

**Culture-dependent and -independent methods reveal diverse methylotrophic communities in terrestrial environments**

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## **Abstract**

One-carbon compounds such as methanol, dimethylsulfide (DMS) and dimethylsulfoxide (DMSO) are significant intermediates in biogeochemical cycles. They are suggested to affect atmospheric chemistry and global climate. Methylotrophic microorganisms are considered as a significant sink for these compounds, therefore we analysed the diversity of terrestrial bacteria that utilise methanol, DMS and DMSO as carbon and energy source using culture-dependent and -independent methods. The effect of habitat type on the methylotrophic community structure was also investigated in rhizosphere and bulk soil. While thirteen strains affiliated to the genera *Hyphomicrobium*, *Methylobacterium*, *Pseudomonas*, *Hydrogenophaga*, *Rhodococcus*, *Flavobacterium*, and *Variovorax* were isolated, denaturing gradient gel electrophoresis revealed the dominance of *Thiobacillus*, *Rhodococcus*, *Flavobacterium* and *Bacteroidetes* species. Furthermore, methylotrophic communities that degrade methanol or DMS are not shaped by terrestrial habitat type. Rhizosphere and soil samples showed dominance of *Methylophilus* spp. and *Methylovorus* spp. for methanol enrichments; *Cytophaga* spp., *Pseudomonas tremae* and *Thiobacillus thioparus* for DMS enrichments.

Keywords: Dimethylsulfide, isolation, methanol, methylotrophy, rhizosphere, soil.

## **Introduction**

Methylotrophy is a metabolic capacity that allows bacteria to utilize reduced one-carbon compounds as their sole source of carbon and energy (Anthony 1982). A wide range of one-carbon compounds such as methane, methanol, methylated amines, methyl halides and methylated sulfur compounds can be substrates for methylotrophs, highlighting the importance of methylotrophic metabolism for the biogeochemical cycling of carbon, nitrogen and sulfur (Schäfer et al. 2007; Trotsenko et al. 2008; Kolb 2009; Vorholt 2012; Stacheter et al. 2013). Methanol and dimethylsulfide (DMS) are two important methylotrophic substrates in the environment. Methanol is the second most abundant organic compound in the atmosphere after methane and affects ozone formation and the oxidizing capacity of the atmosphere (MacDonald and Fall 1993; Kolb and Stacheter 2013). In terrestrial environments, methanol is mainly released from plants during synthesis of cell wall components with an estimated amount of 100 Tg year<sup>-1</sup> (Galbally and Kirstine 2002). Other terrestrial sources of methanol include plant decay, biomass burning and industrial production (Crocco 1994; Nemecek-Marshall et al. 1995). The industrially produced methanol is used as chemical feedstock, fuel, solvent, antifreeze and hydrate inhibitor as well as a substrate for microorganisms during fermentation, growth of C3 plants (i.e. tomato, wheat, cotton) and sewage treatment (Crocco 1997; Ginige et al. 2009). DMS is the most abundant biologically produced sulfur compound emitted to the atmosphere and is suggested to affect the atmospheric chemistry and the global climate (Charlson et al. 1987; Kalyuzhnaya et al. 2009). DMS emission to the atmosphere from terrestrial ecosystems has been estimated at 3.8 Tg year<sup>-1</sup> (Watts 2000). The majority of this is produced through anaerobic degradation of methoxylated aromatic compounds and

degradation of sulfur containing amino acids like methionine and cysteine (Kiene and Hines 1995; Lomans et al. 1997). In addition, DMS is emitted from trees, cruciferous plants, lichens and wheat. Decay of biomass of brassicas is well-known to produce DMS and other volatile sulfur compounds during decomposition, which is useful for biofumigation of soil borne pests (Gamliel and Stapleton 1993; Bending and Lincoln 1999). Dimethylsulfoxide (DMSO) which is widely found in fruit and vegetables are further sources of DMS in terrestrial habitats due to its reduction to DMS by microorganisms (Zinder and Brock 1978; Pearson et al. 1981; Scarlata and Ebeler 1999; Zhang et al. 2006).

Methylotrophic bacteria in terrestrial habitats can mitigate the net flux of volatile one-carbon compounds to the atmosphere by using them as carbon and energy sources (Kolb 2009). Previous studies showed that aerated forest, rice field, grassland soil and rhizosphere soil can harbor diverse methylotrophic communities (Lueders et al. 2004; Radajewski et al. 2002; Turner et al. 2013; Stacheter et al. 2013; Eyice et al. 2015). Furthermore, plants, one of the main sources of one-carbon compounds, can represent an important habitat for methylotrophic bacteria below or above the ground (Vorholt 2012). Using cultivation-dependent and -independent methods, methylotrophs in the plant phyllosphere have indeed been shown to degrade methanol and methyl chloride, thereby acting as plant-associated sinks for these compounds (Gogleva et al. 2010; Knief et al. 2010; Nadalig et al. 2011). Terrestrial one-carbon compound utilization may also be important from an agricultural point of view as microbial degradation of DMS and DMSO in soil may increase the amount of inorganic sulfur available for plants (Kertesz and Mirleau 2004). To date, several methanol, DMS and DMSO degrading bacteria from more than 50 genera have been isolated from terrestrial

