenetically close and taxonomically characterized organisms were acidophilic, facultatively methanotrophic bacteria of the genus *Methylocella* (4.3–4.4% sequence difference) and acidophilic heterotrophic bacteria of the genus *Beijerinckia* (4.3–4.4% sequence difference).

Comparative sequence analysis of *mxaF*, the gene encoding the large subunit of methanol dehydrogenase, placed the deduced MxaF sequences of the two novel strains in a cluster that is distinct from all previously described MxaF sequences of cultivated methylotrophs (Fig. 3). The identity between the deduced MxaF sequences of our isolates and those from known alpha-, beta- and gamma-proteobacterial methylotrophic bacteria was 69–75, 61–63 and 64–67%, respectively. Interestingly, the deduced MxaF sequences of strains BW863[T] and BW872 were closely affiliated (95.6–97.5%) with the inferred peptide sequences of *mxaF* clones retrieved by means of the SIP technique from different acidic soils amended with ^{13}C-methanol (Radajewski *et al.*, 2000; 2002). Thus, our novel isolates are the first representatives of this previously uncultivated group of methylotrophic bacteria, which were earlier detected in acidic terrestrial environments by means of molecular approaches.

Table 2. Enzyme activities in cell extracts of strain BW863[T] grown on methanol

Data are enzyme activities expressed as nmol min^{-1} (mg protein)$^{-1}$.

Enzyme	Cofactor(s)	Activity
Methanol dehydrogenase	PMS	220
Formaldehyde dehydrogenase	PMS	30
	NAD$^+$	10
Formate dehydrogenase	PMS	80
	NAD$^+$	20
3-Hexulosephosphate synthase		0
Hydroxypyruvate reductase	NADH	268
	NADPH	67
Serine-glyoxylate aminotransferase	NADH	0
	NADPH	0
Malyl-CoA synthetase/CoA lyase	ATP, CoA	0
Glycerate kinase	ATP	0
Phosphoribulokinase	ATP	51
Ribulose-1,5-bisphosphate carboxylase		82
Isocitrate dehydrogenase	NAD$^+$	2
	NADP	40
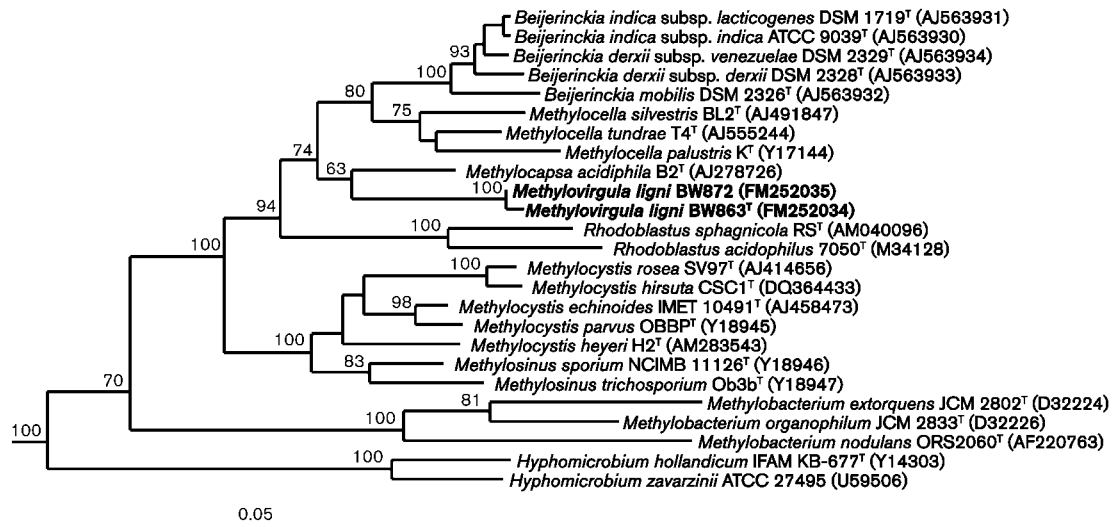
Fig. 2. 16S rRNA gene sequence-based neighbour-joining tree showing the phylogenetic positions of strains BW863[T] and BW872 in relation to other members of the family *Beijerinckiaceae* and some other representatives of the *Alphaproteobacteria*. The sequences of the type I methanotrophs *Methylomicrobium album* ACM 3314[T] (GenBank accession no. X72777), *Methylobacter luteus* NCIMB 11914[T] (AF304195), *Methylomonas methanica* S1[T] (AF304196) and *Methylococcus capsulatus* Texas (AJ563935) were used as an outgroup (not shown). Bootstrap values (percentages of 1000 data resamplings) >50 % are shown. Bar, 0.05 substitutions per nucleotide position.

