4 The Family *Beijerinckiaceae*

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Abstract
To date there are only 18 established species in the family *Beijerinckiaceae* and only 12 if outgroup members are not taken into account. This small number does not impede to consider them quite interesting from the metabolic and evolutionary point of view since it encompasses from obligate methanotrophs to chemoorganoheterotrophs, plus examples of the intermediate states: facultative methylotrophs and facultative methanotrophs. Nitrogen fixation is another remarkable trait of the family, enabling them to thrive in habitats in which other potential sources of nitrogen are scarce. Their global distribution is wide, with a preference for acidic soils of tropical regions (in the case of *Beijerinckia*) or of temperate and even polar regions in the case of *Methylocapsa*, *Methylocella*, *Methyloferula*, *Methylorosula*, and *Methylovirgula*.

Taxonomy, Historical and Current

The family *Beijerinckiaceae* belongs to the order *Rhizobiales* within the *Alphaproteobacteria* (Garrity et al. 2005). At the time of its proposal contained the genera *Beijerinckia*, *Chelatococcus*, *Methylocapsa*, and *Methylocella* and was described as a metabolically diverse family of aerobic bacteria capable of forming poly-β-hydroxybutyrate granules and fixing nitrogen (*Beijerinckia* and *Methylocella*). To date, four more genera have been formally described within this family: *Camelimonas*, *Methyloferula*, *Methylorosula*, and *Methylovirgula*. However, the phylogenetic analysis taking into account the newly described taxa (also within neighboring families) reveals that two of the eight genera, namely, *Camelimonas* and *Chelatococcus*, occupy an uncertain position close to *Bosea* spp. (family *Bradyrhizobiaceae*) and *Microvirga* (family *Methylobacteriaceae*) (◉ *Fig. 4.1*).

From the historical point of view, there is a clear separation in the taxonomic activity of the different genera. The type genus, *Beijerinckia*, named to honor the Dutch pioneering microbiologist Martinus Willem Beijerinck (1851–1931), has deep roots (Starkey and De 1939; Derx 1950a). There is account of intense research going on for decades concerning isolation of *Beijerinckia* sp. from different habitats and characterization by different methods (Becking 2006) and its beneficial role as nitrogen fixers. However, studies become infrequent after the nineties and there is only one species description of *Beijerinckia* in this century (Oggerin et al. 2009). For the other genera it is just the opposite since all descriptions appeared from 2000 on with the only exception of *Chelatococcus asaccharovorans* (Auling et al. 1993). The push of knowledge in recent times, and not only taxonomic, has been especially important in the case of the methanotrophic and methylotrophic aerobic bacteria of the genera *Methylocapsa*, *Methylocella*, *Methyloferula*, *Methylorosula*, and *Methylovirgula*, whose ecological importance and interest for understanding evolutionary processes go beyond the possibilities of this chapter.

Members of the family *Beijerinckiaceae* include chemoorganoheterotrophs, facultative methylotrophs, facultative methanotrophs, and obligate methanotrophs (◉ *Table 4.1*). The ability to utilize multicarbon compounds is variable but is generally broader in the chemoorganoheterotrophs, then facultative methylotrophs, and then facultative methanotrophs. Other important aspects related with C_1 compounds metabolism such as the carbon assimilation pathway or the type of methane monooxygenase, in those species affected, are additional sources of variability (◉ *Table 4.1*).

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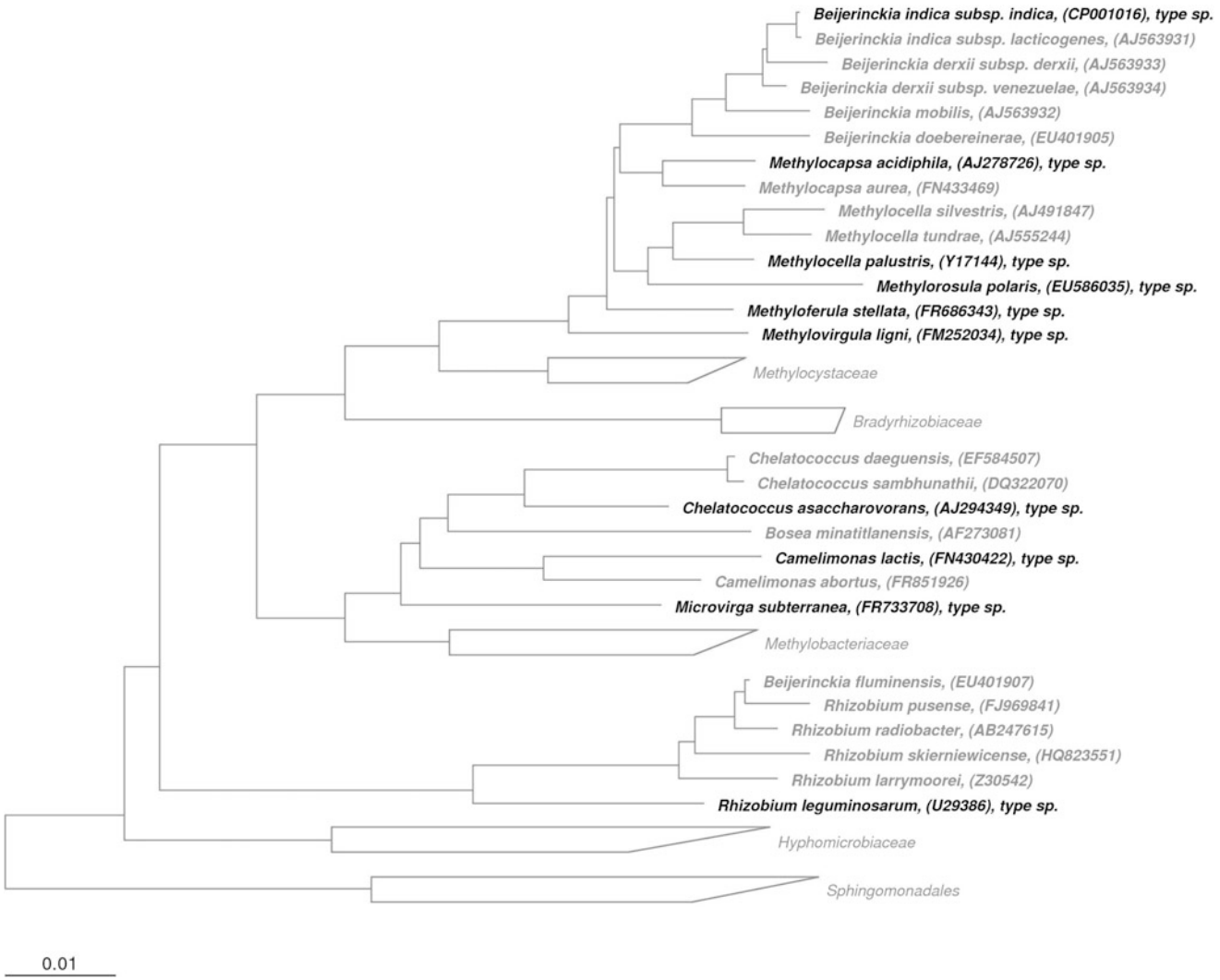

Fig. 4.1

Phylogenetic reconstruction of the family *Beijerinckiaceae* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes–Cantor correction. The sequence data sets and alignments were used according to the All-Species Living Tree Project (*LTP*) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high-quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

Formation of poly-β-hydroxybutyrate granules is still a general trait, followed by exopolysaccharide production and nitrogen-fixing activity, with one and two exceptions known, respectively. Unfortunately, these three traits have not been determined in all species, and the main data gap occurs in the uncertain position genera *Camelimonas* and *Chelatococcus*, with no or only partial data, respectively (*Table 4.1*). In contrast, some of the characteristics determined in them (e.g., polyamine pattern or polar lipid composition) have not been explored in the other genera and therefore comparison is not possible. If we leave aside these two genera, the DNA G+C content is 55–63 mol %, a range that could well fit for a single genus.

All members of the family *Beijerinckiaceae* are aerobic but *C. daeguensis* and *C. sambhunathii* can also grow anaerobically with nitrate (Panday and Das 2010; Yoon et al. 2008). Growth factor requirements have been only reported in two species, *C. asaccharovorans* (Auling et al. 1993) and *M. ligni* (Vorob'ev et al. 2009).

During the course of a research project with free-living nitrogen-fixing bacteria, Oggerin et al. (2009) confirmed that strains *B. fluminensis* UQM 1685^T and *B. fluminensis* CIP 106281^T had the same origin but did not descend from the nomenclatural type of the species since they exhibit important phenotypic and genotypic differences

Table 4.1
Genera of the family *Beijerinckiaceae* and diagnostic traits

	1	2	3	4	5	6	7	8
Cell morphology	Rods	Rods	Cocci or rods	Curved coccoids or rods	Rods or ovoids	Rods	Rods	Rods
Metabolism[a]	COH/ FMlo	COH	COH	OMno/FMno	FMno	OMno	FMlo	FMlo
Multicarbon compounds used	Sugars and others	Sugars and others	Sugars and others	None or acetate	Acetate, ethanol, pyruvate, succinate, malate	None	Sugars and others	Ethanol, pyruvate, malate
C_1 assimilation pathway	RuBP	–	–	Serine	Serine	RuBP	RuBP	RuBP
Methane monooxygenase type	–	–	–	pMMO	sMMO	sMMO	–	–
PHB synthesis	+	ND	+[b]	+	+	+	ND	+
Exopolysaccharides	+	ND	+[b]	+	+	ND	+	–
Nitrogen fixation	+	ND	–[b]	+	+	+	–	+
G+C content (mol%)	54–61	65[c]	63–68	61–63	60–63	56–57	65	62–63