habitats (De Bont et al. 1981; Suylen and Kuenen 1986; Nercessian et al. 2005; Schäfer et al. 2010; Khadem et al. 2011; Giri et al. 2012). However, culture-independent approaches to studying methylotrophic microbial populations have suggested that the diversity of methylotrophs in the environment has not been exhaustively sampled and brought into culture (Mano et al. 2007; Kalyuzhnaya et al. 2008; Chistoserdova et al. 2009). Especially, methanol-utilizing methylotrophs are considered difficult to investigate due to their broad diversity in the environment (Kolb and Stacheter 2013). Also, it is not clear whether there are distinct methylotrophic populations in different terrestrial habitats such as bulk soil and the rhizosphere. Steeghs et al. (2004) showed that *Arabidopsis* roots emit methanol and Galbally and Kirstine (2002) suggested that there may be more methanol in the rhizosphere than in bulk soil due to the release of methanol during plant growth. Presumably, this would lead to higher methylotrophic activity in the rhizosphere compared to bulk soil. In a recent study conducted on soil and rhizosphere of several plants grown in a glass house, Turner et al. (2013) found that distinct methylotrophic microorganisms were selected by different plant species, however it is not known if that would be the case in natural environment. In this study, we sought to explore the diversity of methylotrophs in samples from terrestrial environments using culture-dependent and -independent methods. We were particularly interested to explore the culturable diversity of soil methylotrophs growing on DMS as this aspect has only rarely been addressed in previous studies (Smith and Kelly 1988) and to compare the diversity of enrichment cultures with that of the strains isolated. We also tested whether the habitat type (rhizosphere/bulk soil) has an effect on the methylotrophic community structure in plant-associated environments.

## **Materials and methods**

### *Sampling for isolation of methylotrophs*

Methylotrophic bacteria were isolated from the rhizosphere of greenhouse-grown *Brassica oleracea* (University of Warwick, UK), from moss (*Brachytecium*) and two different soils collected in Warwickshire, UK, in April 2009. Soil samples were obtained from 0 to 10 cm depth of Long Close and Hunts Mill fields of Warwick Crop Centre using ethanol-sterilised trowels and passed through a 3 mm sieve. Long Close soil is a sandy loam soil and had 1.29% carbon and 0.14% nitrogen while Hunts Mill is an organic sandy loam soil and had 1.43% carbon and 0.15% nitrogen (Whitfield 1974). Hunts Mill field has been converted from a conventional cereal rotation and managed as organic soil for 18 years prior to sample collection. All samples were put in plastic bags, transferred to the laboratory immediately and processed within two hours of collection.

### *Sampling for DGGE analysis of plant-associated environments*

Rhizosphere samples from five individual *Brassica oleracea* plants grown in the Hunts Mill field and five bulk soil samples surrounding these plants were obtained from an area of 1.5 x 2.5 m in November 2009. Rhizosphere samples at 5-10 cm soil depth and bulk soil samples within a radius of 10 cm from the centre of the plants were collected and treated as explained above.

### *Enrichment and isolation conditions*

Enrichment cultures were set up in 50-ml sealed serum vials containing 20 ml of basal mineral salts medium (BMS) (Wood and Kelly 1977) and DMS, DMSO or methanol

as the only carbon source to a final concentration of 1 mmol l<sup>-1</sup>, 10 mmol l<sup>-1</sup> and 25 mmol l<sup>-1</sup>, respectively. Approximately 0.5 grams of each soil, rhizosphere and moss sample was used as inoculum. Rhizosphere samples were obtained by shaking the plants to remove the soil not tightly attached to the roots and the roots with the remaining, attached soil were placed in the enrichment bottles. To isolate phyllosphere bacteria, a moss sample was imprinted onto BMS agar plates aseptically and amended with methanol (Chanprame et al. 1996).

The liquid enrichment cultures were incubated for two weeks at 16°C on a 150 rpm rotary shaker, enrichment agar plates were kept in gas-tight jars at 16°C. During this time, the enrichment cultures on plates were periodically aerated and supplied with fresh substrate. Second generation liquid enrichments were set up using 2 ml of the first round of enrichments as the inoculum for a 20 ml culture volume after three additions of methanol, DMS and DMSO. After a further two additions of substrate, 10-fold serial dilutions of liquid samples from the second-generation enrichment bottles were prepared and transferred onto BMS agar plates which were supplemented with the corresponding substrates (methanol, DMS or DMSO). Individual colonies were sampled to represent all distinct colony morphologies, restreaked and isolated. The purity of the isolates was checked on BMS agar plates amended with methanol, DMS or DMSO, on nutrient agar plates, and by microscopic observation. Once pure, each isolate was tested for methanol, DMS and DMSO utilization as the only carbon and energy source.