In summary, our novel isolates from decaying wood possessed a number of characteristics that clearly distinguished them from other methylotrophic members of the family *Beijerinckiaceae* (Table 3). Cell morphology, the absence of a slimy capsule and the requirement for growth factors made strains BW863[T] and BW872 different from all other currently described members of this family. The inability to grow on methane, their

obligate acidophily and the use of the RuBP pathway for C_1 assimilation distinguished them from members of the genera *Methylocapsa* and *Methylocella*. The inability to utilize sugars distinguished the isolates from members of the genus *Beijerinckia*. Therefore, we propose a novel genus and a novel species, *Methylovirgula ligni* gen. nov., sp. nov., to accommodate strains BW863[T] and BW872.
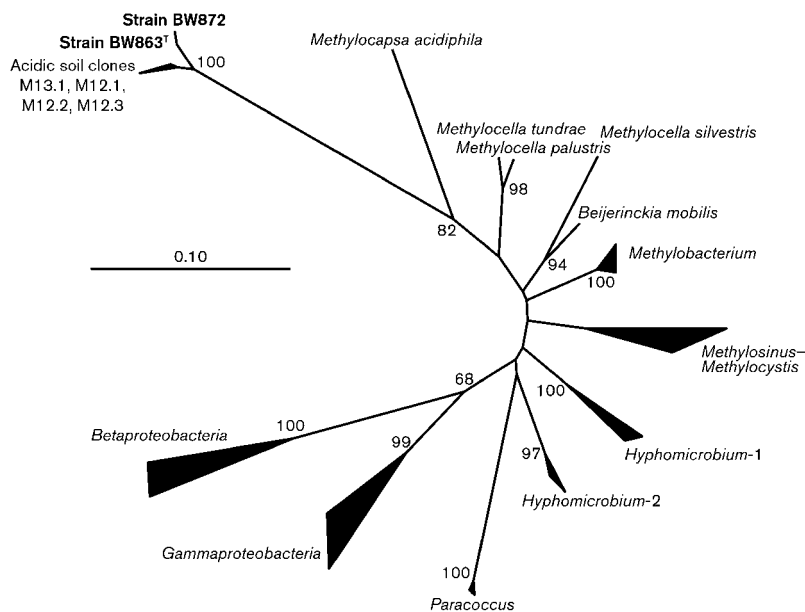
Fig. 3. Unrooted neighbour-joining tree (PAM correction) constructed based on 170 deduced amino acid sites of partial *mxaF* gene sequences, showing the positions of strains BW863[T] and BW872 relative to environmental sequences retrieved in SIP-based studies from acidic soils and other proteobacterial methylotrophs. Bootstrap values (1000 data resamplings) >60 % are shown. Bar, 0.1 substitutions per nucleotide position.

Table 3. Major characteristics that distinguish strains BW863[T] and BW872 (*Methylovirgula ligni* gen. nov., sp. nov.) from other methylotrophic members of the family *Beijerinckiaceae*

Data for reference species were taken from Dedysh *et al.* (2002) (*Methylocapsa acidiphila*), from Dedysh *et al.* (2000, 2004a, 2005b) and Dunfield *et al.* (2003) (*Methylocella*) and from Becking (2006) and Dedysh *et al.* (2005a) (*Beijerinckia mobilis*). V, Variable (present in *Methylocella palustris* and *Methylocella silvestris* and absent in *Methylocella tundrae*); ND, not determined/no data available.

Characteristic	*Methylovirgula ligni*	*Methylocapsa acidiphila*	*Methylocella*	*Beijerinckia mobilis*
Cell morphology	Straight or curved rods	Curved coccoids	Bipolar straight or curved rods	Straight, curved or pear-shaped rods
Cell size (μm)	0.3–0.65 × 1.2–2.5	0.7–1.0 × 0.8–1.2	0.6–1.0 × 1.0–2.5	0.6–1.0 × 1.6–3.0
Rosette formation	+	−	−	−
Capsule	−	+	V	+
Resting cells	None	Cysts	None	None
Type of metabolism	Facultative methylotrophy	Obligate methanotrophy	Facultative methanotrophy	Facultative methylotrophy
Growth on:				
Methane	−	+	+	−
Methanol (% v/v)	Good (0.01–2)	Poor (<0.05)	Good (0.01–5)	Good (0.01–3)
Multicarbon compounds utilized	Pyruvate, malate, ethanol	−	Acetate, pyruvate, succinate, malate, ethanol	Most sugars, some organic acids, alcohols, aromatic compounds
C_1-utilization pathway	RuBP	Serine	Serine	RuBP
Growth factor requirement	+	−	−	−
Growth at/in:				
35 °C	−	−	−	+
pH 7.0	−	+	+	+
pH 3.5	+	−	−	+
0.5 % NaCl	+	−	−	+
Major phospholipid(s)*	PE, PC, PG	PG	PME	ND

*PC, Phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PME, phosphatidylmethylethanolamine.

