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Characterization of Ni-tolerant methylobacteria associated with the hyperaccumulating plant *Thlaspi goesingense* and description of *Methylobacterium goesingense* sp. nov.

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Abstract

Various pink-pigmented facultative methylotrophic (PPFM) bacteria (strains iEII3, iEIV1, iEI6, iEII1, iEIII3 iEIII4, iEIII5, iRII1, iRII2, iRIII1, iRIV1 and iRIV2) were obtained from the rhizosphere and endosphere of hyperaccumulating plant *Thlaspi goesingense* grown in Redschlag, Austria [R. Idris, R. Trifonova, M. Puschenreiter, W.W. Wenzel, A. Sessitsch, Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*, Appl. Environ. Microbiol. 70 (2004) 2667–2677]. Due to their unexpected diversity, abundance and nickel tolerance they were further characterized by detailed 16S rRNA gene analysis, DNA–DNA hybridization, fatty acid analysis, heavy metal tolerance, screening for known Ni resistance genes and phenotypic analysis. These strains were found to exhibit different multiple heavy metal resistance characteristics to Ni, Cd, Co, Zn and Cr. On the basis of their physiological and genotypic properties, strains could be grouped with *Methylobacterium extorquens* and *M. mesophilicum*. One endophyte, strain iEII3, was found to belong to a novel species for which the name *M. goesingense* is proposed.
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Introduction

Bacteria of the genus *Methylobacterium* are strictly aerobic, Gram-negative, rod-shaped facultative methylotrophs, which utilize methanol and other reduced one-carbon compounds via the serine pathway [13]. Methylobacteria are classified as α-Proteobacteria and most species show pink to red pigmentation due to the synthesis of carotenoids [14]. These pink-pigmented facultative methylotrophs (PPFMs) are widely distributed in nature, but are particularly known for their close association with plants [1,7,15,23,24]. Methylobacteria have been found on surfaces of a wide variety of plants [7,8,15] and they probably utilize methanol emitted by stomata of plants [32]. In addition, *Methylobacterium* strains have been shown to colonize the intercellular spaces and/or vascular tissues of plants as endophytes

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[1,16,25] and have been even identified as endosymbionts within the cells of the buds of Scotch pine [33]. *Methylobacterium* strains have further shown to nodulate legume roots and to establish a nitrogen-fixing symbiosis similar to rhizobia [19,35]. A high abundance of PPFMs in the rhizosphere has been reported [16]. Sequencing the genome of *Methylobacterium extorquens* revealed a high number of open reading frames with high homology to genes involved in plant association in rhizobia and *Agrobacterium* [23] further demonstrating the close relationship and interaction with plants.

PPFMs are not pathogenic to their plant hosts. The plant provides methanol or methylated pectin as nutrients for their associated methylobacteria, whereas the bacteria can benefit the plant in various ways. Various plant growth promoting activities have been reported for PPFMs. They have been shown to stimulate seed germination and plant development, probably due to hormone or vitamin production [2,14]. Some *Methylobacterium* strains have been described with the ability to produce indole acetic acid [18] or cytokinins [17]. Furthermore, some strains showed ACC deaminase activity potentially leading to a better stress tolerance in plants [16]. Methylobacteria may also contribute to a better iron nutrition of plants by the production of siderophores [3,16]. Evidence exists that methylobacteria contribute to the flavour development of strawberries [41].

In a previous study we analysed rhizosphere and endophytic bacterial communities of Ni-hyperaccumulating plant *Thlaspi goesingense* [16]. Methylobacteria showed high abundance and diversity among rhizosphere and endophyte isolates, and were characterized by high Ni tolerance, siderophore production and in some strains ACC deaminase activity. Rhizosphere methylobacteria showed highest homology to *M. extorquens* and *M. mesophilicum*, whereas some endophytes were identified by partial 16S rRNA gene analysis as *M. mesophilicum*. Other endophytic methylobacteria could not be clearly affiliated with any described *Methylobacterium* species [16]. The objective of the present study is to characterize the *Methylobacterium* strains associated with *T. goesingense* in more detail by using a polyphasic approach and to describe a novel species for which the name *M. goesingense* sp. nov. is proposed.