#### *DMS consumption*

The DMS concentration in the headspace of culture and enrichment bottles was measured using a gas chromatograph (Agilent 5973, Agilent Technologies, Cheshire, UK) by manually injecting 100  $\mu$ l of headspace gas. A flame ionisation detector and a 30 m x 0.32 mm column (DB-1) were used at a column temperature of 200°C.

#### *Identification of bacterial isolates*

Isolates were identified by sequencing of the 16S rRNA genes amplified by PCR using primers 27F and 1492R (Lane 1991). PCR reactions were performed in a total volume of 50  $\mu$ l which contained 10  $\mu$ mol l<sup>-1</sup> of each primer (Invitrogen Life Technologies, Paisley, UK), 0.2 mmol l<sup>-1</sup> dNTPs, 1U Taq polymerase enzyme (Invitrogen Life Technologies, Paisley, UK) and biomass was taken from a single colony as template. PCR conditions were as follows: initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 1min, 55°C for 1 min, 72°C for 1.5 min, a final elongation step at 72°C for 5 min. PCR products were cleaned up (QIAquick PCR purification kit, Qiagen, UK), quantified using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, DE, USA) and sequenced with primers 27F and 1492R using BigDye Terminator v3.1 cycle sequencing kit and ABI Prism 7900HT sequence detection system (Applied Biosystems, UK). The sequences were assembled using SeqMan, DNA Star Lasergene 2.0 and analysed using BLAST at <http://blast.ncbi.nlm.nih.gov/Blast.cgi> (Altschul et al. 1990). Sequences were aligned and a neighbour-joining phylogenetic tree was constructed using ARB (Ludwig et al. 2004).

#### *Genomic DNA extraction from methylotrophic enrichment cultures*

Two ml aliquots of second-generation enrichment samples were centrifuged at 13,000xg for 10 minutes and the pellets were used for DNA extraction. Total DNA from the enrichment biomass pellets was extracted on the day of sampling using a FastDNA Spin Kit for Soil (MP Bioscience, Derby, UK) following the manufacturer's instructions.

#### *Denaturing gradient gel electrophoresis (DGGE)*

DNA extracted from the second-generation enrichment cultures was used for PCR and DGGE analysis. PCR amplification of 16S rRNA genes using primers 341F-GC and 907R (Muyzer et al. 1998) was performed using the PCR conditions described above. DGGE was applied using a Bio-Rad DGGE system (Bio-rad, Hercules, CA, USA) with 6% (w/v) polyacrylamide (37.5:1 acrylamide: bisacrylamide) gels with a 30-70% linear denaturant gradient (Schäfer and Muyzer 2001). Gels were stained with SYBR Green (Sigma-Aldrich Inc., UK) 1:10000 diluted in 1X TAE buffer and images were taken using Kodak Gel Logic 200 Imaging System (Carestream Health Inc., NY, USA). DGGE patterns were analyzed and compared based on band presence/absence data (Dice coefficient) using GelCompar II (Applied Maths NV, St.Martens-Latern, Belgium). Similarity between DGGE profiles was calculated as Pearson product moment correlation coefficients. Analysis of variance (ANOVA) was performed to identify the significance of similarities between the DGGE profiles ( $P < 0.05$ ) using GenStat (12<sup>th</sup> edition, VSN International Ltd.).

DGGE bands were cut from the gel and incubated in 10 µl distilled water overnight at 4°C. One µl of the solution was used as template in a PCR with primers 341F-GC/907R. Their purity was confirmed by DGGE analysis prior to sequencing with

primers 341F and 907R. Sequencing of the bands was done using BigDye Terminator v3.1 cycle sequencing kit and ABI Prism 7900HT sequence detection system (Applied Biosystems, UK). The sequences obtained were assembled using SeqMan, DNA Star Lasergene 2.0 and analysed using BLAST at <http://blast.ncbi.nlm.nih.gov/Blast.cgi> (Altschul et al. 1990).

## Results

### *Methylotrophic bacteria isolated from the enrichment cultures*

Using three methylotrophic substrates (methanol, DMS and DMSO) as the only carbon and energy sources, several aerobic bacteria were isolated from samples of soil, *Brassica oleracea* rhizosphere and moss tissue. Sequence analysis of the 16S rRNA genes of single colonies obtained from  $10^{-3}$ ,  $10^{-4}$  dilutions and the moss leaf enrichment plates revealed that the isolates were affiliated to the genera *Hyphomicrobium*, *Methylobacterium*, *Pseudomonas*, *Hydrogenophaga*, *Rhodococcus*, *Flavobacterium*, and *Variovorax* with sequence identities ranging between 96% to 99% (Table 1). The most commonly isolated bacteria were from the genus *Hyphomicrobium* and were closely related to *H. methylovorum*, *H. facile*, *H. vulgare* and *H. sulfonivorans*.