1, *Beijerinckia* (Derx 1950a, b; Döbereiner and Ruschel 1958; Oggerin et al. 2009, 2011; Starkey and De 1939; Tchan 1957; Thompson and Skerman 1979; Dedysh et al. 2005b); 2, *Camelimonas* (Kämpfer et al. 2010, 2012); 3, *Chelatococcus* (Auling et al. 1993; Panday and Das 2010; Yoon et al. 2008); 4, *Methylocapsa* (Dedysh et al. 2002; Dunfield et al. 2010); 5, *Methylocella* (Dedysh et al. 2000, 2004a; Dunfield et al. 2003); 6, *Methyloferula* (Vorobev et al. 2011); 7, *Methylorosula* (Berestovskaya et al. 2012); 8, *Methylovirgula* (Vorob'ev et al. 2009)
+ positive, *ND* not determined, – negative
[a]*COH* chemoorganoheterotrophs (non-methylotrophic), *FMno* facultative methanotroph, *FMlo* facultative methylotroph, *OMno* obligate methanotroph
[b]Reported for *Chelatococcus asaccharovorans* but not for *C. daeguensis* or *C. sambhunathii*
[c]Reported for *Camelimonas lactis* but not for *C. abortus*

when compared with the descriptions of *B. fluminensis* or any of the other species of the genus *Beijerinckia*. As a matter of fact, they were identified as *Rhizobium radiobacter* (Fig. 4.1). All other available equivalent strains (DSM 2327^T and CCUG 53676^T) were confirmed too as *R. radiobacter* (Oggerin et al. 2011). Subsequent attempts to find older deposits of the type strain, hopefully derived from the original isolate, or other existing strains of *B. fluminensis* that could be proposed as a neotype strain were fruitless, and the bibliographic search of the key publications (Döbereiner and Ruschel 1958; Hilger 1965; Skerman et al. 1980; Thompson and Skerman 1979) did not provide enough traceability or hints to locate such biological material. According to Rule 18c of the International Code of Nomenclature of Bacteria (Lapage et al. 1992), if a suitable replacement type strain or a neotype cannot be found or proposed, respectively, within 2 years of the publication of the request for an opinion (Oggerin et al. 2011), the Judicial Commission of the International Committee on Systematic of Prokaryotes is entitled to place the name *Beijerinckia fluminensis* on the list of rejected names. The next foreseeable meeting of the Judicial Commission is during the celebration of the 14th International Congress of Bacteriology and Applied Microbiology to be held in Montreal, Canada, from July 27 to August 1, 2014.

Subspeciation has only been proposed in the genus *Beijerinckia*, namely, in the species *B. derxii* and *B. indica* (Thompson and Skerman 1979).

Molecular Analyses

DDH

To the best of our knowledge, DDH methods have never been reported in the genus *Beijerinckia*, in spite of having the largest number of species, not in the monospecific genera *Methyloferula*, *Methylorosula*, and *Methylovirgula*. In the remaining four genera of the family *Beijerinckiaceae*, we find several examples.

Thus, the proposal of *Chelatococcus asaccharovorans* as novel genus and species (Auling et al. 1993) was based on two strains, TE1 and TE2^T, that could not be discriminated by DDH. However, much lower values (less than 30 %) were obtained against other nine nitrilotriacetate (NTA)-utilizing strains that were proposed in the same study as *Chelatobacter heintzii* gen. nov., sp. nov. For about 15 years, the genus contained one single species, but recently it was enlarged twice and in both cases DDH was considered necessary. The mean value obtained between

C. daeguensis K106^T and *C. asaccharovorans* DSM 6462^T was 13 % (Yoon et al. 2008), whereas *C. sambhunathii* HT4^T displayed 52 and 20 % to *C. daeguensis* CCUG 54519^T and *C. asaccharovorans* DSM 6462^T, respectively (Panday and Das 2010).

The description of *Methylocapsa acidiphila* (Dedysh et al. 2002) included the DDH value between the type strain B2^T and *Methylocella palustris* K^T (7 %). However, in the description of the second species of the genus, *M. aurea* (Dunfield et al. 2010), this information was absent.

In the case of the genus *Methylocella*, DDH was included in the proposal of the second and third species: *M. silvestris* BL2^T was confronted to *M. palustris* K^T resulting in 21–22 % values (Dunfield et al. 2003) and, later on, *M. tundrae* T4^T was hybridized to *M. silvestris* BL2^T and *M. palustris* K^T resulting in values of 36 and 27 %, respectively.

When proposing *Camelimonas lactis* as new genus and species based on strains M-2040^T, M 1973, and M 1878-SK2, Kämpfer et al. (2010) performed DDH experiments and obtained relatedness values among them ranging from 89 % to 100 %. The three strains where also confronted to *Chelatococcus asaccharovorans* DSM 6462^T and *Chelatococcus daeguensis* CCUG 54519^T resulting in values from 22.3 % to 49.2 % and 18.5 % to 49.6 %, respectively (reciprocal measurements included). Later, the proposal of the second species of the genus *Camelimonas* was supported by DNA–DNA relatedness values of 18.0 % and 13.6 % (reciprocal analysis) between *C. abortus* UK34/07-5^T and *C. lactis* M 2040^T (Kämpfer et al. 2012).

Molecular Phylogenetic Markers

An important metabolic ability in many *Beijerinckiaceae*, as has been already mentioned, is dinitrogen fixation that allows them to thrive in environments depleted in available nitrogen compounds. The sequences of *nifH* and *nifD* gene fragments from representatives of the genera *Methylocella*, *Methylocapsa*, *Beijerinckia*, together with some outgroup methanotrophs have been used to withdraw phylogenetic relationships (Dedysh et al. 2004b). The trees constructed for the inferred amino acid sequences of *nifH* and *nifD* were highly congruent. The phylogenetic relationships among methanotrophs in the NifH and NifD trees also agreed well with the corresponding 16S rRNA-based phylogeny, but two discrepancies were found. One suggested that an ancient event of lateral gene transfer was responsible for the aberrant branching of *Methylococcus capsulatus*. On the other hand, the identity values of NifH and NifD sequences between *Methylocapsa acidiphila* B2^T and representatives of *Beijerinckia* were clearly higher (98.5 % and 96.6 %, respectively) than would be expected from their 16S rRNA-based relationships. This can be taken as an indication that these two bacteria originated from a common acidophilic dinitrogen-fixing ancestor and were subject to similar evolutionary pressure with regard to nitrogen acquisition. This interpretation is corroborated by the observation that, in contrast to most other diazotrophs, *M. acidiphila* and *Beijerinckia* spp. are capable of active growth on nitrogen-free media under fully aerobic conditions.

In addition to these genes, the key genes of C_1 metabolism have also been widely used as phylogenetic and functional markers in a number of taxonomic and ecologic studies (Berestovskaya et al. 2012; Dedysh et al. 1998b, 2000, 2001b, 2002, 2004a; Dunfield et al. 2003, 2010; Lau et al. 2013; McDonald and Murrell 1997a, b; Vorob'ev et al. 2009; Vorobev et al. 2011).

The first step of bacterial methane oxidation is catalyzed by methane monooxygenase (MMO), which occurs in two forms: a membrane-bound or particulate (pMMO) and a cytoplasmic or soluble (sMMO). Virtually all known methanotrophs except perhaps, the *Methylocella* species, possess pMMO that consists of three membrane-associated polypeptides encoded by *pmoC*, *pmoA*, and *pmoB* (McDonald and Murrell 1997b; Murrell et al. 2000; Pacheco-Oliver et al. 2002). In addition to pMMO, several methanotrophs possess sMMO. The enzyme from strains belonging to *Methylosinus*, *Methylocystis*, and *Methylococcus* has been thoroughly studied, and the nucleotide sequence of the sMMO gene cluster *mmoX*, *mmoY*, *mmoB*, *mmoZ*, *mmoC*, and *mmoD* appears to be highly conserved (Trotsenko and Khmelenina 2005). The *pmoA* gene encoding a 26-kDa subunit that harbors the active site of the pMMO and *mmoX* gene coding for α-subunit of the sMMO hydroxylase component has been used as appropriate gene markers for the occurrence of the enzymes in various methanotrophs.

Methanol dehydrogenase (MDH), the second enzyme involved in methane oxidation, is present in all Gram-negative methylotrophs including methane and methanol utilizers, and *mxaF* is an appropriate indicator gene for their occurrence in the natural environment (McDonald and Murrell 1997a). Its potential as a functional and phylogenetic marker for methanotrophs has been recently addressed (Lau et al. 2013). This gene codes for the large α-subunit of methanol dehydrogenase and is highly conserved among distantly related methylotrophic species in the alpha-, beta-, and gammaproteobacteria. Topology tests revealed that *mxaF* and 16S rRNA genes of methanotrophs do not show congruent evolutionary histories. Moreover, the *mxaF* gene has poor resolving power for methanotrophs within the *Beijerinckiaceae* (genera *Methylocapsa*, *Methylocella*, and *Methyloferula* in this study), although it performed well for the families *Methylocystaceae* and *Methylococcaceae*. The polyphyletic nature seen for the *Beijerinckiaceae* and the fact that it includes obligate and facultative methylotrophs give rise to various hypothesis: (i) methanotrophy arose once in the group and was lost by some methylotrophic taxa, (b) methanotrophy arose separately in more than one taxon in the group, and/or (c) multiple occurrences of horizontal gene transfers have occurred in the common ancestor of methanotrophic *Beijerinckiaceae*. It therefore is difficult to ascertain whether microorganisms with *mxaF* gene sequences placed near the polyphyletic *Beijerinckiaceae* genera *Methylocapsa*, *Methylocella*, and *Methyloferula* are indeed methanotrophic.

Genomic Data

The first two complete genome sequences to be published in the family came in 2010, both obtained from type strains of separate species and genera.

On one hand, *Methylocella silvestris* BL2^T (Chen et al. 2010) was a judicious choice since it brought as well the first genome available for an alphaproteobacterial methanotroph. Moreover, the organism itself is interesting because it was the first fully authenticated example of facultative methanotroph growing not only on methane and other one-carbon (C_1) substrates but also on some compounds containing carbon–carbon bonds, such as acetate, pyruvate, propane, and succinate (Dedysh et al. 2005a). The genome is a circular chromosome of 4.31 Mbp (CP001280) and a G+C content of 63.1 mol%. In total, 3,917 candidate genes were predicted and 99 pseudogenes were found. Functionality was assigned to 67.9 % of the genes, while 30.9 % of the genes could not be assigned any known function. All tRNA-encoding regions were identified, and two identical rRNA operons were found (Chen et al. 2010). In agreement with previous knowledge of the metabolic capabilities of the organism, the genome analysis permitted to conclusively verify (i) the absence of any *pmoCAB* genes encoding a pMMO enzyme that is present in all other genera of methanotrophs and (ii) the presence of a complete operon encoding sMMO (*mmoXYBZDC*), a complete operon encoding methanol dehydrogenase (*mxaFJGIRSACKLDEH*), and all genes necessary for fixation of methane-derived carbon via the serine cycle. Genes encoding key enzymes in both the tetrahydrofolate and the tetrahydromethanopterin-mediated formaldehyde oxidation pathways were found. Acetate kinase- and phosphotransacetylase-encoding genes are present, allowing acetate to be fed into the tricarboxylic acid (TCA) cycle. Genes encoding glyoxylate bypass enzymes (i.e., isocitrate lyase and malate synthase), essential for bacteria growing on C_2 compounds, were also identified. A full gene set encoding enzymes of the TCA cycle is also present, including genes encoding α-ketoglutarate dehydrogenase, which are lacking in some methanotrophs. Interestingly, a gene cluster encoding di-iron-containing multicomponent propane monooxygenase is also present (Chen et al. 2010).