Description of *Methylovirgula* gen. nov.

Methylovirgula (Me.thy.lo.vir′gu.la. N.Gr. n. *methyl* the methyl group from Gr. n. *methu* wine and Gr. n. *hulê* wood; L. fem. n. *virgula* a little rod; N.L. fem. n. *Methylovirgula* methyl-using rod-shaped cell).

Gram-negative, aerobic, colourless, non-motile, thin rods that occur singly or are arranged in rosettes and misshapen cell clusters. Reproduce by binary fission. Colonies are small, circular, smooth and convex. The colony colour varies from white to cream. Produce poly-β-hydroxybutyrate. Obligately acidophilic and mesophilic. Prefer dilute media of low salt content. Restricted facultative methylotrophs. Assimilate methanol-derived carbon via the RuBP pathway. Capable of atmospheric nitrogen fixation. The major phospholipid fatty acid is $C_{18:1}\omega7c$ and the major phospholipids are phosphatidylethanolamine, phosphatidylcholine and phosphatidylglycerols. The major quinone is Q-10. The G+C content of the DNA varies between 61.8 and 62.8 mol%. Member of the class *Alphaproteobacteria*, family *Beijerinckiaceae*. The type and only species is *Methylovirgula ligni*. Strains have been isolated from acidic soils and decaying wood.

Description of *Methylovirgula ligni* sp. nov.

Methylovirgula ligni (lig′ni. L. gen. n. *ligni* of wood, referring to the isolation of known strains from wood material).

Description as for the genus plus the following traits. Cells are 0.3–0.65 μm wide and 1.2–2.5 μm long. Resting cell forms are absent. Carbon sources include methanol, ethanol, pyruvate and malate. Grows optimally at methanol concentrations of 0.5–1 %. Nitrogen sources used (at 0.05 %, w/v) include ammonia, nitrate and yeast extract. Growth factors are required. Capable of growth at pH 3.1–6.5 (optimum pH 4.5–5.0) and at 4–30 °C (optimum 20–24 °C). NaCl inhibits growth at concentrations above 0.7 % (w/v).

The type strain is strain BW863[T] (=DSM 19998[T] =NCIMB 14408[T]), which was isolated from decaying beechwood blocks incubated on an acidic forest soil from the central part of the Netherlands.

Acknowledgements

References

Ander, P. & Eriksson, K.-E. (1984). Methanol formation during lignin degradation by *Phanerochaete chrysosporium*. *Appl Microbiol Biotechnol* **21**, 96–102.

Becking, J. H. (2006). The genus *Beijerinckia*. In *The Prokaryotes*, 3rd edn, vol. 5, pp. 151–162. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. New York: Springer.

Collins, M. D. (1985). Analysis of isoprenoid quinones. *Methods Microbiol* **18**, 329–366.

Costello, A. M. & Lidstrom, M. E. (1999). Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. *Appl Environ Microbiol* **65**, 5066–5074.

Dedysh, S. N., Liesack, W., Khmelenina, V. N., Suzina, N. E., Trotsenko, Y. A., Semrau, J. D., Bares, A. M., Panikov, N. S. & Tiedje, J. M. (2000). *Methylocella palustris* gen. nov., sp. nov., a new methane-oxidizing acidophilic bacterium from peat bogs, representing a novel subtype of serine-pathway methanotrophs. *Int J Syst Evol Microbiol* **50**, 955–969.

Dedysh, S. N., Khmelenina, V. N., Suzina, N. E., Trotsenko, Y. A., Semrau, J. D., Liesack, W. & Tiedje, J. M. (2002). *Methylocapsa acidiphila* gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from *Sphagnum* bog. *Int J Syst Evol Microbiol* **52**, 251–261.

Dedysh, S. N., Berestovskaya, Y. Y., Vasylieva, L. V., Belova, S. E., Khmelenina, V. N., Suzina, N. E., Trotsenko, Y. A., Liesack, W. & Zavarzin, G. A. (2004a). *Methylocella tundrae* sp. nov., a novel methanotrophic bacterium from acidic tundra peatlands. *Int J Syst Evol Microbiol* **54**, 151–156.

Dedysh, S. N., Ricke, P. & Liesack, W. (2004b). NifH and NifD phylogenies: a molecular basis for understanding nitrogen fixation capabilities of methanotrophic bacteria. *Microbiology* **150**, 1301–1313.

Dedysh, S. N., Smirnova, K. V., Chmelenina, V. N., Suzina, N. E., Liesack, W. & Trotsenko, Y. A. (2005a). Methylotrophic autotrophy in *Beijerinckia mobilis*. *J Bacteriol* **187**, 3884–3888.