All isolated strains were checked for utilization of all three substrates as the only carbon and energy source (Table 2). Results revealed that only *Hyphomicrobium* strains were able to grow using the three substrates individually as the only carbon and energy source, similar to results of previous studies (De Bont et al. 1981; Pol et

al. 1994). The other isolates grew on methanol as their sole carbon and energy source, but were unable to grow on DMS and DMSO.

#### *Bacterial diversity of enrichment cultures*

DGGE profiles of the enrichment cultures showed that the highest diversity was observed within the DMS enriched samples with more than five dominant bands in each sample (Figure 1). The cluster analysis suggested that the greatest similarity was between DMSO enriched Long Close soil sample, methanol enriched Long Close soil sample and methanol enriched rhizosphere sample (100%).

Four dominant bands from the DGGE gels were excised and sequenced (Figure 1). The closest neighbours of the nucleotide sequences of three of these bands were species from three distinct families, namely uncultured bacterium clone NarTC10 from *Flavobacteriaceae*, *Thiobacillus thioparus* API from *Hydrogenophilaceae* and *Rhodococcus* sp. AH21 from *Nocardiaceae* (96 to 100% similarity). One band was identified as uncultured bacterium clone MA00070D11 from the phylum *Bacteroidetes* with 99% similarity (Table 3). The *Rhodococcus* strain was the only isolate which was also identified by DGGE band sequencing. Figure 2 shows the phylogenetic tree of the 16S rRNA sequences from the isolates and the DGGE bands.

#### *Comparison of bacterial diversity in rhizosphere and bulk soil enrichment cultures*

A second set of enrichments was established to investigate the effects of the terrestrial habitat on the methylotrophic populations in plant-associated environments. This was carried out with rhizosphere soil of field grown *B. oleracea* and bulk soil sampled from the same plot. DGGE results demonstrated that the bacterial communities did

not cluster with respect to the sample type when enriched with either methanol or DMS (Figure 3). Evaluation of the DGGE profiles of methanol enrichments showed that the mean similarity between the DGGE profiles of the rhizosphere enrichments ( $84.96 \pm 3.34$ ,  $n = 10$ ) was not greater than the similarity between the bulk soil enrichments ( $74.19 \pm 6.29$ ,  $n = 10$ ,  $p = 0.330$ ). Comparison of DMS-amended samples did not show a statistically significant difference between the similarities of the rhizosphere enrichments ( $75.59 \pm 2.22$ ,  $n = 10$ ) and bulk soil enrichments ( $70.28 \pm 6.02$ ,  $n = 10$ ,  $p = 0.884$ ).

Sequences of the dominant bands from the methanol enrichments showed 99% or 100% similarity to species of the genera *Methylophilus* and *Methylovorus*, while the band sequences from the DMS enrichments were affiliated to a *Cytophaga* sp., *Pseudomonas tremae* and *Thiobacillus thioparus* with 97% or 99% similarity (Table 4).

## **Discussion**

Terrestrial ecosystems are significant sinks for one-carbon compounds, however the microbial populations that have a role in one-carbon compound cycling are not well documented, there is a particular lack of studies of DMS degrading microorganisms as this substrate has often been considered to be toxic for microorganism (Suylén and Kuenen 1986). Therefore, methylotrophic bacteria that degrade methanol, DMS and DMSO as the only carbon and energy source were isolated from terrestrial samples and the bacterial diversities of the enrichment cultures were compared.

The bacterial strains isolated in this study as well as the populations identified in enrichments by DGGE belonged to genera known to have methylotrophic members (Sivelä and Sundman 1975; De Bont et al. 1981; Omori et al. 1995; Borodina et al. 2000; Schäfer et al. 2010). Two of the DGGE bands were not closely related to cultured bacteria. This suggests that these sequences may represent novel methylotrophs in the enrichments. The most frequently isolated genus was *Hyphomicrobium* which had been found repeatedly from several habitats (De Bont et al. 1981; Suylen and Kuenen 1986; Holm et al. 1996; Kim et al. 2013). Likewise, *Methylobacterium* isolated from the moss phyllosphere were reported to be one of the dominant and stable methylotrophic genera on plant leaves (Delmotte et al. 2009; Knief et al. 2010). However, the diversity of culturable phyllosphere bacteria can change with the leaf age, plant species, location and the season (Lindow and Brandl 2003; Wellner et al. 2011). Knief et al. (2010) demonstrated that sampling site and plant type did have an effect on *Methylobacterium* community structure which were detected on the plant leaves collected from *Arabidopsis thaliana* and *Medicago truncatula*. The abiotic and biotic factors might have also lead to the cultivation of a less abundant methylotrophic phyllosphere bacterium, *Flavobacterium* spp., from the moss tissue (Corpe and Rheem 1989; Hirano and Upper 1991). It is interesting to note that isolated *Hydrogenophaga*, *Rhodococcus* and *Variovorax* species did not grow on either DMS or DMSO although they were cultivated from samples amended with these substrates. With the exception of *Rhodococcus*, which based on the DGGE analysis is assumed to have been a dominant population in these enrichments, these bacteria may have been rare members of the enrichments and played a role in carbon cycling by breaking down the complex organics in the samples or by using the