On the other hand, *B. indica* subsp. *indica* ATCC 9039^T was also a wise choice for its phylogenetic proximity yet different metabolism. Its genome consists of a chromosome of 4,170,153 bp and two plasmids of 181,736 and 66,727 bp (CP001016, CP001017, and CP001018, respectively). There are a total of 3,982 open reading frames predicted, of which 3,784 are predicted protein-coding genes and 2,695 (70 %) have been assigned a predicted function (Tamas et al. 2010). There are 134 pseudogenes, 52 tRNA genes, and three operons each containing 16S, 23S, and 5S rRNA genes. The G+C content is 57.0 % (56 % and 54 % in the plasmids). The initial analysis (Tamas et al. 2010) revealed that this strain lacks phosphofructokinase, the key enzyme of the Embden–Meyerhof pathway. Instead, it uses the Entner–Doudoroff or pentose phosphate pathway to catabolize sugars, which is typical of free-living *Rhizobiales*. The majority of the genes involved in N_2 fixation are clustered in two genomic islands (10 and 51 kb), with the notable exception of the *nifS* gene encoding cysteine desulfurase. *B. indica* is a metabolically versatile bacterium capable of growth on a variety of organic acids, sugars, and alcohols, while *M. silvestris* is a highly specialized methanotrophs capable of growth on very few substrates (Dedysh et al. 2005a). However, their genome sizes (4.17 vs. 4.30 Mbp, respectively) and the numbers of predicted protein-encoding genes (3,788 vs. 3,917, respectively) are remarkably similar. A BLAST analysis indicated that the 57 % of the genes in the genome of *B. indica* have homologues in *M. silvestris* (Tamas et al. 2010). Some key pathways of one-carbon metabolism (such as the tetrahydromethanopterin and serine pathways of formaldehyde metabolism) that are present in *M. silvestris* appear to be absent or incomplete in *B. indica*, which confirms previous experiments showing that the organism is incapable of methylotrophic growth (Dedysh et al. 2005b). However, an operon encoding a putative propane monooxygenase homologous to soluble propane/methane monooxygenases of *M. silvestris* BL2^T was identified. More in-depth comparison of these genomes will help elucidate what defines their distinct lifestyles.

Meanwhile three more genomic sequences (scaffolds or contigs) have become available. One corresponds to *Chelatococcus* sp. GW1, a crude-oil degrading strain that has 5.21 Mb and a G+C content of 68.7 mol%. The other two are from the types of two obligate methanotrophs, *Methylocapsa acidiphila* B2^T and *Methyloferula stellata* AR4^T, with sizes of 4.10 and 4.24 Mb and G+C contents of 61.8 and 59.5 mol%, respectively. This expands considerably the possibilities of performing comparative genome analyses for the study and understanding of methanotrophy.

Using a combination of proteomic, biochemical, and genetic approaches, the identification and characterization of trimethylamine (TMA) monooxygenase gene (*tmm*) from *M. silvestris* have been reported (Chen et al. 2011). This enzyme is an example of bacterial flavin-containing monooxygenase, a system much better studied in eukaryotes and with many important physiological functions. It contained the conserved sequence motif and typical flavin adenine dinucleotide and nicotinamide adenine dinucleotide phosphate-binding domains. It was highly expressed in TMA-grown *M. silvestris* and absent during growth on methanol. Indeed, the ability to use TMA as a sole carbon and/or nitrogen source is directly linked to the presence of *tmm* in the genomes as shown by recombination and mutagenesis experiments. Close homologues of *tmm* occur in many *Alphaproteobacteria*, in particular *Rhodobacteraceae* (marine *Roseobacter* clade) and the marine SAR11 clade (*Pelagibacter ubique*). It is highly abundant in the metagenomes of the Global Ocean Sampling expedition, and it has been estimated that 20 % of the bacteria in the surface ocean contain *tmm*, playing an important role in the global carbon and nitrogen cycles (Chen et al. 2011).

By comparing the genomes of the generalist organotroph *B. indica*, the facultative methanotroph *M. silvestris*, and the obligate methanotroph *M. acidiphila*, Tamas et al. (2014) obtained the first insights into the evolution of the family *Beijerinckiaceae*

Table 4.2

Differential traits between *Beijerinckia* species (Becking 2006; Dedysh et al. 2005b; Derx 1950a, b; Döbereiner and Ruschel 1958; Kennedy 2005; Oggerin et al. 2009, 2011; Starkey and De 1939; Tchan 1957; Thompson and Skerman 1979)

	B. indica	*B. derxii*	*B. doebereinerae*	*B. fluminensis*	*B. mobilis*
Cell size (μm)	0.5–1.2 × 1.6–3.0	1.5–2.0 × 3.5–4.5	1.0 × 3.25	1.0–1.5 × 3.0–3.5	0.6–1.0 × 1.6–3.0
Water-soluble, green fluorescent pigment	–	+	–	–	–
Colony color after aging	Pink	Beige	Cream	Amber brown	Brown
Motility	–/w[a]	–	–	–/w[a]	+
pH range for growth (optimal)	3.0–10.0 (4.0–10.0)	4.0–9.0 (6.0–7.0)	3.0–10.0 (6.5)	3.5–9.2 (ND)	3.0–10.0 (4.0–5.0)
Urease	+	+	–	–	+
Assimilation of					
Fructose	+	+	+	+	–
Maltose	–	+	+	v	–
Mannose	+	–	+	v	–
Sorbose	+	v	–	+	+
Xylose	–	–	–	+	–
Glycerol	+	–	–	+	+
Sorbitol	+	+	–	+	–
DNA G+C content (mol%)	54.7–58.8	57.5–60.7	57.1	54.4–58.0	57.3

[a]Negative or weak and only in young cultures

Fatty acid methyl ester profiles of *B. indica* LMG 2817^T, *B. doebereinerae* LMG 2819^T, and *B. mobilis* LMG 3912^T were obtained by Oggerin et al. (2009). The only major fatty acid was C18:1 ω7c accounting 86.0–92.4 %. C16:0 was the only minor fatty acid present in all three strains (at amounts between 2.8 and 6.9 %), the rest being present only in one or two strains and representing up to 7.0 % of the total composition: C17:0 iso, C18:0, C16:1 and/or C15:0 iso 2-OH, and C17:1 iso and/or anteiso

and into the trade-offs required for a specialist methanotrophic lifestyle compared with a generalist chemoorganotrophic lifestyle. Thus, highly resolved phylogenetic construction based on 29 universally conserved genes demonstrated that the *Beijerinckiaceae* forms a monophyletic cluster with the *Methylocystaceae* (each represented by three genomes in this study), the only other family of alphaproteobacterial methanotrophs. For methanotrophy and methylotrophy genes, a vertical inheritance pattern within these families could be seen. At the same time, many lateral gene transfer events were detected in the genome of *B. indica* for genes encoding carbohydrate transport and metabolism, energy production and conversion, and transcriptional regulation, suggesting that they have been acquired recently. *B. indica* also had a larger abundance of transporter elements, particularly periplasmic-binding proteins and major facilitator transporters. Thus, the authors conclude that the most parsimonious scenario for the evolution of methanotrophy in the *Alphaproteobacteria* is that it occurred only once, when a methylotroph acquired methane monooxygenases via lateral gene transfer. This was supported by a compositional analysis suggesting that all methane monooxygenases in *Alphaproteobacteria* methanotrophs are foreign in origin. Some members of the *Beijerinckiaceae* subsequently lost methanotrophic functions and regained the ability to grow on multicarbon energy substrates. According to that, *B. indica* is a recidivist multitroph, the only known example of a bacterium having completely abandoned an evolved lifestyle of specialized methanotrophy (Tamas et al. 2014).

Phenotypic Analyses

Beijerinckia

Formally, the description of the genus is that of Derx (1950a), although some traits have been studied later (Becking 2006; Kennedy 2005). *Beijerinckia* species are nonsymbiotic, aerobic, chemoheterotrophic bacteria with the ability to fix atmospheric dinitrogen. They utilize a wide range of multicarbon compounds, but sugars are the preferred growth substrates. Members of this genus are straight or slightly curved rods with rounded ends containing polar lipoid bodies (PHB). Cells occur singly or appear as dividing pairs. Individual cells measure 0.5–2.0 × 1.6–4.5 μm (*Table 4.2*), although larger misshapen cells can also be seen. Cysts (enclosing one cell) and capsules (enclosing several cells) may occur in some species. Gram negative. Motile by peritrichous flagella or nonmotile. Aerobic, having a strictly respiratory type of metabolism. N_2 is fixed under aerobic or microaerobic conditions. Growth occurs between 10°C and 35 °C (optimal 20–30 °C); no growth occurs at 37 °C. Growth occurs between pH 3.0 and 10.0. Liquid cultures can become a highly viscous, semitransparent mass; in

some species the whole medium becomes opalescent and turbid, and adhering slime is not produced. On agar media, especially under N_2-fixing conditions, copious slime is produced and giant colonies with a smooth, folded, or plicate surface develop; some strains form slime having a more granular consistency. Catalase positive. Glucose, fructose, and sucrose are utilized by all strains and oxidized to CO_2. No growth occurs on peptone medium. Glutamate is utilized poorly or not at all. Negative for indole production. The G+C of the DNA is 54.4–60.7 mol%.

The chemical structure of the water-soluble polysaccharide, CV-70, produced by one strain of *Beijerinckia* sp. was defined applying different analytical methods. Glucose, galactose, and fucose were identified as the components in the CV-70 polysaccharide, in a 3:1:3 ratio (Scamparini et al. 1997).

A recent study (Jendrossek et al. 2007) has shown that the location of poly-3-hydroxybutyrate granules in *B. indica* and other bacteria is nonrandom. The early stages of formation were examined by confocal laser scanning fluorescence microscopy of Nile red-stained cells and by transmission electron microscopy. Cells of this species harbored one PHB granule at each cell pole, often close to or even in physical contact with the cytoplasmic membrane.