Materials and methods

Bacterial strains and growth conditions

Strains, which were previously isolated as endophytes (iEI6, iEII1, iEII3, iEIV1, iEIII3, iEIII4, iEIII5) and rhizosphere bacteria (iRII1, iRII2, iRIII1, iRIV1, iRIV2) from *T. goesingense* grown in an ultramafic soil containing high levels of Ni [16], were investigated in this study. Reference strains included *M. organophilum*^{T} (DSM 760), *M. extorquens*^{T} (DSM 1337), *M. extorquens* (DSM13060), *M. extorquens* (DSM 1340), *M. mesophilicum*^{T} (DSM 1708), *M. radiotolerans*^{T} (DSM 1819), *M. fujisawaense*^{T} (DSM 5686) and *M. nodulans* (ORS2060) (kindly provided by Catherine Boivin-Masson). These strains were grown in minimal salts medium [21] containing 0.5% methanol as carbon source.

DNA extraction and DNA–DNA hybridization

DNA was isolated from bacterial cultures using the QIAamp® DNA Mini kit (QIAGEN) with minor modifications. DNA–DNA hybridization was performed with selected strains as described previously [20]. Briefly, using 25 µl samples containing 400 ng genomic DNA was blotted onto Nylon (Amersham Biosciences) membranes. Membranes were hybridized with 4 µg genomic DNA per dot previously digested with *Alu*I and labelled with α-[^{32}P] dCTP by using the Megaprime DNA labelling system (Amersham Biosciences). Hybridization was carried out under relaxed conditions at 55 °C for 2 h in Rapid-Hyb buffer (Amersham Biosciences). Then, membranes were washed two times for 15 min under stringent conditions (0.15 M NaCl, 0.015 M sodium citrate, 0.1% (w/v) SDS) at 65 °C. Hybridized membranes were incubated with a detection screen (Molecular Dynamics, Sunnyvale, CA) for 16–20 h. Probe signals were detected and quantified using a PhosphorImager SF (Molecular Dynamics) and image analysis software Image Quant V3.3 (Molecular Dynamics).

Analysis of 16S rRNA genes

16S rRNA gene sequences were amplified and sequenced using the conditions described by Idris et al. [16]. For sequencing the primers 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) [10] and 1520r (5′-TGCGGCTGGATCACCTCCTT-3′) [10] in addition to three internal primers 518rev (5′-CCAGCAGCCGCGGTAAT-3′), 926rev (5′-AAACTCAAAGGAATTGACGG-3′) [22] and 799f (3-AGGGTATCTAATCCGTGTT-5) [5] were used to obtain nearly 800 bp (strains iEI6, iEIII3, iEIII5, iEIII4, iEII1, iRII1 and iRIV2) or full-length (strains iEII3, iEIV1, iRIV1, iRIII1 and iRII2) 16S rRNA sequence information. Sequences were deposited in GenBank under accession number AY364016, AY364025, AY364027, AY364026, AY364019, AY357996, AY358006, AY364020, AY369236, AY358005, AY358000 and AY357997, respectively.

Phylogenetic analysis was carried out using the ARB software package [26]. Sequences were aligned to the official ARB release of 16S rRNA database and a neighbour joining tree was generated with 100 bootstrap values.

Phenotypic analysis

The Gram reaction was investigated after growth in minimal salts medium with 0.5% methanol. Fatty acid analysis was carried out as described by Frostegård et al. [11].

For metabolic characterization, nitrate reduction, methyl red reaction, starch hydrolysis, ammonia production, halotolerance, temperature tolerance and pH optima were analysed in appropriate liquid media as described by Doronina et al. [9]. Starch hydrolysis was determined with an iodine indicator solution on solid medium K [9] containing 0.2% of soluble starch after 10 days of incubation. Arginine dihydrolase, trypsine deaminase ornithin decarboxylase, lysin decarboxylase, β-galactosidase, gelatinase and urease activity as well as the production of indole, acetoin and hydrogen sulphide were tested with the API 20E system (Biomérieux). Moreover, API 20E was used to determine the utilization of inositol, D-sorbitol, D-sacchararose, D-melibiose, L-rhamnose, L-arabinose, D-mannitol and amygdaline.