intermediates of DMS metabolism (e.g. formate, methanethiol, or organic matter from the inoculum or lysed bacteria).

Overall results showed a discrepancy between the microbial diversities revealed by the culture-dependent and -independent analyses. Out of 13 isolates, the strain closely related to *Rhodococcus* was the only isolate that was detected by DGGE (98% pairwise identity). However, the isolated *Rhodococcus* strain was from the methanol enrichment while the DGGE showed the dominance of *Rhodococcus* strain in the DMS enrichment. This might be due to the fact that the isolated strain have better adapted to laboratory conditions during isolation with methanol and might have used the intermediates of DMS metabolism in DMS enrichments as mentioned previously. In a recent study, methylotrophic *Actinobacteria* and *Flavobacteria* were cultivated from soil samples although they were not detected using cultivation-independent methods (Stacheter et al. 2013). Here, several strains of *Hyphomicrobium* were isolated from the enrichments, which might have suggested that *Hyphomicrobium* spp. might dominate the enrichments, but DGGE analysis showed that this was not the case, as none of the dominant band sequences was related to *Hyphomicrobium*. The high growth rate of *Hyphomicrobium* species might have favoured their growth in laboratory conditions. Hayes et al. (2010) estimated the growth rate of *Hyphomicrobium* spp. growing on DMS to be  $0.099 \text{ h}^{-1}$ . In contrast, the growth rate of *Thiobacillus thioparus* TK-m on DMS was found to be  $0.05 \text{ h}^{-1}$  (Kanagawa and Kelly 1986). *T. thioparus* detected in the methanol-enrichments using DGGE was possibly outcompeted during cultivation or might have not been well adapted to the growth conditions. Earlier studies also reported similar limitations of growing *Acidithiobacillus ferrooxidans* on agar plates (Johnson et al. 1987).

The bacterial populations degrading methanol and DMS in enrichments of plant-associated samples were compared to those from surrounding bulk soil samples. Results demonstrated that the habitat type did not have an observable effect on the outcome of the bacterial community structure in the enrichments, which is in agreement with other studies (Duineveld et al. 2001; Normander and Prosser 2006). On the contrary, Turner et al. (2013) suggested that different plants may select distinct methylotrophs such as uncultured *Methylophilaceae*, *Variovorax* and *Flavobacterium* according to their metabolic capabilities. Our results imply that the methylotrophs dominating the rhizosphere-derived enrichments might have been recruited from the surrounding bulk soil. However, it is challenging to evaluate the ‘rhizosphere effect’ on DMS-degrading bacteria as the reproducibility was low within the replicates, which might indicate that the distribution of the DMS-degrading population was less homogeneous in the original sample and that the diversity of DMS degrading bacteria was lower than that of methanol degrading bacteria. Nevertheless, the possibility of spatial variation of the community structure in the original samples cannot be precluded as temporal variation in the physicochemical characteristics of soils such as pH, temperature and moisture may provide a variety of microhabitats and support dissimilar bacterial diversity (Roesch et al. 2007; Kolb 2009; Stacheter et al. 2013).

Members of *Methylophilaceae* were found to be dominant across all methanol-enriched soil and rhizosphere samples from the *Brassica oleracea* field. There are several reports indicating these bacteria as dominant methylotrophs in various terrestrial environments such as lake sediment, cereal rhizosphere and rice field soil (Lomans et al. 2001; Lueders et al. 2004; Nercessian et al. 2005; Kalyuzhnaya et al. 2009; Turner et al. 2013; Eyice et al. 2015). Interestingly, this family was not

abundant in the first set of methanol enrichment of the sample from the same field (Hunts Mill soil). The variation might be due to the seasonal shift between different sampling times. Advanced methods in microbial ecology such as next-generation sequencing techniques will help further understand the structure of methylotrophic communities in different terrestrial environments.