Dedysh, S. N., Knief, C. & Dunfield, P. (2005b). *Methylocella* species are facultatively methanotrophic. *J Bacteriol* **187**, 4665–4670.

Dedysh, S. N., Belova, S. E., Bodelier, P. L. E., Smirnova, K. V., Khmelenina, V. N., Chidthaisong, A., Trotsenko, Y. A., Liesack, W. & Dunfield, P. F. (2007). *Methylocystis heyeri* sp. nov., a novel type II methanotrophic bacterium possessing 'signature' fatty acids of type I methanotrophs. *Int J Syst Evol Microbiol* **57**, 472–479.

Dunfield, P. F., Khmelenina, V. N., Suzina, N. E., Trotsenko, Y. A. & Dedysh, S. N. (2003). *Methylocella silvestris* sp. nov., a novel methanotrophic bacterium isolated from an acidic forest cambisol. *Int J Syst Evol Microbiol* **53**, 1231–1239.

Fang, J., Barcelona, M. J. & Semrau, J. D. (2000). Characterization of methanotrophic bacteria on the basis of intact phospholipid profiles. *FEMS Microbiol Lett* **189**, 67–72.

Folman, L. B., Klein Gunnewiek, P. J. A., Boddy, L. & de Boer, W. (2008). Impact of white-rot fungi on numbers and community composition of bacteria colonizing beech wood from forest soil. *FEMS Microbiol Ecol* **63**, 181–191.

Holmes, A. J., Costello, A., Lidstrom, M. E. & Murrell, J. C. (1995). Evidence that particulate methane monooxygenase and ammonium monooxygenase may be evolutionarily related. *FEMS Microbiol Lett* **132**, 203–208.

Kates, M. (1972). *Techniques of Lipidology*. New York: Elsevier.

Kato, Y., Asahara, M., Arai, D., Goto, K. & Yokota, A. (2005). Reclassification of *Methylobacterium chloromethanicum* and *Methylobacterium dichloromethanicum* as later subjective synonyms of *Methylobacterium extorquens* and of *Methylobacterium lusitanum* as a later subjective synonym of *Methylobacterium rhodesianum*. *J Gen Appl Microbiol* **51**, 287–299.

Kato, Y., Asahara, M., Goto, K., Kasai, H. & Yokota, A. (2008). *Methylobacterium persicinum* sp. nov., *Methylobacterium komagatae* sp. nov., *Methylobacterium brachiatum* sp. nov., *Methylobacterium tardum* sp. nov. and *Methylobacterium gregans* sp. nov., isolated from freshwater. *Int J Syst Evol Microbiol* **58**, 1134–1141.

Kolb, S., Knief, C., Stubner, S. & Conrad, R. (2003). Quantitative detection of methanotrophs in soil by novel *pmoA*-targeted real-time PCR assays. *Appl Environ Microbiol* **69**, 2423–2429.

Lidstrom, M. E. (2006). Aerobic methylotrophic prokaryotes. In *The Prokaryotes*, 3rd edn, vol. 2, pp. 618–634. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. New York: Springer.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G. & other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.

McDonald, I. R. & Murrell, J. C. (1997). The methanol dehydrogenase structural gene *mxaF* and its use as a functional gene probe for methanotrophs and methylotrophs. *Appl Environ Microbiol* **63**, 3218–3224.

Owen, R. J., Lapage, S. P. & Hill, L. R. (1969). Determination of base composition from melting profiles in dilute buffers. *Biopolymers* **7**, 503–516.

Radajewski, S., Ineson, P., Parekh, N. R. & Murrell, J. C. (2000). Stable-isotope probing as a tool in microbial ecology. *Nature* **403**, 646–649.

Radajewski, S., Webster, G., Reay, D. S., Morris, S. A., Ineson, P., Nedwell, D. B., Prosser, J. I. & Murrell, J. C. (2002). Identification of active methylotroph populations in an acidic forest soil by stable-isotope probing. *Microbiology* **148**, 2331–2342.

Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J Cell Biol* **17**, 208–212.

Spiridonova, E. M., Berg, I. A., Kolganova, T. V., Ivanovsky, R. N., Kuznetsov, B. B. & Tourova, T. P. (2004). An oligonucleotide primer system for amplification of the ribulose-1,5-bisphosphate carboxylase/oxygenase genes of bacteria of various taxonomic groups. *Microbiology* (English translation of *Mikrobiologiia*) **73**, 316–325.

Warneke, C., Karl, T., Judmaier, H., Hansel, A., Jordan, A., Lindinger, W. & Crutzen, P. J. (1999). Acetone, methanol, and other partially oxidized volatile organic emissions from dead plant matter by abiological processes: significance for atmospheric chemistry. *J Geophys Res* **13**, 9–18.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.