The utilization of 34 additional carbon substrates (as listed in Table 2) was analysed in 8 ml volumes of mineral medium K [9]. Carbon sources were filter sterilized and added separately in concentrations of 10 mM (dichlormethane and dimethylsulphoxide (DMSO)), 3 mM (formaldehyde and dimethylsulphone (DMS)), or 0.35% (alcohols and carbohydrates). Alternative nitrogen sources were tested in mineral medium by replacing $(NH_4)_2SO_4$ (30 mM N) with either methylamine, ethylene diamine tetracetate (EDTA), L-aspartate or, L-glutamic acid. To insure optimal growth in liquid medium, inocula were taken from fresh cultures on solid medium K as well as from liquid precultures. Precultures were grown in mineral medium K containing 0.5% (v/v) methanol and spun down before transfer to the test media. Methane assimilation was assessed in 500 ml Erlenmeyer flasks containing 30 ml of mineral medium K, 100 ml of methane and 370 ml air. The test cultures were incubated for 14 days at room temperature with shaking.

In order to determine the relatedness of strains based on metabolic activities, a binary matrix was generated recording the absence and presence of activities. Estimation of genetic distances as suggested by Nei and Li [29] in combination with the unweighted pair group with mathematical averages (UPGMA) method was used for comparisons. For analysis and tree generation (based on 100 bootstrap replications) the TREECON software package [38] was applied.

Biochemical analysis

The hydroxypyruvate reductase (HPR) enzyme assay was determined according to the method described earlier [21] except that cell disruption was done by sonication (3×30 s) with 15-s intermittent bursts.

Analysis of heavy metal resistance

For the determination of minimal inhibitory concentrations (MICs), selected *Methylobacterium* isolates and the reference strains were tested for the ability to grow in increasing concentrations of the metal cations Ni (1–15 mM), Zn (1–15 mM), Cd (0.5–5 mM), Co (1–5 mM) and Cr(III) (0.5–3 mM) supplemented to (a phosphate-poor morpholinepropanesulphonic acid (MOPS)) medium [31].

Screening for known Ni resistance genes was done by PCR. Primers targeting four known genes involved in nickel resistance were selected: *cnrY* present on *Ralstonia metallidurans* CH34 plasmid pMOL28 [37], *nccB* and *nccN* present on *R. metallidurans* 31A plasmid pTOM9 [34], and *nreB* present on both plasmids [12,28,34]. The following primers were designed using Primer3 software from the Whitehead Institute/MIT (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi): cnrY_f (5′-GGCAATGGATCTGGATGGAG), cnrY_r (5′-GAAAGCCGTCGGGTACGA), nccB_f (5′-GGCGTTGGTTGGATTTGG), nccB_r (5′- TTGTTCATTTCCGCCTTGAT), nccN_f (5′-CAATGGGATGACACCTGGAT), nccN_r (5′-CCAGTTCCACTGCGTTCAC), nreB_f (5′-GTATTGCGGAACCGAACCTA), nreB_r (5′-AGCGAGAAATCCGACAACAG). PCR reaction conditions were as follows: initial denaturing step of 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s annealing temperature ranging from 55 to 58 °C (depending on the primer sequence used), and 3 min extension at 72 °C (AB APPLIED BIOSYSTEM "pcr system 2700"). PCR reaction mixtures (50 µl) contained 300 ng chromosomal DNA, 5 µl of $10\times$ reaction buffer, 2.5 µl dNTP (0.2 mM), 5 µl (0.3 µM) each primer pair and 0.5 µl (0.01 U) Taq polymerase (Roche). As positive control two samples from strains *R. metallidurans* CH34 and *R. metallidurans* 31A were included in these amplification reactions.

Results

16S rDNA sequence and phylogenetic analysis

Phylogenetic analysis of partial and full 16S rRNA gene sequences are shown in Fig. 1. Based on the results of this analysis, strains iRIII1, iRII2 and iRIV1 belong to a group of *M. extorquens* and related isolates/

Fig. 1. 16S rRNA neighbour tree showing the phylogenetic positions of the isolates obtained. The tree is based on a partial alignment stretching nucleotides 100–823 (*Escherichia coli* numbering). For isolates iEIV1, iEII3, iRII2, iRII1 and iRIV1 the same analysis was also performed based on the full 16S rDNA sequence (nucleotide positions 100–1491, *E. coli* numbering), resulting in the same branching pattern (data not shown). The scale bar indicates the estimated number of base changes per nucleotide sequence position.