## **Conclusion**

The findings presented here underline that the diversity of methylotrophic bacteria in terrestrial environments exceeds that represented by frequently isolated bacteria. Also, it was shown that methylotrophic communities that degrade methanol or DMS are not shaped by terrestrial habitat type. This study reemphasizes the significance of culture-dependent diversity analysis. Although isolated bacteria from an enrichment culture may not represent the actual diversity in the same enrichment due to the biases at the isolation step, pure bacterial cultures are prerequisite to explore the functions of bacteria in the environment and to complete the reference databases used in culture-independent diversity analysis.

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## **Conflict of interest**

No conflict of interest declared.

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## List of Figures

**Fig 1** Negative image of the DGGE gel of PCR-amplified soil, rhizosphere and moss samples enriched with methanol, DMS and DMSO. Numbers show the dominant bands sequenced.

**Fig 2** Phylogenetic tree constructed by neighbour-joining method. It shows the affiliation of the 16S rRNA sequences of the isolates (in grey boxes) and the bands excised from the DGGE gels (in boxes). Scale bar represents 10% sequence divergence.

**Fig 3** DGGE analysis of PCR-amplified soil, rhizosphere and bulk soil samples enriched with (A) methanol and (C) DMS and dendograms (B and D) produced based on the similarities (Pearson product moment correlation coefficients) of the DGGE banding patterns. R1 to R5 refer to the rhizosphere replicates and B1 to B5 refer to the bulk soil replicates. The numbers with arrows show the dominant bands sequenced.

**Table 1** Identification of bacterial strains isolated from enrichment cultures and isolation conditions. Closest relatives according to BLAST search and the similarity percentages are presented.

Strain Name	Sample source	Enrichment substrate	Closest hit	Accession number	Identity (%)
Bras 1	<i>Brassica</i> rhizosphere	25 mM Methanol	<i>Hyphomicrobium methylovorum</i>	AB016812	96
Bras 2	<i>Brassica</i> rhizosphere	25 mM Methanol	<i>Pseudomonas plecoglossicida</i>	EU594553	100
LC 1	Long Close soil	25 mM Methanol	<i>Pseudomonas synxantha</i> F127	EF204250	99
HM 1	Hunts Mill soil	25 mM Methanol	<i>Methylobacterium</i> sp. GW2	EF126748	100
Bras 3	<i>Brassica</i> rhizosphere	1 mM DMS	<i>Hyphomicrobium facile</i>	NR027611	96
Moss 1	Moss	1 mM DMS	<i>Hydrogenophaga palleronii</i>	AM922191	100
Moss 2	Moss	1 mM DMS	<i>Hyphomicrobium vulgare</i>	Y14302	99
Moss 3	Moss	1 mM DMS	<i>Rhodococcus</i> sp. AH21	AJ551145	98
Bras 4	<i>Brassica</i> rhizosphere	10 mM DMSO	<i>Variovorax paradoxus</i> SFWT	EU 441166	96
Bras 5	<i>Brassica</i> rhizosphere	10 mM DMSO	<i>Hyphomicrobium sulfonivorans</i> 25S	AY305006	96
Moss 4	Moss leaf surface	10 mM DMSO	<i>Hyphomicrobium sulfonivorans</i> 25S	AY305006	99
Moss 5	Moss leaf surface	25 mM Methanol	<i>Methylobacterium</i> sp. F38	AM910536	96
Moss 6	Moss leaf surface	25 mM Methanol	<i>Flavobacterium</i> sp. WB 4.3.15	AM177628	99

**Table 2** Use of one-carbon compounds by methylotrophic isolates as the only carbon and energy source. (+) growth, (-) no growth

Strain Name	Closest hit	Growth substrate		
		Methanol	DMS	DMSO
Bras 1	<i>Hyphomicrobium methylovorum</i>	+	+	+
Bras 3	<i>Hyphomicrobium facile</i>	+	+	+
Bras 5	<i>Hyphomicrobium sulfonivorans</i> 25S	+	+	+
Moss 2	<i>Hyphomicrobium vulgare</i>	+	+	+
Moss 1	<i>Hydrogenophaga palleronii</i>	+	-	-
HM 1	<i>Methylobacterium</i> sp. GW2	+	-	-
Moss 5	<i>Methylobacterium</i> sp. F38	+	-	-
Moss 6	<i>Flavobacterium</i> sp. WB 4.3.15	+	-	-
Bras 2	<i>Pseudomonas plecoglossicida</i>	+	-	-
LC 1	<i>Pseudomonas synxantha</i> F127	+	-	-
Bras 4	<i>Variovorax paradoxus</i> SFWT	+	-	-
Moss 3	<i>Rhodococcus</i> sp. AH21	+	-	-

**Table 3** Identities of the dominant DGGE bands from the enrichments of terrestrial samples with DMS, DMSO and methanol by BLAST search.