As methanotrophy was being uncovered in the family *Beijerinckiaceae* and given the close phylogenetic distance between *Beijerinckia* and the new genera, and the fact that some of the new species were facultative and not obligate methanotrophs, it became more imperative to check for C_1 metabolism in the genus *Beijerinckia*. Dedysh et al. (2005b) tested five type strains of different *Beijerinckia* species/subspecies (*B. mobilis* DSM 2326^T, *B. indica* subsp. *indica* ATCC 9039^T, *B. indica* subsp. *lacticogenes* DSM 1719^T, *B. derxii* subsp. *derxii* DSM 2328^T, and *B. derxii* subsp. *venezuelae* DSM 2329^T) for their ability to grow on methanol as the sole carbon and energy source in either nitrogen-free medium or supplemented with KNO_3. The only species capable of growth on methanol was *B. mobilis*. Growth occurred under a wide range of methanol concentrations ranging from 0.01 % to 3 % (vol/vol) and was optimal between 0.05 % and 0.5 % (vol/vol) CH_3OH. Growth was obtained under both nitrogen-fixing and nitrogen-sufficient conditions and could be maintained continuously without loss of viability. The enzymatic activities in the metabolism of methanol and glucose have been measured (Smirnova et al. 2005) confirming that *B. mobilis* possesses all the enzymes necessary for oxidation of methanol to CO_2 via formaldehyde and formate coupled with the ribulose bisphosphate (RuBP) pathway of C_1 assimilation. Therefore, *B. mobilis* is a facultative chemoautotrophic methylotroph that in addition to growth on C_1-reduced compounds (methanol and formate) has the ability to use a wide range of multicarbon substrates. These studies also demonstrate that there is more metabolic versatility within representatives of the genus *Beijerinckia* than was previously thought. Although methylotrophic autotrophy could be attributed to only one species of this genus, future studies may extend the number of *Beijerinckia* species capable of C_1 metabolism. In this sense, it is important to mention that *B. doebereinerae* (Oggerin et al. 2009) was described after the publication of these studies and occupies a position that is intermediate between *B. mobilis* and *Methylocapsa* spp. (Fig. 4.1).

Table 4.3
Differential traits between *C. lactis* (Kämpfer et al. 2010) and *C. abortus* (Kämpfer et al. 2012)

	C. lactis	*C. abortus*
Growth on MacConkey agar	+	–
Utilization of		
Trehalose	–	+
4-Aminobutyrate	+	–
Citrate	+	–
Fumarate	+	–
Itaconate	+	–
DL-Lactate	+	–
Mesaconate	+	–
L-Alanine	+	–
Presence of		
Unidentified glycolipid	+	–
Unidentified phospholipid	–	+

Camelimonas

Cells of *Camelimonas* (Kämpfer et al. 2010) are Gram-negative, nonmotile, nonspore-forming rods, approximately 2 µm long and 1 µm wide. Oxidase-positive, showing an oxidative metabolism. Good growth occurs on R2A agar, TSA, PYE agar, and nutrient agar at 25–30 °C. Beige, translucent, and shiny colonies with entire edges are formed within 24 h, with a diameter of about 2 mm. The characteristic peptidoglycan diamino acid is meso-diaminopimelic acid. The predominant compound in the polyamine pattern is spermidine, and sym-homospermidine is absent. The quinone system is ubiquinone Q-10. The polar lipid profile includes the major compounds phosphatidylcholine and diphosphatidylglycerol and moderate amounts of phosphatidylethanolamine, phosphatidylglycerol, an unidentified glycolipid, and two unidentified aminolipids. The major fatty acids are C19:0 cyclo ω8c, C18:1 ω7c, and C16:0, with C18:0 3-OH as the major hydroxylated fatty acid. The G+C content of the DNA of the type strain of the type species is 65 mol%.

The genus contains two species, *C. lactis* (type species) and *C. abortus*, that can be differentiated according to the traits in Table 4.3.

Chelatococcus

Description of the genus *Chelatococcus* is based on Auling et al. (1993) and the emendation by Yoon et al. (2008). Cells are cocci

Table 4.4
Differential traits between *C. asaccharovorans* (Auling et al. 1993), *C. daeguensis* (Yoon et al. 2008), and *C. sambhunathii* (Panday and Das 2010)

	C. asaccharovorans	*C. daeguensis*	*C. sambhunathii*
Cell morphology	Cocci	Rods	Rods
Motility	–	+	+
Hydrolysis of gelatin	+	–	+
NaCl tolerance range (w/v)	0.0–2.5	0.0–0.5	0.0–3.0
Utilization of			
Mannose, xylose, gluconate, D-arabinose, cysteine	+	+	–
Glycerol, salicin, inositol, adonitol, cellobiose, xylitol, aesculin, rhamnose, galactose, methionine, threonine, tyrosine, lysine	–	+	–
Glycine	+	–	+
Nitrilotriacetate	+	–	+
Acid production from			
Glucose, inositol, arabinose, rhamnose	–	+	–
pH range for growth	5.5–9.5	5.5–10.0	6.0–8.5
pH optimum for growth	7.0–8.0	7.0–7.5	7.5–8.0

or rods (Table 4.4). Gram negative. Nonmotile. Obligately aerobic. Optimal growth temperature is 35–37 °C and temperature range for growth is 4–41 °C. Poly-β-hydroxybutyrate is accumulated within the cells. Utilization of the metal-chelating nitrilotriacetic acid (NTA) as sole source of carbon, nitrogen, and energy is positive or negative. Ubiquinone Q-10 is present. The major fatty acids are C18:1 ω7c and C19:0 cyclo ω8c. Major polar lipids are phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, two aminolipids, and two unidentified phospholipids. Major polyamines are sym-homospermidine, spermidine, and putrescine. The DNA G+C content is 63.3–68.3 mol%.

The type species, *C. asaccharovorans*, requires one or more vitamins (Auling et al. 1993), whereas growth factors are not needed by the two other established species of the genus. Besides the typical Gram-negative cell envelope (cytoplasmic membrane, murein layer, and outer membrane), *C. asaccharovorans* is surrounded by an additional proteinaceous S-layer approximately 15 nm wide.

There are some traits of the genus description, not formally emended though, that are discrepant in *C. daeguensis* and *C. sambhunathii*. These include temperature growth range (20–50 °C on both species), anaerobic growth with nitrate (both species), and motility (*C. sambhunathii* has one polar flagellum).

Methylocapsa

The description of *Methylocapsa* is that of Dedysh et al. (2002) later emended by Dunfield et al. (2010). Gram-negative, curved coccoids or thick curved rods (Table 4.5) that occur singly or in conglomerates, but do not form rosettes. Surrounded by an extracellular polysaccharide matrix (the etymologic meaning of the genus name is methyl-using cell covered by a capsule). Reproduce by normal cell division. Nonmotile. Produce intracellular poly-β-hydroxybutyrate granules. Resting cells are *Azotobacter* type cysts. Cells are not lysed by 2 % SDS. Cells possess a well-developed intracytoplasmic membrane system of type III which appears as stacks of membrane vesicles packed in parallel on only one side of the cell membrane. Strictly aerobic. Possess pMMO and do not express sMMO. Moderately acidophilic and mesophilic. Prefers dilute media of low salt content. Some members of the genus are obligate utilizers of C_1 compounds via the serine pathway, while others are also capable of growth on acetate, but sugars are not utilized. Does not contain the ribulose monophosphate and ribulose bisphosphate enzymes. Tricarboxylic acid cycle is complete. Capable of atmospheric nitrogen fixation. The major cellular fatty acid is C18:1 ω7c and the major phospholipids are phosphatidylglycerols. The G+C content is 61.4–63.1 mol%.

Methylocella

The isolation of acidophilic methane-oxidizing bacteria from northern peat wetlands (Dedysh et al. 1998a) paved the way for the description of the new genus and species *Methylocella palustris* (Dedysh et al. 2000). Two more species proposals followed, *M. silvestris* (Dunfield et al. 2003) and *M. tundrae* (Dedysh et al. 2004a), each time including an emendation of the genus description. Several characteristics of the genus make

Table 4.5
Differential traits between *M. acidiphila* (Dedysh et al. 2002) and *M. aurea* (Dunfield et al. 2010)

	M. acidiphila	*M. aurea*
Cell morphology	Curved coccoids	Thick curved rods
Cell size	0.5–0.8 × 0.8–1.2 μm	0.7–1.2 × 1.8–3.1 μm
Growth on solid media	Not achieved	Yellow colonies
Carbon sources	Methane and methanol (obligate methanotrophy)	Methane, methanol, formate, and acetate (facultative methanotrophy)
pH range for growth (optimal)	4.2–7.2 (5.0–5.5)	5.2–7.2 (6.0–6.2)
Temperature range for growth (optimal)	10–30 °C (20–24 °C)	2–33 °C (25–30 °C)
Growth in nitrogen-free medium under fully aerobic conditions	+	–
Growth inhibited by NaCl (w/v)	0.5 %	0.3 %

it unique compared with other methanotrophic bacteria. Representatives of this genus do not contain an intracytoplasmic membrane system like that in all other known methanotrophs and appear to possess only a soluble form of methane monooxygenase (sMMO).

Methylocella spp. are Gram-negative, polymorphic rods or ovoids that are 0.6–1.0 μm wide by 1.0–2.5 μm long with rounded ends. Produce large, highly refractile, intracellular poly-β-hydroxybutyrate granules. Reproduce by normal cell division. Cells occur singly or in irregularly shaped aggregates, but do not form rosettes. Nonmotile. Encapsulated. Cells are not lysed by 2 % SDS. Cells lack an extensive ICM system typical of both type I and type II methanotrophic bacteria but contain a vesicular membrane system composed of singular flattened or ovoid vesicles connected to the cytoplasmic membrane. Possess sMMO. The temperature range for growth is 4–30 °C with an optimum at 15–25 °C; no growth occurs at 37 °C. Growth occurs between pH 4.2 and 7.5. Highly sensitive to salt stress; prefer diluted media with a low salt content. Utilize C_1 compounds via the serine pathway. Do not contain the enzymes of the ribulose monophosphate and ribulose bisphosphate pathways. The tricarboxylic acid cycle is complete. Fix atmospheric nitrogen via an oxygen-sensitive nitrogenase. The major phospholipid fatty acids are 18:1 ω7c acids. The G+C content of the DNA is 60–63 mol%.