environmental clones. Isolates iEIV1, iRII1, iEIII4, iEIII3, iEII1, iEIII5, iRIV2 and iEI6 all belong to the well-defined, distinct clade of *M. mesophilicum*. Finally, iEII3 belongs to another, well-separated clade harboring so far yet undescribed isolates derived from very different environments. This group is also clearly part of the *Methylobacterium* genus, but most likely represents a new species therein. Based on these results as well as those published previously [16] representative group isolates were selected for further analysis. The level of similarity between the representative group isolate strain iEIV1 and *M. mesophilicum* was 99.8%, whereas that between strain iRIII1 and *M. extorquens* was 100%. According to the 16S rRNA gene analysis *M. goesingense* sp. nov. strain iEII3 was most closely related to *M. organophilum* (95.8%) and *M. fujisawaense* (94.2%).

DNA–DNA hybridization

The levels of DNA–DNA hybridization between the strains iEII3, iEIV1, iRIII1 and the reference species of the genus *Methylobacterium* ranged from 6% to 83% (Table 1). Strain iEIV1 showed lowest homology (11%)

Table 1. DNA–DNA hybridization (% DNA relatedness) between strains iEII3, iEIV1 and iRIII1 and related methylobacteria

Strain	iEII3	IEIV1	iRIII1
M. mesophilicum DSM1708	21	83	13
M. extorquens DSM1337	40	23	80
M. organophilum DSM760	31	52	19
M. nodulans ORS2060	17	20	6
M. radiotolerans DSM1819	25	40	8
M. *fujisawaense* DSM5686	60	11	16

to *M. fujisawaense* and high homology (83%) to *M. mesophilicum*. Strain iRIII1 showed the lowest similarity (6%) to *M. nodulans* and high similarity (80%) to *M. extorquens*. Strain iEII3 showed to all methylobacteria analysed homology values lower than or equal to 60% indicating that this strain belongs to a novel species within the genus *Methylobacterium*. Highest similarity of this strain was found to *M. fujisawaense*.

Physiological and biochemical properties

As shown in Table 2 isolates iEII3, iEIV1 and iRIII1 and reference strains grew well in mineral liquid medium K with methanol, glycerol and L-arabinose. Other carbon sources investigated (ethanol, D-fructose, L-rhamnose, malate, fumarate, α-ketoglutarate and formaldehyde) supported the growth of isolate iEII3. Isolate iRIII1 with highest 16S rRNA gene homology to *M. extorquens* showed a C-source utilization pattern nearly identical with that of *M. extorquens* (Table 2, Fig. 2). Isolate iEIV1 with highest 16S rRNA gene homology to *M. mesophilicum* showed a C-source utilization pattern different from those of iEII3 and iRIII1 and all investigated reference strains, however, was most similar to *M. mesophilicum* and iEII3 (Fig. 2). Strain iEIV1 showed a broad C-source utilization capability including in addition to methanol, glycerol and L-arabinose also the utilization of D-glucose, D-fructose, galactose, D(+)mannose, maltose, D(−)ribose L-arabinose, L-rhamnose, D-saccharose, D-melibiose, inositol, D-sorbitol, fumarate, α-ketoglutarate and L-alanine. In all isolates investigated, urease activity was found. Ammonium salts and nitrate served as nitrogen sources, nitrate was reduced to nitrite and ammonia production was observed in isolate iEII3 but not in iEIV1 and iRIII1 (Table 3). All isolates under study showed no activity of arginine dihydrolase, tryptophan desaminase, ornithine decarboxylase, lysine decarboxylase and β-galactosidase (Table 3). Similarly, starch hydrolysis, H_2S formation, indol formation, acetoin formation and methyl red reaction were not observed in any of the strains investigated. The optimal growth temperature of isolate iEII3 was 25 °C, whereas isolates iEIV1 and iRIII1 grew best in a temperature range of 25–30 °C. The pH optimum for all the isolates was 6.5–7.9. Assimilation of C1 compounds for iEII3, iEIV1 and iRIII1 is via the serine pathway. Cluster analysis of all metabolic activities indicated a close relationship of iRIII1 and *M. extorquens* (Fig. 2). Strains iEII3 and iEIV1 were in this analysis only distantly related to other species, but showed highest relatedness to *M. mesophilicum*.