Band number	Closest hit	Accession number	Isolation source	Identity (%)
1	Uncultured bacterium clone NarTC10	GQ401691	Polychlorinated biphenyl contaminated soil	96
2	Uncultured bacterium clone MA00070D11	FJ772390	Lake basin	99
3	<i>Thiobacillus thioparus</i> API	EU591536	Petrochemical environment	96
4	<i>Rhodococcus</i> sp. AH21	JN819591	Forest soil	100

**Table 4** Identities of the dominant DGGE bands from the enrichments of rhizosphere and bulk soil of *Brassica oleracea* samples with methanol and DMS by BLAST search.

<b>Band number</b>	<b>Enrichment substrate</b>	<b>Closest hit</b>	<b>Accession number</b>	<b>Isolation source</b>	<b>Identity (%)</b>
1	Methanol	<i>Methylophilus</i> sp. CBMB162	EU194894	Rice field	99
2	Methanol	<i>Methylovorus</i> sp. MM	HQ380796	Corn mint rhizoplane	99
3	Methanol	<i>Methylovorus</i> sp. MM	HQ380796	Corn mint rhizoplane	100
4	Methanol	<i>Methylovorus</i> sp. MM	HQ380796	Corn mint rhizoplane	100
5	Methanol	<i>Methylovorus</i> sp. MM	HQ380796	Corn mint rhizoplane	99
6	Methanol	<i>Methylophilus flavus</i> Ship	FJ872108	<i>Rosa cinnamomea</i> phyllosphere	100
7	DMS	<i>Cytophaga</i> sp. SSL03	EU395843	Chinese cabbage	97
8	DMS	<i>Pseudomonas tremae</i> Ht3-25	JF899280	Tobacco phyllosphere	99
9	DMS	<i>Thiobacillus thioparus</i> Pankhurst T4	HM173633	Thiosulfate-oxidising mixed	99

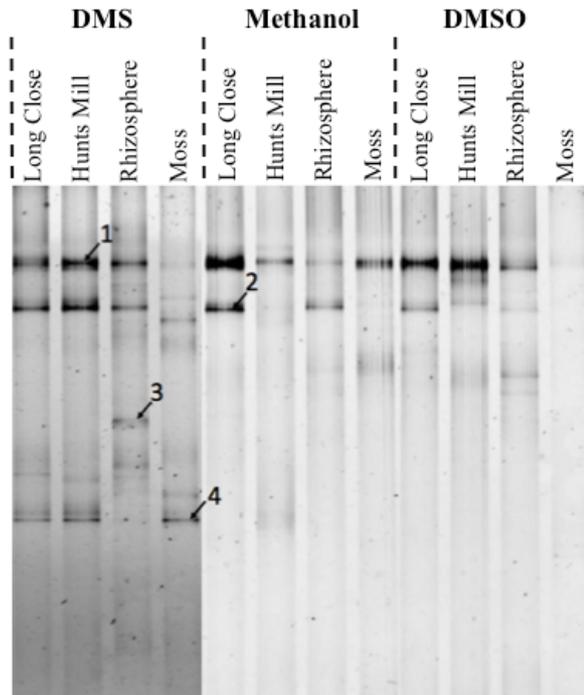


Figure 1.