The intact phospholipid profiles of *M. palustris* using liquid chromatography/electrospray ionization/mass spectrometry (Fang et al. 2000) revealed it has a preponderance of phosphatidylmethylethanolamine phospholipids with C18:1 fatty acids. The relative abundance of C18:1 is one of the features that help to discriminate species of the genus (*Table 4.6*).

Methylocella species are considered the first fully authenticated examples of facultative methanotrophs (Dedysh et al. 2005a). In addition to methane and methanol, they can utilize as well multicarbon compounds, such as acetate, pyruvate, succinate, malate, and ethanol. This ability does not include other compounds tested, urea, glucose, fructose, sucrose, lactose, galactose, xylose, sorbose, maltose, raffinose, arabinose, ribose, lactate, oxalate, citrate, mannitol, or sorbitol, but clearly provides a competitive advantage in natural environments where methane production is temporally heterogeneous due to fluctuations in temperature, water content, and water table level (Segers 1998). The molecular regulation of methane oxidation in *M. silvestris* $BL2^T$ during growth on methane and acetate has been assessed (Theisen et al. 2005).

Methyloferula

Shortly after the description of *Methylovirgula* (Vorob'ev et al. 2009), another genus of rosette-forming bacteria was proposed in the family *Beijerinckiaceae*. *Methyloferula* (Vorobev et al. 2011), an obligate methanotroph, was described as Gram-negative, aerobic, colorless, nonmotile rods that occur singly or are arranged in rosettes and misshapen cell clusters. Reproduce by irregular fission. Colonies are small, circular, and smooth. The colony color varies from white to cream. Produce poly-β-hydroxybutyrate. Mesophilic and moderately acidophilic. Prefer dilute media of low salt content. Obligate utilizers of methane and methanol; the latter is the preferred growth substrate. Possess only a soluble MMO. Positive to RubisCO activity. Capable of atmospheric nitrogen fixation. The major fatty acid is 18:1 ω7c. The major quinone is Q-10. The G+C content of the DNA is 55.6–57.5 mol%.

The type species *M. stellata* is the only one of the genus to date. Its cells are 0.4–0.65 μm wide and 1.1–3.0 μm long. Carbon sources include methane and methanol. Grows optimally at methanol concentrations of 0.5–1 %. Nitrogen sources (0.05 %, w/v) include ammonia, nitrate, urea, and yeast extract. Capable of growth at pH 3.5–7.2 (optimum pH 4.8–5.2) and at 4–33 °C (optimum 20–23 °C). NaCl inhibits growth at concentrations above 0.7 % (w/v).

Methylorosula

Methylorosula (Berestovskaya et al. 2012) is described as Gram-negative rods that are not capable of nitrogen fixation. Aerobic, facultative methylotrophic bacteria.

Table 4.6
Differential traits between *M. palustris* (Dedysh et al. 2000), *M. silvestris* (Dunfield et al. 2003), and *M. tundrae* (Dedysh et al. 2004a). Substrate utilization from (Dedysh et al. 2005a)

	M. palustris	*M. silvestris*	*M. tundrae*
Cell morphology	Straight or curved short rods	Straight or curved short rods	Curved ovoids
Macrocapsule (slimy colonies)	+	+	–
pH range for growth (optimal)	4.5–7.0 (5.5)	4.5–7.0 (5.5)	4.2–7.5 (5.5–6.0)
Temperature range for growth (optimal)	10–28 °C (20 °C)	4–30 °C (15–25 °C)	5–30 °C (15 °C)
Growth inhibited by NaCl (w/v)	0.5 %	0.8 %	0.8 %
Concentration range of methanol (v/v) utilization	0.01–0.1 %	0.01–5 %	0.01–2 %
Substrate utilization			
Formate	–	–	w
Methylamine	w	+	+
Fatty acids (%)			
16:1 ω7t	5.8	0	0
17:0 cyclo	0	0	5.0–6.5
18:1 ω7c	78.6	82.2	59.2–61.7
19:0 ω8c cyclo	0	0	7.9–13.6

Chemoorganoheterotrophic. Use the RuBP pathway for carbon assimilation. The dominant cellular fatty acid is C18:1 ω7c.

M. polaris is the type species, and only species thus far, of the genus. Its cells are long, bipolar rods (0.5–0.6 × 1.3–4.5 μm) that are single or form rosettes. Reproduces by irregular fission. Young cells are motile by means of a subpolar flagellum. Forms pale, non-translucent slimy (mucoid) colonies with an uneven edge. Resting cells are not known. After 2 weeks of growth on methanol at 20 °C, colonies are 1.5–2.0 mm in size. Utilizes C_1 compounds, methanol and methylamines, sugars, polysaccharides, sugar alcohols, and amino acids as a carbon and energy source. The temperature and pH ranges for growth are 4–30 °C (optimum, 20–25 °C) and pH 4.0–7.8 (optimum, pH 5.5–6.0), respectively. NaCl is not required for growth and is tolerated up to a concentration of 2.0 % (w/v). The major fatty acid is C18:1 ω7c. The DNA G+C content of the type strain is 65.2 mol%.

Methylovirgula

The genus *Methylovirgula* (Vorob'ev et al. 2009) contains Gram-negative, aerobic, colorless, nonmotile, 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 color 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 C18:1 ω7c, 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%.

The type and only species is *M. ligni*, whose 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).

Enrichment, Isolation, and Maintenance Procedures

Strategies for enrichment and isolation are different depending on the metabolism and physiology considered.

Beijerinckia spp. are chemoheterotrophic diazotrophs, mesophilic, able to grow on a wide range of pH (including values as low as 3.0 units), and known to show a preference for sugars as carbon sources. Complex media such as peptone medium are even inhibitory and yield no growth. Thus, nitrogen-free media with low pH are considered selective for members this genus. A simple formulation used in early studies (Becking 1961; Derx 1950a, b) contains (g/l of distilled water) glucose, 20.0; KH_2PO_4, 1.0; and $MgSO_4 \cdot 7H_2O$, 0.5 (pH is adjusted to 5.0). Aeration is important to inhibit the development of nitrogen-fixing anaerobes. So, instead of using tubes, the medium can be poured as thin layers (2–3 mm) into Petri dishes. Approximately 0.5 g of soil per plate can be used as inoculum. This provides also the necessary trace elements. For water samples, for example,

irrigation water, larger inocula might be needed to compensate the much lower presence of *Beijerinckia* in this type of habitat. Enrichment can be then approached mixing the water sample with sterile double-strength medium before dispensing the dishes as before. Samples collected from the phyllosphere can be immersed in the enrichment medium for the same purpose. Recommended incubation is at 30 °C for 2 or more weeks. Positive enrichments cultures can be streaked on the following isolation nitrogen-free, mineral agar medium (Becking 2006) that contains (g/l of distilled water) glucose, 20.0; K_2HPO_4, 0.8; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.5; $FeCl_3 \cdot 6H_2O$, 0.025; $Na_2MoO_4 \cdot 2H_2O$, 0.005; $CaCl_2$, 0.05; and agar 15.0 (pH is adjusted to 6.9) that can also be used for routine maintenance. Development of exopolysaccharide, a common trait of *Beijerinckia*, can be easily recognized by the slimy appearance of the colonies or by the viscosity of liquid-aged cultures. An easy confirmation step is plating on peptone medium because *Beijerinckia* will not develop, whereas other free-living diazotrophs such as *Azotobacter* or *Azomonas* will grow readily.

Camelimonas have been isolated from two different farm animals in different countries but using very similar media. Strains *C. lactis* M 2040^T, M 1973, and M 1878-SK2 were initially cultivated on *Brucella* agar (Difco) from milk of different camels of a camel milk production farm in the United Arab Emirates during routine bacteriological testing for *Brucella* species in 2008 (Kämpfer et al. 2010). *C. abortus* UK34/07-5^T was initially cultured on Farrell's medium (Farrell 1974) from placental tissue following a case of abortion in a Holstein–Friesian cow in Derbyshire, UK, in 2007 (Kämpfer et al. 2012). Farrell's medium was manufactured using a *Brucella* medium base (CM0169; Oxoid) and a Brucella selective supplement (SR0209E; Oxoid). So, it seems that an adequate formulation is (g/l of distilled water) peptone, 10.0; "Lab-Lemco" powder, 5.0; glucose, 10.0; NaCl, 5.0; and agar, 15.0 (pH 7.5). Antibiotic combinations used as selective supplements for *Brucella* can also favor the growth of *Camelimonas* sp., as occurred with *C. abortus*. In both cases, the classical methods for identification of *Brucella* species failed because they are phenotypically very distinct. Subcultivation was done in tryptone soy agar (Kämpfer et al. 2010, 2012) and other common general media can be also employed.

The two strains that served for the description of ***Chelatococcus*** *assacharovorans* were isolated with a batch enrichment procedure using nitrilotriacetic acid (NTA) as the only source of carbon, energy, and nitrogen (Egli 1988). However, they were not as easily enriched as members of the genus *Chelatobacter*. The fact that the optimal growth temperature of *Chelatococcus* strains (35–37 °C) is considerably higher than that of *Chelatobacter* strains, together with their resistance to vancomycin, was proposed to enrich for *Chelatococcus* when using NTA as the only source of carbon and nitrogen for growth (Egli and Auling 2005). However, the next two species of the genus, each based on a single strain, were obtained following a more general approach. *C. daeguensis* K106^T (Yoon et al. 2008) was isolated from wastewater of a textile dye works in Korea, by means of the standard dilution-plating technique on trypticase soy agar (TSA; Difco) at 30 °C. *C. sambhunathii* HT4^T (Panday and Das 2010) was retrieved from sediment of an Indian hot spring that was inoculated into a 250 ml conical flask containing 50 ml nutrient broth (Difco) and incubated on a shaker at 200 r.p.m. and 45 °C. After 2 days incubation, the contents of the flasks were serially diluted and plated onto nutrient agar medium (Difco) and incubated at 37 °C for 4 days, after which colonies were picked and purified by repeated streaking on the same medium. Interestingly, *C. sambhunathii* HT4^T showed a nutritional pattern closer to *C. asaccharovorans* than to *C. daeguensis* (Table 4.4) and was also able to grow on NTA as single carbon, nitrogen, and energy source although it had not been used for the enrichment and isolation.