Fatty acid analysis showed again a high relatedness between strain iRIII1 and *M. extorquens* (Table 4). Strain iEIV1 showed considerably higher amounts of 16:1ω7c and lower amounts of 18:1ω7c fatty acids than the *M. mesophilicum* reference strain and its fatty acid profile indicated a high relatedness to *M. fujisawaense*. Strain iEII3 showed in comparison to most other *Methylobacterium* spp. high levels of 16:1ω7c and 16:0 fatty acids, whereas levels of 18:1ω7c fatty acids were lower, but the pattern was generally very similar to that of *M. fujisawaense*.

Minimal inhibitory concentration (MIC) of heavy metals

In general, all isolates tested showed higher resistance to nickel than the reference strains, only the *M. organophilum* reference strain was also resistant up to 7 mM Ni (Table 5). Isolates, which were identified as *M. mesophilicum*, showed in addition to Ni resistance to Cd, Co, and Zn. The reference strain *M. mesophilicum* did not tolerate Ni and Cd, but showed resistance to Co and Zn. Isolates, which were identified as *M. extorquens*, showed higher resistance to Ni and Co as well as some resistance to Cr. The type strain of *M. extorquens* did not tolerate Ni and Cr, but was resistant to comparable levels of Zn, whereas the other *M. extorquens* reference strains tested showed a slightly different resitance pattern. Strain iEII3 showed resistance to Ni, Cd and Zn.

Heavy metal resistance genes

Plasmid borne nickel resistance determinants such as *cnrY* [36,37], *nccB* and *nccN* [34] and *nreB* [12,34] have been investigated for their presence in the Ni-tolerant *Methylobacterium* strains associated with *T. goesingense*. No PCR mediated amplification was obtained for all stringency conditions tested. However, it may be possible that primers were not adequately suited to detect variable regions of orthologs.

Table 2. Various carbon and nitrogen sources utilized by selected *Methylobacterium* isolates and by six reference strains

	Isolates			Reference strains					
	iEII3	iEIV1	iRIII1	*M. organophilicum* DSM760	*M. nodulans* ORS2060	*M. mesophilicum* DSM1708	*M. radiotolerans* DSM1819	*M. extorquens* DSM1337	*M. fujisawaense* DSM5686
Carbon sources									
Methanol	+	+	+	+	+	+	+	+	+
Methylamine	−	−	+	−	−	−	−	+	−
Ethanol	+	−	+	−	+	−	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+
D-glucose	−	+	−	−	−	+	+	−	−
D-fructose	+	+	−	−	−	+	−	−	−
Galactose	−	+	−	+	−	+	+	−	+
D(+)mannose	−	−	−	−	−	−	−	−	−
Maltose	−	−	−	−	−	−	−	−	−
D(−)ribose	−	−	−	−	+	−	−	−	−
L-arabinose	+	+	+	+	+	+	+	+	+
L-rhamnose	+	+	+	+	−	−	+	+	+
D-saccharose	−	+	−	−	−	−	−	−	−
D-melibiose	−	+	−	−	+	−	−	−	+
Inositol	−	+	−	−	−	−	−	−	−
D-sorbitol	−	+	−	−	+	−	−	−	−
NH_4 acetate	−	−	−	+	+	−	−	−	−
Malate	+	−	+	+	+	−	+	+	+
Fumarate	+	+	+	+	+	+	+	+	−
α-ketoglutarate	+	+	−	+	+	+	+	−	+
Betaine	−	−	+	+	+	−	+	+	−
Acetamide	−	−	+	−	−	−	−	+	−
L-aspartate	−	−	−	−	−	−	−	−	+
L-alanine	−	+	−	+	−	+	+	−	+
L-glutamic acid	−	−	−	+	−	−	+	−	−
Formaldehyde	+	−	−	−	−	+	−	+	+
Nitrogen sources									
$(NH_4)_2SO_4$	+	+	+	+	+	+	+	+	+
NH_4Cl	+	+	+	+	+	+	+	+	+
KNO_3	+	+	+	+	+	−	+	+	+
Methylamine	−	−	+	+	−	−	−	+	+
L-aspartate	−	−	−	−	−	−	−	−	+
L-glutamic acid	−	−	−	+	−	−	−	−	+

Utilized (+), not utilized (−).