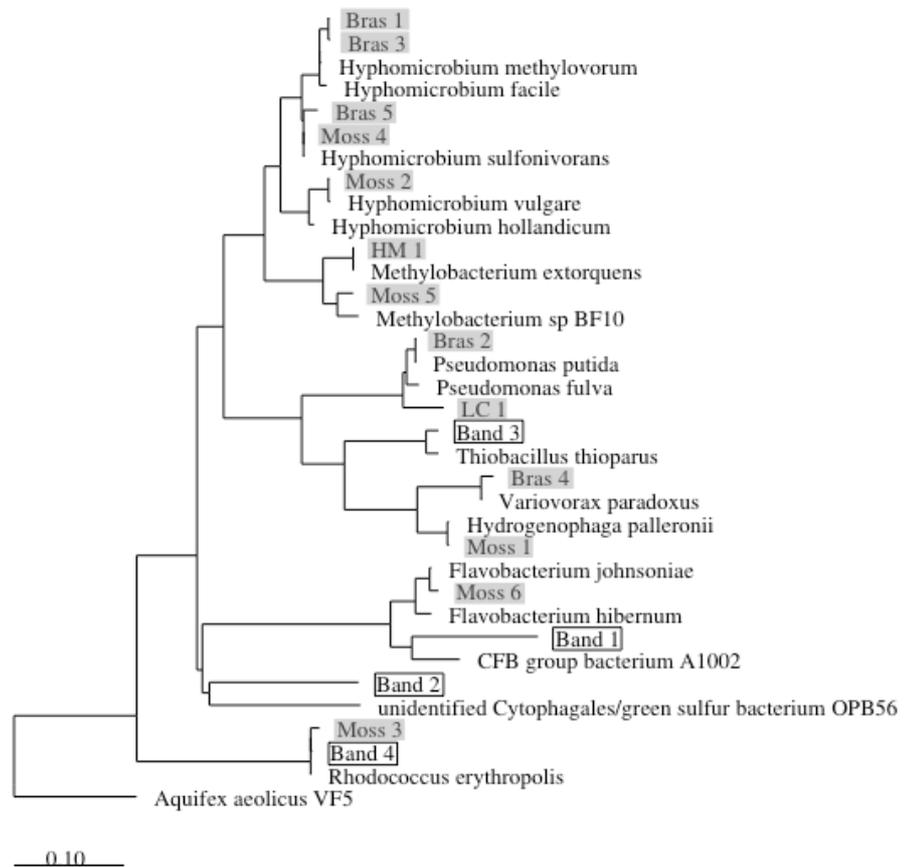


Figure 2.

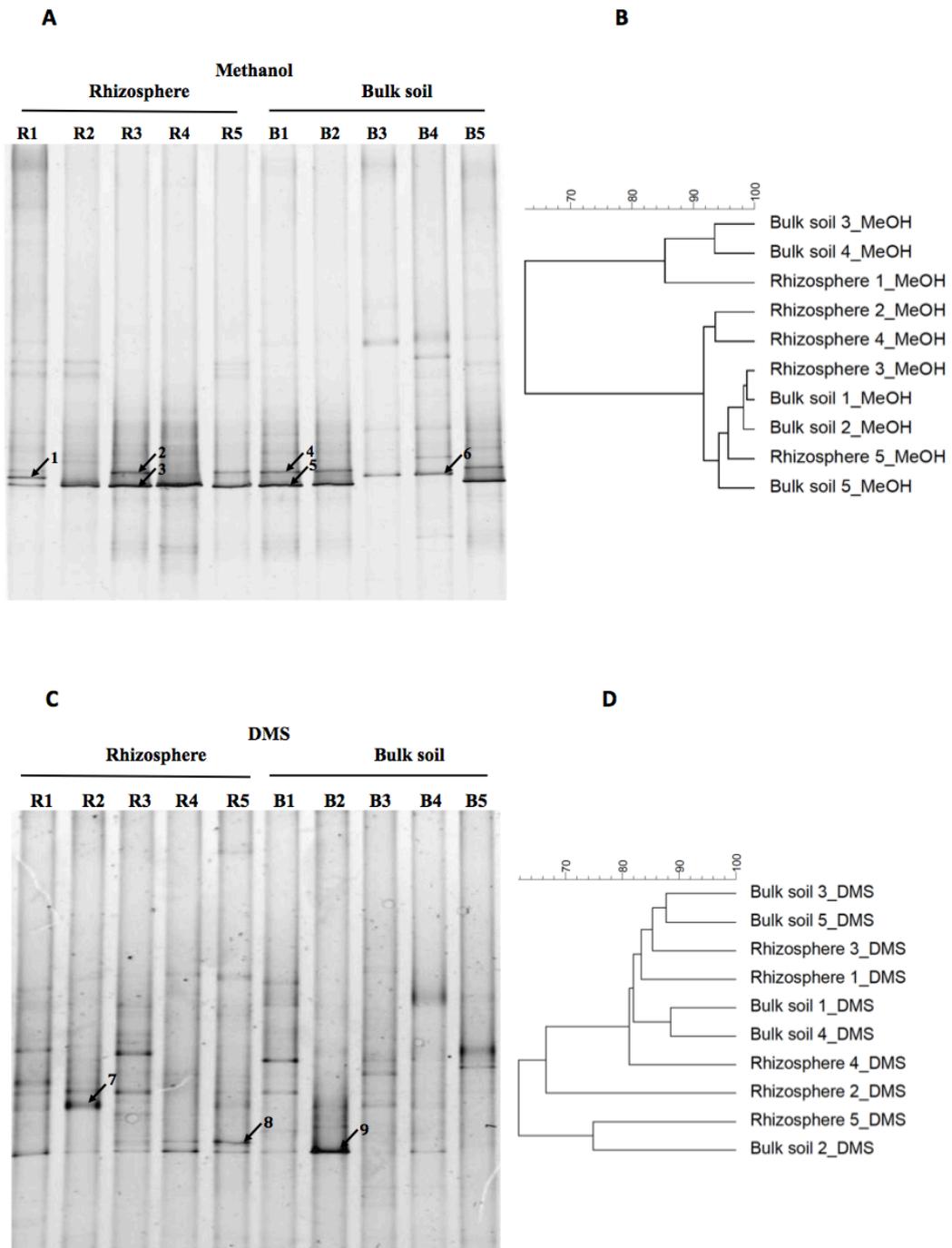


Figure 3.