Methylocapsa, ***Methylocella***, and ***Methyloferula*** are methanotrophs, either facultative or obligately, that can be cultivated using liquid or solid mineral media with methane as growth substrate (Dedysh and Dunfield 2011). Facultative methanotrophic species can utilize multicarbon compounds, but its use for isolation or even maintenance is not recommended since these substrates give a selective advantage to chemoorganoheterotrophs that will contaminate and surpass the methanotrophic cultures. Two media have been successfully applied for the enrichment and isolation of most strains of these three genera:

- Nitrogen-free medium M2 (Dedysh et al. 1998b) containing (in grams per liter of distilled water) KH_2PO_4, 0.04; $MgSO_4 \cdot 7H_2O$, 0.02; and $CaCl_2 \cdot 2H_2O$, 0.004, with the addition of 0.1 % (by volume) of a trace elements stock solution containing (in grams per liter) EDTA, 5; $FeSO_4 \cdot 7H_2O$, 2; $ZnSO_4 \cdot 7H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.03; $CoCl_2 \cdot 6H_2O$, 0.2; $CuCl_2 \cdot 5H_2O$, 0.1; $NiCl_2 \cdot 6H_2O$, 0.02; and Na_2MoO_4, 0.03. The initial pH of the medium can be adjusted with concentrated phosphoric acid from 6 to 3.
- Dilute nitrate mineral salts (DNMS) medium (Dunfield et al. 2003) containing (in grams per liter of distilled water) KNO_3, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; $Na_2HPO_4 \cdot 12H_2O$, 0.143; KH_2PO_4, 0.054; and $CaCl_2 \cdot 6H_2O$, 0.04 and containing 1 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 5.8).

For growth in liquid medium, screw cap serum bottles are used with a headspace/liquid space ratio of 4:1. After inoculation, the bottles are sealed with silicone rubber septa, and methane is added aseptically using a syringe equipped with a disposable filter (0.22 μm) to achieve a 15–20 % mixing ratio in the headspace. Bottles can be incubated on a rotary shaker (120 r.p.m.) at 20–25 °C. Solid media are prepared adding agar or gellan gum as solidifying agents (15 g per liter). These media are incubated in closed vessels or desiccators containing air supplemented with (v/v) CH_4 (10–30 %) and CO_2 (5 %) and can be used for maintenance.

Of course, other formulations are possible (Vorobev et al. 2011; Dedysh and Dunfield 2011) with variations in the presence or the amount of minerals and/or adjusted pH. Initial incubation times are long (up to 2 months). Methanotrophic cultures are frequently contaminated by heterotrophic bacteria that survive on metabolic by-products of methanotrophs. The presence of only a few satellite cells in a culture may lead to false

conclusions regarding substrate utilization, and several early reports of facultative methanotrophy are likely attributable to impure cultures. Another recurring mistake is the misidentification of non-methanotrophic facultative methylotrophs as facultative methanotrophs. Valuable advice on how to differentiate and identify facultative and obligate methanotrophs, not necessarily members of the family *Beijerinckiaceae*, is given by Dedysh and Dunfield (2011).

Media based on methane as carbon source are not suited for the facultative methylotrophic genera ***Methylorosula*** and ***Methylovirgula*** because they lack MMO. But actually, the three strains that conformed *Methylorosula polaris* (Berestovskaya et al. 2012) were isolated from acidophilic, methanotrophic enrichments obtained from *Sphagnum* peat of three tundra wetland sites of Northern Russia. Cell suspensions of these enrichments were spread onto medium 2A (Berestovskaya et al. 2002) agar plates, containing 2 % (w/v) agar (Difco) and amended with 0.5 % methanol as the sole carbon source. Mineral medium 2A had been specially developed to cultivate microorganisms from ultrafresh habitats due to its low mineralization (mg/l): NH_4SO, 500; $MgCl_2$, 40; KH_2PO_4, 70; and a solution of microelements, 1 ml/l (Berestovskaya et al. 2002). In the case of *Methylovirgula ligni*, each of the two strains that served for its description was recovered from a nutrient-rich, although diluted, medium. One of them, designated medium W5, had the following composition (per liter of demineralized water): NaCl, 1 g; yeast extract, 0.1 g; 2-[*N*-morpholino]ethane sulfonic acid (MES) as a buffering compound, 1.95 g; and agar, 20 g. Medium T5 contained (per liter) NaCl, 1 g; trypticase soy broth (Oxoid), 3 g; MES, 1.95 g; and agar, 20 g. Attempts to maintain both strains on their respective isolation media proved they were not suited for optimal growth and a slightly modified medium M2 (designated medium MM2) resulted in good development of the strains 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 (mg per liter distilled water): $(NH_4)_2SO_4$, 200; KH_2PO_4, 100; $MgSO_4$, 50; $CaCl_2 \cdot 2H_2O$, 20; yeast extract, 10; and 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.

In addition to periodic transfer using the maintenance media referred above, members of the family can be successfully maintained using the common long-term preservation protocols and techniques, that is, lyophilization and deep freezing of cell suspensions in the presence of lyo-/cryoprotectants. The possibility of preserving *Beijerinckia* cultures by encapsulation using a spray drier, for use in biotechnological processes in the production of biopolymers, has been addressed (Boza et al. 2003). Among different wall (coating) malt dextrin presented the greatest stability with respect to fermentative activity, although the glucose-encapsulated cells showed the highest percentage of viability during spray drying and during the storage period.

Ecology

Members of the type genus ***Beijerinckia*** are commonly found as free-living bacteria in acidic soils and also in plant rhizosphere and phyllosphere environments. The earliest recorded isolations (Becking 2006) are from a Malaysian quartzite soil (pH 4.5) by Altson (1936), from a Dacca, Bangladesh, soil (pH 4.9), and from a soil of Insein, Burma (pH 5.2), by Starkey and De (1939), although considered at that time *Azotobacter* spp. Later it was found to be widely distributed in the more acidic soils of mainly tropical regions, and it was also isolated from neutral and slightly alkaline soils and outside the tropics. In a large soil survey covering 392 soils of worldwide distribution, *Beijerinckia* was found in some temperate and subtropical soils of Europe (former Yugoslavia), South Africa, India, China, and Japan (Becking 1959, 1961). Other authors mention its isolation from other nontropical soils, i.e., from an acidic volcanic ash soil of Sendai, Tohoku District, Japan (lat. 38°N) (Suto 1954, 1957); from some Indian nontropical soils (up to lat. 27–30°N) (Barooah and Sen 1959); in some subtropical and nontropical soils in Australia (lat. 15–43°S) (Tchan 1953; Thompson 1968; Thompson and Skerman 1979); and in two soils of Egypt (Kharga Oasis, lat. 25°N) (Vančura et al. 1965). In addition, it was isolated from some Pacific Northwest soils in North America (Snake River Plain, Idaho, USA; lat. 44°N), from soil of the Squamish Bay Area, British Columbia, Canada (lat. 50°N) by Anderson (1966). Also, Jordan and McNicol (1978) reported the isolation of *Beijerinckia* from three sites of a permanently cold, high Arctic soil (Devon Island, Northwest Territories; lat. 75°N).

Distribution in the more tropical soils of the African continent was largely explored. In addition to the South African soils already mentioned (Becking 1959, 1961) *Beijerinckia* was observed to occur in soils of Zimbabwe (Meiklejohn 1968), Ivory Coast (Kauffmann and Toussaint 1951a, b), Sudan (Hegazi and Ayoub 1979), Sierra Leone, Nigeria, Ethiopia, Uganda, Kenya, Tanzania, and Congo/Zaire (Becking 1961). *Beijerinckia* was also found in many soil samples of South America: Argentina (Amor Asunción et al. 1980), Trinidad Island, Surinam, Venezuela, Bolivia, and Brazil (Becking 1961).

Decades of research on the biological nitrogen fixation in graminaceous plants in Brazil (Baldani and Baldani 2005) have provided many insights on ecology of *Beijerinckia*. It was demonstrated that the size of the populations was related to vegetation, physical, and chemical characteristics of the soil (Döbereiner and Castro 1955). Further studies on the occurrence of this genus in soil of several Brazilian States (Rio de Janeiro, São Paulo, Pernambuco and Paraná) led to the description of *B. fluminensis* (Döbereiner and Ruschel 1958). Analysis of 158 samples collected in different regions of Brazil showed that this species occurred predominantly in soils where sugarcane was cultivated (Döbereiner 1959a) and a direct influence of

the plant on the development of the bacteria was suggested (Döbereiner 1959b). Additional studies showed that roots as well as leaves and stems had a positive influence on *Beijerinckia* populations. This was influenced by the exudation of substances into the soil by the roots during rainfall (Döbereiner and Alvahydo 1959). The population of *Beijerinckia* was much more pronounced in the rhizoplane region (refers to the soil adherent to the root surface) than in the rhizosphere. In addition, it was shown that removal of the aerial part of the plant significantly reduced the population of bacteria in both the rhizoplane and rhizosphere regions (Döbereiner 1961). Rice plants grown in greenhouse and inoculated with *Beijerinckia* showed the establishment of the bacteria as well as an increase in the yield (Döbereiner and Ruschel 1961). The measurement of the nitrogenase activity in roots of sugarcane, thanks to the introduction of acetylene reduction methodology, showed that it was much higher than that observed in the rhizosphere and in soil between the plant rows. *B. indica* was the most abundant bacterial species in both roots and soil samples (Döbereiner et al. 1972). Quantification of nitrogen fixation in sugarcane based on the extrapolation of the nitrogenase activity data indicated a contribution of 50 kg N/ha/year to the soil/plant system (Döbereiner et al. 1973).

Concerning habitats other than soil, Ruinen (1956, 1961) found *Beijerinckia* as a regular component of the phyllosphere of tropical plants. Diem et al. (1978) have used the fluorescent antibody technique to study the behavior of a *Beijerinckia* isolate in the rhizosphere and seed region (spermosphere) of rice seedlings in Camargue (France). Also in rice, Karkhanis and Tikhe (1980) and Karkhanis (1987) reported not only its presence in the rhizosphere but also its intracortical occurrence within the rice root. Murty (1984) measured nitrogenase activities on the leaf surfaces of different varieties of cotton (*Gossypium hirsutum* L. and *G. herbaceum* L.) plants, and *Beijerinckia* sp. was observed to be the predominant nitrogen-fixing microorganism in the phyllosphere of these varieties.

Jackson and Denney (2011) studied the annual and seasonal variation in the phyllosphere bacterial community associated with leaves of the southern Magnolia (*Magnolia grandiflora*) using multivariate analysis of denaturing gradient gel electrophoresis profiles of 16S rRNA gene fragments. Temporal changes had a much greater variation than leaf to leaf. This was confirmed by sequencing and analysis of 16S rRNA gene clone libraries generated for each sample date. All phyllosphere communities were dominated by *Alphaproteobacteria*, with a reduction in the representation of *Beijerinckiaceae* related to *B. indica* and *M. silvestris* during the summer and a concurrent increase in the *Methylobacteriaceae* being the most significant seasonal change.