D-mannitol, maleic acid, pyruvate, propionate, citrate, amygdaline, soluble starch, D/L serine, glycine, valine, dichlormethane, dimethylamine, trimethylamine, methane, dimethyl sulfone and dimethyl sulfoxide were not used as carbon source by any of the isolates and reference strains. Similarly, none of the strains utilized ethylene-diamine-tetra-acetic acid (EDTA) as nitrogen source.

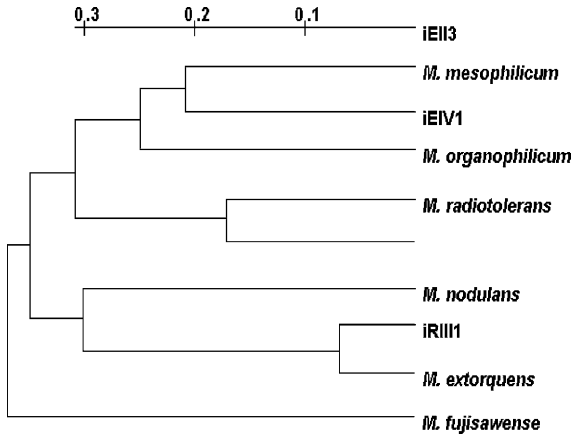

Fig. 2. Phylogenetic tree showing the relatedness of the strains based on metabolic characteristics (Table 3) and carbon and nitrogen sources (Table 2) utilized by selected *Methylobacterium* isolates and six reference strains. Estimation of the genetic distances as suggested by Nei and Li [29] in combination with the UPGMA method for comparisons. The TREECON software package [38] was applied for analysis and tree generation. The bar indicates the Jukes–Cantor distance.

Discussion

Hyperaccumulating plants such as *T. goesingense* accumulate large amounts of heavy metals in their shoots and therefore contain endophytes, which are adapted to this specific environment. Similarly, the rhizosphere of hyperaccumulators contains high concentrations of easily bioaccessible heavy metal concentrations [27] favouring the growth of heavy metal resistant bacteria. The total Ni concentration in the ultramafic serpentine soil at the site Redlschlag is 2580 mg kg^{-1} [40], whereas the concentration in the exchangeable soil fraction (regarded as bioavailable) was found to be 7.72 mg kg^{-1} in the non-rooted bulk soil, but may decrease due to plant uptake to 5.06 mg kg^{-1} in *Thlaspi* rhizosphere soil. Typical plant concentrations in shoots (stems + leaves) range between 2560 and 12,400 mg kg^{-1}. In a previous study [16] the microflora associated with *T. goesingense* was analysed by using a cultivation-dependent and -independent approach, which revealed a high microbial diversity in the rhizosphere as well as in the plant apoplast. Cultivation of bacteria on Ni-containing media resulted in a high diversity of strains belonging to the genus *Methylobacterium*, which were further analysed in this study. 16S rRNA gene sequence analysis clearly indicated that strains belonged to three species, *M. mesophilicum*, *M. extorquens* and a novel species, for which the name *M. goesingense* is proposed. This classification correlates well with the resistance patterns

Table 3. Metabolic characteristics of selected *Methylobacterium* isolates and of six reference strains

	Isolates			Reference strains					
	iEII3	iEIV1	iRIII1	*M. organophilicum* DSM1708	*M. nodulans* ORS2060	*M. mesophilicum* DSM1708	*M. radiotolerans* DSM1819	*M. extorquens* DSM1337	*M. fujisawaense* DSM5686
Arginine dihydrolase	−	−	−	+	−	+	−	−	+
Tryptophan deaminase	−	−	−	−	−	−	−	−	−
Ornithine decarboxylase	−	−	−	+	−	−	+	−	+
Lysine decarboxylase	−	−	−	+	−	−	+	−	−
Urease	+	+	+	+	+	+	+	−	−
Gelatinase	−	−	−	−	+	−	−	−	+
NO_3 reduction	+	+	+	+	+	+	+	+	+
NH_3 production	+	−	−	+	+	+	+	−	+
Tolerance to NaCl (%)	1	2	1	1	<1	1	1	1	1
Growth at 4 °C	+	−	−	−	−	−	−	+	−
Growth at 37 °C	−	−	+	−	+	−	+	+	+

Present (+), absent (−).