The knowledge about the ecology of ***Camelimonas*** is limited to the information of the habitat from where the two species described were isolated: milk of camels at a camel milk production farm in the United Arab Emirates for *C. lactis* (Kämpfer et al. 2010) and placental tissue following a case of abortion in a Holstein–Friesian cow in the UK for *C. abortus* (Kämpfer et al. 2012). This limitation does not occur with ***Chelatococcus***; the type species *C. asaccharovorans* was isolated from wastewater and soil in Switzerland (Auling et al. 1993), but its ubiquitous distribution in surface waters and sewage treatment plants was shown by immunofluorescence (Wilberg et al. 1993). *C. daeguensis* was indeed isolated from wastewater, in this case, from textile dye works in the Republic of Korea (Yoon et al. 2008), while *C. sambhunathii* originated from a hot spring sediment in India (Panday and Das 2010). In addition to water, soils can also be inhabited by *Chelatococcus*. In a recent study (Fredrickson et al. 2008) dealing with bacterial cultures obtained from irradiated soils in Washington (USA), 6 out of 47 were identified as *Chelatococcus* sp. For extremely ionizing radiation-resistant bacteria, survival has been attributed to protection of proteins from oxidative damage during irradiation, with the result that repair systems survive and function with far greater efficiency during recovery than in sensitive bacteria. The relationship between survival of dry climate soil bacteria and the level of cellular protein oxidation induced by desiccation was examined. Desiccation-resistant isolates accumulated high intracellular manganese and low iron concentrations compared to sensitive bacteria. In vivo, proteins of desiccation-resistant bacteria were protected from oxidative modifications that introduce carbonyl groups in sensitive bacteria during drying. Members of *Chelatococcus* have also been identified among other genera present in the headbox water of a paper machine in Canada (Prince et al. 2009), using an amplified ribosomal DNA restriction analysis.

Acidic wetlands, particularly *Sphagnum* peat bogs, from different sites of Russia and forest soils from Germany, Russia, and the Netherlands were the habitats from where the species of ***Methylocapsa***, ***Methylocella***, ***Methyloferula***, ***Methylorosula***, and ***Methylovirgula*** were originally isolated (Berestovskaya et al. 2012; Dedysh et al. 2000, 2002, 2004a; Dunfield et al. 2003, 2010; Vorob'ev et al. 2009; Vorobev et al. 2011).

Wetlands with peat-forming soils (peatlands) provide habitat for anaerobic archaeal methanogens that produce CH_4 under strictly anoxic conditions, usually below the water table. In contrast aerobic methane-oxidizing bacteria (methanotrophs) are active primarily above the water table in aerated portions of the soil and play an important role in wetlands by limiting CH_4 emissions to the atmosphere. Given the global scope of bogs, the number of molecular studies of methanotrophs in these sites is still small. Gupta et al. (2012) investigated the active methanotroph community in two contrasting North American peatlands, a nutrient-rich sedge fen and nutrient-poor *Sphagnum* bog using in vitro incubations and DNA stable-isotope probing (DNA-SIP) with ^{13}C–CH_4. This approach avoids cultivation biases or issues associated with facultative methanotrophs such as members of the genus *Methylocella* being active, but not necessarily using CH_4 as a substrate. Rates of CH_4 oxidation were slightly, but significantly, faster in the bog, and methanotrophs belonged to the class *Alphaproteobacteria* and were similar to other methanotrophs of the genera *Methylocystis*, *Methylosinus*, and *Methylocapsa* or *Methylocella* detected in, or isolated from, European bogs. The fen had a greater phylogenetic diversity of organisms that had

assimilated ^{13}C, including methanotrophs from both the *Alpha-* and *Gammaproteobacteria* classes and other potentially non-methanotrophic organisms that were similar to bacteria detected in a UK and Finnish fen. Based on similarities between bacteria in North American sites and those in Europe, including Russia, it was concluded that site physicochemical characteristics rather than biogeography controlled the phylogenetic diversity of active methanotrophs and that differences in phylogenetic diversity between the bog and fen did not relate to measured CH_4 oxidation rates (Gupta et al. 2012). Using a different approach, fluorescent in situ hybridization with 16S rRNA targeted oligonucleotide probes; Dedysh et al. (2003) detected *Methylocystis* spp. as the numerically largest methanotroph group in peat from Siberia and Germany, followed by *Methylocapsa acidiphila* and *Methylocella palustris*. *M. acidiphila* was enumerated in both samples at greater than 10^5 cells g^{-1} of peat. This accounted for 5 % and 2 % of total methanotroph cells detected by FISH in peat from Siberia and Germany, respectively. In contrast, the population sizes of *M. palustris* were significantly different in *Sphagnum* peat from West Siberia (10^6 cells g^{-1} of peat) and northern Germany (10^4 cells g^{-1} of peat). But, the possibility that some unknown species of acidophilic *Methylocella* and *Methylocapsa* escaped detection by FISH with the available set of oligonucleotide probes cannot be excluded. Besides, these values refer to single-time, single peat soil depth sample measurements. Significant variations in methanotroph community structure and abundance might occur in different subsites and depths as well as on a seasonal scale. In addition, some environmental factors (variation in temperature, aeration conditions, substrate availability) might also have a strong impact on population dynamics of different methanotrophs in acidic peatlands. Indeed, a previous study at Bakchar bog, Plotnikovo field station in west Siberia, 56°N, 82°E, revealed *M. palustris* as predominant methanotroph well above *Methylocystis* (Dedysh et al. 2001a).

Radajewski et al. (2002) applied DNA-SIP to characterize the active methylotroph populations in microcosms in an oak (*Quercus petraea*) forest soil (pH 3.5) collected from the Gisburn Forest Experiment, UK. The microcosms were exposed to $^{13}CH_3OH$ or $^{13}CH_4$ and distinct ^{13}C-labeled DNA fractions were resolved from total community DNA by CsCl density gradient centrifugation. Subsequent analysis of amplified 16S rRNA sequences from these ^{13}C-DNA revealed bacteria related to the genera *Methylocella*, *Methylocapsa*, *Methylocystis*, and *Rhodoblastus*. Enrichments targeted towards the active proteobacterial CH_3OH utilizers were successful, although none of these bacteria were isolated into pure culture. Chen et al. (2008) investigated the active methanotroph community in heather (*Calluna*)-covered moorlands and *Sphagnum-*/*Eriophorum*-covered UK peatlands. Direct extraction of mRNA from these soils facilitated detection of expression of methane monooxygenase genes, which revealed that particulate methane monooxygenase, and not soluble methane monooxygenase, was probably responsible for CH_4 oxidation in situ, because only *pmoA* transcripts (encoding a subunit of particulate methane monooxygenase) were readily detectable. Differences in methanotroph community structures were observed between both habitats. In *Calluna*-covered moorlands, in addition to *Methylocella* and *Methylocystis*, a unique group of peat-associated type I methanotrophs (*Gammaproteobacteria*) and a group of uncultivated type II methanotrophs (*Alphaproteobacteria*) were also found. The *pmoA* sequences of the latter were only distantly related to *Methylocapsa* and also to the RA-14 group of methanotrophs, which are believed to be involved in oxidation of atmospheric CH_4. Soil samples were also labeled with $^{13}CH_4$, and subsequent analysis of the ^{13}C-labeled phospholipid fatty acids showed that C16:1 ω7, C18:1 ω7, and C18:1 ω9 were the major labeled fatty acids. Since C18:1 ω9 is not a major fatty acid of any established species of methanotroph, it suggests the presence of novel species in this peatland. The *Sphagnum-*/*Eriophorum*-covered gullies were dominated by *Methylocystis*, but *Methylocella*- and *Methylocapsa*-related species were also present.

Corsican pine (*Pinus nigra*) roots colonized by the ectomycorrhizal (ECM) fungi, *Suillus variegatus* and *Tomentellopsis submollis*, were examined analyzing the diversity of bacterial nitrogenase genes (*nifH*) present and their mRNA transcription (Izumi et al. 2006). DNA-derived *nifH* PCR products were obtained from all samples, but only a few reverse transcription PCRs for *nifH* mRNA were successful, suggesting that nitrogenase genes were not always transcribed. Putative nitrogenase amino acid sequences revealed that more than half of the *nifH* products were derived from methylotrophic bacteria, such as *Methylocella* spp.

Methanotroph abundance was analyzed in control and long-term nitrogen-amended pine and hardwood soils in Massachusetts, USA (42°13′N, 72°11′W) using rRNA-targeted quantitative hybridization (Lau et al. 2007). Family-specific 16S rRNA and *pmoA*/*amoA* genes were analyzed via PCR-directed assays to elucidate methanotrophic bacteria inhabiting soils undergoing atmospheric methane consumption. Quantitative hybridizations suggested methanotrophs related to the family *Methylocystaceae* were one order of magnitude more abundant than *Methylococcaceae* and more sensitive to nitrogen addition in pine soils. 16S rRNA gene phylotypes related to known *Methylocystaceae* and acidophilic methanotrophs and *pmoA*/*amoA* gene sequences, including three related to the upland soil cluster *Alphaproteobacteria* (USCalpha) group, were detected across different treatments and soil depths. The result suggested that methanotrophic members of the *Methylocystaceae* and *Beijerinckiaceae* may be the candidates for soil atmospheric methane consumption (Lau et al. 2007).

Landfills are also a suitable habitat for methanotrophs with important ecological implications. The identity of active methanotrophs in Roscommon (Ireland) landfill cover soil, a slightly acidic peat soil, was assessed by DNA-SIP (Cébron et al. 2007). Landfill cover soil slurries were incubated with ^{13}C-labeled methane and under either nutrient-rich nitrate mineral salt medium or water. 16S rRNA gene analysis revealed that the cover soil was mainly dominated by Type II methanotrophs closely related to the genera *Methylocella* and *Methylocapsa* and to *Methylocystis* species. These results were supported by analysis

of *mmoX* genes in ^{13}C-DNA. Analysis of *pmoA* gene diversity indicated that a significant proportion of active bacteria were also closely related to the Type I methanotrophs, *Methylobacter*, and *Methylomonas* species. Phylogenetic analysis of mRNA recovered from the soil indicated that *Methylobacter*, *Methylosarcina*, *Methylomonas*, *Methylocystis*, and *Methylocella* were actively expressing genes involved in methane and methanol oxidation. Transcripts of *pmoA*, but not *mmoX*, were readily detected by reverse transcription polymerase chain reaction (RT-PCR), indicating that particulate methane monooxygenase may be largely responsible for methane oxidation in situ (Chen et al. 2007).

Another study (Han et al. 2009), involving 16S rRNA gene and functional gene clone libraries and microarray analyses using *pmoA*, investigated the methanotroph community structure in alkaline soil from a Chinese coal mine. This environment contained a high diversity of methanotrophs, including the type II *Methylosinus* and *Methylocystis*, type I *Methylobacter*, *Methylosoma*, *Methylococcus*, and a number of as yet uncultivated methanotrophs. In order to identify the metabolically active methane-oxidizing bacteria from this alkaline environment, DNA-SIP experiments using $^{13}CH_4$ were carried out. This showed that both type I and type II methanotrophs were active, together with methanotrophs related to *Methylocella*, which had previously been found only in acidic environments. This finding opened the question if *Methylocella* species are widely distributed in nature and not restricted to acidic environments. The affirmative answer came through another study (Rahman et al. 2011) employing a real-time quantitative PCR method and primers targeting *Methylocella mmoX*.

Application

Beijerinckia have received attention due to their plant growth-promoting properties (Miyasaka et al. 2003; Thuler et al. 2003) and for their abundant production of exopolysaccharide with potential biotechnological uses (Scamparini et al. 1997).

Nitrogen-fixing bacteria are probably among the most extensively studied soil microorganisms. Several genera of free-living, N_2-fixing bacteria occur in high numbers in the rhizosphere, rhizoplane, and phyllosphere, and *Beijerinckia* is one of the most notable genera. Inoculation of rice seeds with *Beijerinckia* showed that this genus is able to multiply in the soil, establishing itself in large numbers and reducing the number of other competing microorganisms (Döbereiner and Ruschel 1961). It has been observed (Miyasaka et al. 2003) that *B. derxii* maintains an increasing nitrogenase-specific activity during the stationary growth phase. To verify the destination of the nitrogen fixed during this phase, intra- and extracellular nitrogenated contents were analyzed. Organic nitrogen and amino acids were detected in the supernatant of the cultures. An increase in intracellular content of both nitrogen and protein occurred. Cytoplasmic granules indicated the presence of arginine. The ability of a non-diazotrophic bacterium (*Escherichia coli*) to use *B. derxii* proteins as a source of nitrogen was observed concomitantly with *E. coli* growth. This suggested that *B. derxii* contributes to the environment by both releasing nitrogenated substances and accumulating substances capable of being consumed after its death. Moreover, the ability of *B. derxii* to release certain plant growth regulators (indoleacetic acid, ethylene and polyamines) and amino acids into the growth medium has also been addressed (Thuler et al. 2003). The production of those substances was compared using cultures in which nitrogenase was active (N-free medium) and cultures in which nitrogenase was repressed (combined-N cultures) and with and without agitation. Total indoleacetic acid production was higher in agitated, N-free cultures, but specific production was greater in combined-N cultures under agitation. Putrescine and spermidine were detected under all conditions tested. Ethylene was produced in both N-free and combined-N cultures. Although a greatest diversity of amino acids was released in N-free cultures, it is important that production of growth regulators was not inhibited under conditions where nitrogenase was inactive, and so are independent activities. The fact that indoleacetic acid was released by *B. derxii* in three of the four tested conditions suggests that it is likely to be produced in the rhizosphere and therefore influence plant growth. The diversity of amino acids released by *B. derxii* was higher than that observed for *B. indica* (Pati et al. 1994) but in lower concentrations. The high concentration of glutamic acid detected under all conditions tested suggests that *B. derxii* actively excretes amino acids as the CFU number remained stable in N-free medium under agitation. The excretion of amino acids may be seen as a way for the bacteria to maintain a low intracellular N level, necessary for active N_2 fixation. However, this phenomenon may be particularly advantageous to the naturally surrounding environment where these amino acids may be directly assimilated and incorporated into the proteins of plants and a number of other living organisms. The growth-promoting effect of *B. mobilis* and *Clostridium* sp. cultures isolated from the pea rhizosphere on some agricultural crops was studied (Polianskaia et al. 2002). Seed soaking in bacterial suspensions followed by the soil application of the suspensions or their application by means of foliar spraying was found to be the most efficient method of bacterization. The application of both bacteria in combination with mineral fertilizers increased the crop production by 1.5–2.5 times. The study of the population dynamics of *B. mobilis* by genetic marking showed that it quickly colonized the rhizoplane of plants.

It is important to mention that a number of research articles that appeared between 1973 and 1996 dealing with *Beijerinckia* able to degrade polycyclic aromatic hydrocarbons such as anthracene, biphenyl, dibenzofuran, and phenanthrene, among others, correspond indeed to a different organism. The original strain, isolated from a polluted stream, is designated as B1 or Bwt and its mutant B8/36 (Gibson et al. 1973), but a polyphasic study concluded that it is a member of *Sphingomonas yanoikuyae* (Khan et al. 1996). It is interesting to note that two independent studies, employing apparently other isolates of *Beijerinckia*, do also claim polycyclic aromatic hydrocarbon degradative activity. One is strain *B. mobilis* 1f isolated from a soil contaminated

with creosote (Surovtseva et al. 1999), and the other one is a strain of *B. indica* proposed as a biomaterial for aerobic biofilm barriers for the removal of phenanthrene from groundwater (Lim et al. 2010).

As for the production of EPS, Lee et al. (2007) have characterized the water-soluble extracellular polysaccharide produced by *B. indica*, heteropolysaccharide-7 or PS-7, a gellan-like biopolymer. It is composed of β-D-glucose, α-L-rhamnose, and β-D-galacturonic acid in a molar ratio of approximately 5:1:1.3. The aqueous solution of PS-7 is highly viscous and shows high thermal stability over the temperature range from 4 °C to 93 °C. This PS-7 dispersion is stable in the wide pH range from 3.0 to 12.0 as well. The PS-7 dispersion shows distinct pseudoplastic behavior, which means the viscosity of PS-7 dispersion decreases as the shear rate increases. In the presence of cationic or polyvalent ions at high pH, PS-7 dispersion easily produced a gel matrix. Unique chemical structure and physical properties of PS-7 make them to be useful for a wide range of applications to the oil industries and potentially to dripless water-based latex well-joint cement adhesives and textile printing.

Chelatococcus has been considered for a number of applications mostly related with biodegradation and bioremediation. Aminopolycarboxylic acids are used in a broad range of household products and industrial applications to control the solubility and precipitation of metal ions. The two most widely used are ethylenediaminetetraacetate (EDTA) and nitrilotriacetate (NTA). Because their application is mostly water-based, they are disposed off in wastewater from where they reach the environment if not sufficiently well eliminated. A number of NTA- and EDTA-degrading bacterial strains have been isolated. The NTA-degrading genera *Chelatobacter* and *Chelatococcus* appear to be well represented in most aerobic environments, and their key enzymes involved in NTA and EDTA catabolism have been studied in considerable detail (Egli 2001).

Also related with environment protection but this time through waste treatment is the recent report of an effective candidate for simultaneous nitrification and denitrificaton at high temperature (Yang et al. 2013). Strain, *Chelatococcus daeguensis* TAD1, isolated from the biofilm of a biotrickling filter at a coal-fired power plant in China has the ability to denitrify at 50 °C under aerobic condition without accumulation of nitrite, nitric oxide, and nitrous oxide and with nitrogen gas as its terminal product. It can utilize not only nitrate and nitrite but also ammonium at this temperature. The denitrification genes were identified (*napA*, *nirK*, *cnorB*, and *nosZ*) and sequenced. Heterotrophic nitrification and aerobic denitrification by one single organism has attractive advantages over the two-step process involving anaerobic denitrification because of its easy operation, cost benefit, and relatively accelerated rate.

Another thermophilic member of the genus *Chelatococcus*, strain E1 isolated from a compost sample, is capable of low-molecular-weight polyethylene degradation, and this activity has been thoroughly assessed in a recent study (Jeon and Kim 2013).

In the field of energy production and diversification of strategies with less environmental concerns, the biotransformation of coal into a cleaner fuel is being explored to extend the utilization of coal-derived biofuels as an alternative source of energy. This requires isolation of microbes with the capability to degrade complex coal into simpler substrates to support methanogenesis in the coal beds. For that purpose aerobic bacteria able to solubilize and utilize coal as the sole source of carbon were searched in samples from an Indian coal bed (700 m depth). Two isolates fulfilling these requirements, strains BHU2 and BHU6, were characterized and identified as *Chelatococcus* spp. (Singh and Tripathi 2011).

Polyhydroxybutyrate (PHB) production by *Chelatococcus* has also been approached. Ibrahim and Steinbüchel (2010) examined different fermentation strategies that were employed for the cultivation of *Chelatococcus* sp. strain MW10, a new PHB-accumulating thermophilic bacterium, with the aim of achieving high-cell-density growth and high PHB productivity. Enhanced cultivation was achieved by a cyclic fed-batch fermentation technique (42 l scale), and maximal productivity (16.8 ± 4.2 g/l) was obtained during the second cycle. Strain MW10 has the advantage of accumulating PHB using cheap media made with renewable resources (Ibrahim et al. 2010), but also being a moderate thermophile can help to reduce the costs of bioreactor operation since cooling is not required during the exothermic fermentation process.

Motivated by the need to establish an economical and environmentally friendly methanol control technology for the pulp and paper industry, a bench-scale activated carbon biofiltration system was developed (Babbitt et al. 2009) that supported a diverse community of methanol-degrading bacteria, with high similarity to species in the genera *Methylophilus*, *Hyphomicrobium*, and ***Methylocella***. The system was evaluated for its performance in removing methanol from an artificially contaminated air stream and characterized for its bacterial diversity over time, under varied methanol loading rates, and in different spatial regions of the filter. The biofilter system, composed of a novel packing mixture, provided an excellent support for growth and activity of methanol-degrading bacteria, resulting in approximately 100 % methanol removal efficiency for loading rates of 1–17 g/m3 packing/h, when operated both with and without inoculum containing enriched methanol-degrading bacteria. Although bacterial diversity and abundance varied over the length of the biofilter, the populations present rapidly formed a stable community that was maintained over the entire 138-day operation of the system and through variable operating conditions, as observed by PCR–DGGE methods that targeted all bacteria as well as specific methanol-oxidizing microorganisms.

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