Table 4. Cellular fatty acid composition of strains iEII3, iEIV1, iRIII1 and reference strains (% of total fatty acids)

Fatty acid	Strains								
	iEII3	iEIV1	RIII1	*M. organ.* DSM1708	*M. nodul.* ORS2060	*M. meso.* DSM1708	*M. radio.* DSM1819	*M. extorq.* DSM1337	*M. fujisa.* DSM5686
16:1ω7c	12.0	11.1	4.6	2.0	0.2	1.2	0.6	5.2	12.0
16:0	3.3	0.9	0.7	1.5	2.5	0.7	1.8	1.2	0.0
17:0	0.3	0.0	0.5	0.0	1.0	0.0	0.0	0.5	0.0
Unknown	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.4	0.0
18:1ω7c	82.0	86.7	90.3	93.1	82.8	96.8	95.1	88.4	86.4
18:0	2.4	1.4	3.1	3.4	8.8	1.2	2.5	4.5	1.6
cy19:0	0.0	0.0	0.0	0.0	4.7	0.0	0.0	0.0	0.0

Table 5. Minimum inhibitory concentrations (MICs) of various heavy metals

Isolate	Tentative species	Ni (mM)	Cd (mM)	Co (mM)	Zn (mM)	Cr (mM)
iEII3	*M. goesingense*	10	1	—	2	—
iEI6	*M. mesophilicum*	12	1	3	6	—
iEIV1	*M. mesophilicum*	12	1	3	6	—
iEII1	*M. mesophilicum*	10	1	3	3	—
iRII1	*M. mesophilicum*	10	—	3	3	—
iEIII3	*M. mesophilicum*	12	1	3	2	—
iRIV2	*M. mesophilicum*	10	1	3	3	—
iEIII4	*M. mesophilicum*	12	1	3	6	—
iRII2	*M.* extorquens	10	—	—	2	1
iRIII1	*M. extorquens*	5	—	—	2	1
iRIV1	*M. extorquens*	10	—	—	2	1
Reference strain						
DSM1708	*M. mesophilicum*^{T}	—	—	3	3	—
DSM1337	*M. extorquens*^{T}	—	—	—	2	—
DSM13060	*M. extorquens*	2	—	1.5	2	—
DSM1340	*M. extorquens*	—	—	1.5	2	—
DSM760	*M. organophilum*^{T}	7	2	3	>15	—
DSM5686	*M. fujisawaense*^{T}	1	1	3	10	—
DSM1819	*M. radiotolerans*^{T}	—	—	3	6	—
ORS2060	*M. nodulans*	1	—	1	5	—

—, indicates that the MIC is <1.

of the analysed strains to different heavy metals. Both environments, the rhizo- and endosphere contained a high number of methylobacteria, however, inside shoots a higher number of different *Methylobacterium* strains was found than in the rhizosphere [16]. Similarly, 20% of endophytes obtained from *T. caerulescens*, a Zn hyperacccumulating plant, were shown to belong to the genus *Methylobacterium* [24]. Most endophytic strains belonged to *M. mesophilicum*, whereas in the rhizosphere both *M. mesophilicum* and *M. extorquens* were present in similar numbers. For the proposed *M. goesingense* only one strain was found, which was an endophyte. Interestingly, different *M. mesophilicum* and *M. extorquens* strains were found to colonize the rhizosphere and the plant apoplast. This is in good agreement with previous studies, which reported the presence of distinct subpopulations adapted to the different conditions existing externally and internally to the plant [4,39].

A polyphasic approach was used to characterize representative members of *M. mesophilicum*, *M. extorquens*, and the proposed species *M. goesingense*, iEIV1, iRIII1 and iEII3, respectively, in order to confirm their taxonomic position. Isolate iEIV1 showed high amounts of 16:1ω7c and lower amounts of 18:1ω7c fatty acids as compared to the *M. mesophilicum* type strain and based on fatty acids only this strain would be classified as *M. fujisawaense*. The carbon source utilization pattern showed a particular dominance of D-sugars utilization different from the *M. mesophilicum* type

strain and all other strains investigated. On the other hand, this strain exhibited a similar nitrogen utilization pattern, other metabolic characteristics and a DNA similarity of 83% to *M. mesophilicum*. These criteria in addition to the phylogenetic analysis of 16S rRNA gene sequences seem sufficient to place iEIV1 in the *M. mesophilicum* species. Strain iRIII1 can be clearly classified as *M. extorquens* due to similar metabolic properties, fatty acid composition and a DNA similarity of 80% to *M. extorquens*. This conclusion is confirmed by phylogenetic analysis of 16S rRNA gene sequences (Fig. 1).

16S rDNA sequence analysis placed strain iEII3 clearly into the genus *Methylobacterium*, in which this strain fell into a clade harbouring so far only environmental clones and no described bacteria. Similarly, a DNA hybridization value of only 60% and below to other *Methylobacterium* spp. was insufficient to place strain iEII3 in any known species of this genus. In addition, this isolate showed distinct metabolic activities and fatty acids. Consequently, strain iEII3, was assigned to a novel *Methylobacterium* species with the proposed name *M. goesingense* sp. nov.

Ecosystems characterized by toxic concentrations of heavy metals trigger the growth and development of bacteria, which are resistant to one or a whole series of metal ions. All isolates investigated showed considerably higher resistance to nickel as compared to the reference strains, which can be explained by the adaptation of these isolates to the Ni containing environment they were obtained from. The correlation between methylobacteria and heavy metal polluted environments was also observed in the *Methylobacterium* species isolated from the Zn hyperaccumulator *T. caerulescens* grown in a soil from an abandoned Zn–Pb mine and smelter in Belgium with high resistance capacities to Zn, Cd, Co and Ni [24]. Also all isolates analysed in this study showed multiple metal resistances such as the resistances (Ni^R, Co^R) found in *Ralstonia metallidurans* CH34 isolated from an industrial biotope [37], or those (Ni^R, Co^R, Cd^R) in *Achromobacter xylosoxidans* 31A [34]. *M. mesophilicum* isolates tolerated in addition to Ni also Co and Zn, and some isolates also Cd, whereas *M. extorquens* strains were resistant to Ni, Zn and Cr. *M. goesingense* tolerated Ni, Cd and Zn. Genetic determinants of these resistance combinations have not been reported. Similarly, known nickel resistance genes could not be identified by PCR suggesting the presence of very different orthologs or even novel genes involved in Ni-resistance.

Striking was the tight correlation between resistance patterns found in different species. This indicates no or at least restricted horizontal gene transfer of heavy metal determinants. Isolates may originally have derived from single strains belonging to the different species found, which adapted with time to the specific conditions encountered. Alternatively, heavy metal resistance genes may be located on plasmids, which are only transferred within but not between species.

Methylobacteria have been shown to have intimate contact with plants and may show beneficial functions regarding plant growth. They seem to be particularly abundant in association with plants growing in heavy metal accumulating soils and have shown functions such as siderophore and ACC deaminase production [16,24], which may help the plant to face heavy metal stress. Further research on the ecology and genomics of *Methylobacterium* strains will help to understand the mechanisms involved in their heavy metal resistance and interaction with plants.

Description of *Methylobacterium goesingense* sp. nov.

Methylobacterium goesingense (goe.sing.en'se M.L.adj. *goesingense* referring to Goesing, Austria, the source of the type strain).

Cells are Gram-negative and pink pigmented. Cells are motile rods, 0.3–0.8 × 0.8–1.2 μm in size, which occur singly or in pairs. Type strain showed resistance to Ni, Cd and Zn. Urease is positive. Reduces nitrate to nitrite. Ammonia production is positive. Indole, acetoin and H_2S are not produced. Methyl red test is negative. Does not hydrolyse gelatine and starch. Isolate iEII3 shows no activity of arginine dihydrolase, tryptophan desaminase, ornithine decarboxylase, lysine decarboxylase and β-galactosidase. Assimilation of C1 compounds is via serine pathway. Cells utilize methanol, glycerol, L-arabinose, ethanol, D-fructose, L-rhamnose, malate, fumarate, α-ketoglutarate and formaldehyde. Good growth between pH 6.5 and 7.9 at 25 °C. The main cellular fatty acid is 18:1ω7c. The type strain is iEII3.

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