Methylobacillus pratensis sp. nov., a novel non-pigmented, aerobic, obligately methylotrophic bacterium isolated from meadow grass

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Strain F31^T was isolated from meadow grass (*Poa trivialis* L.) sampled from the city park in Helsinki. Analysis of phenotypic and genotypic properties showed the strain to be related to the group of obligately methylotrophic non-methane utilizing bacteria (methylobacteria) with the ribulose monophosphate pathway of formaldehyde assimilation. Phylogenetic analysis showed the strain to be closely related to the genus *Methylobacillus*, and analysis of fatty acid composition confirmed this association. Thus, on the basis of its genotypic and phenotypic properties, the isolate is proposed as a novel species of the genus *Methylobacillus*, *Methylobacillus pratensis* sp. nov., with F31^T as the type strain (= VKM B-2247^T = NCIMB 13994^T).

To date, four genera have been described for the group of obligately methylotrophic non-methane utilizing bacteria (methylobacteria) with the ribulose monophosphate (RuMP) pathway of formaldehyde assimilation. All known terrestrial strains of obligate methylobacteria were placed into three genera: *Methylobacillus* (Yordy & Weaver, 1977; Urakami & Komagata, 1986), *Methylophilus* (Jenkins *et al.*, 1987) and *Methylovorus* (Govorukhina & Trotsenko, 1991). Marine obligate methylobacteria assigned to the genus *Methylophaga* (Urakami & Komagata, 1987) are clearly distinguished from members of the genera *Methylobacillus*, *Methylophilus* and *Methylovorus* by their tolerance to NaCl (up to 10–12%, w/v), some physiological characteristics and the low DNA G+C content (38.0–49.0 mol%). However, terrestrial and marine strains of obligate methylobacteria possess similar morphology and metabolic organization. Thus, the main criteria used to classify obligate methylobacteria into separate genera and species are their genomic and phylogenetic characteristics. Here, we present a formal taxonomic description of the newly isolated,

obligately methylotrophic strain F31^T, which has the RuMP pathway. The name *Methylobacillus pratensis* sp. nov. is proposed for this isolate.

Strain F31^T was isolated from meadow grass (*Poa trivialis* L.) sampled from the city park in Helsinki (Finland) and grown on medium K (Doronina *et al.*, 1998). Analyses of phenotypic and genotypic properties of the novel isolate were performed as described previously (Doronina *et al.*, 2003). The 16S rRNA gene of strain F31^T was amplified by PCR, sequenced and screened against sequences within the GenBank database by using BLAST (<http://www.ncbi.nlm.nih.gov/blast>). The 16S rRNA gene sequence of strain F31^T was then aligned with a representative set of 16S rRNA gene sequences obtained from recent GenBank releases, using CLUSTAL W software (Thompson *et al.*, 1994). Positions of sequence and alignment uncertainty were omitted, and a total of 1308 nucleotides was used in the phylogenetic analysis. Phylogenetic trees were constructed by using various algorithms implemented in TREECON (Van de Peer & De Wachter, 1994).

Cells of strain F31^T are Gram-negative, asporogenous rods (0.5–0.7 × 0.9–1.8 µm) that are motile by means of a polar flagellum, and occur singly or (rarely) in pairs (Fig. 1a–c). Reproduction occurs by binary fission.

As shown in Table 1, the dominant cellular fatty acids of

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Abbreviation: RuMP, ribulose monophosphate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain F31^T is AY298905.

Fig. 1. Electron micrographs of strain F31^T. (a) Negatively stained cell. (b, c) Ultra-thin sections showing cell wall structure and polyphosphate granules (arrows). Bars, 1 µm (a), 0.2 µm (b, c).

strain F31^T are unsaturated C_{16:1}ω7 acid (46.6%) and straight-chain saturated C_{16:0} acid (41.6%). The presence of 3-hydroxy fatty acids was observed, but no 2-hydroxy fatty acids were found. The data indicate a considerable

similarity in the fatty acid composition of strain F31^T and the type cultures of the genus *Methylobacillus*, i.e. *Methylobacillus glycogenes* ATCC 29475^T and *Methylobacillus flagellatus* DSM 6875^T. Analysis of the cellular phospholipid

Table 1. Cellular fatty acid composition of strain F31^T and members of the genus *Methylobacillus*

Strain: 1, *Methylobacillus pratensis* F31^T; 2, *Methylobacillus glycogenes* ATCC 29475^T; 3, *Methylobacillus flagellatus* DSM 6875^T. Results are given as a percentage of total fatty acids. C_{19:0} (cyclopropane acid) was not present in any of the strains.

Fatty acid	1	2	3
Straight-chain acids			
C _{14:0}	1.1	1.1	1.0
C _{15:0}	0.9	0.5	0.6
C _{16:0}	41.6	43.4	41.4
C _{16:1}	46.6	42.5	44.6
C _{17:0}	0.7	0.5	0.5
C _{18:0}	1.2	0.8	0.7
C _{19:1}	1.3	4.6	4.0
Cyclopropane acid			
C _{17:0}	1.4	1.3	2.1
Hydroxy acid			
3-OH C _{10:0}	5.2	5.3	5.1

composition revealed the presence of phosphatidylethanolamine (65 %), phosphatidylglycerol (20 %), diphosphatidylglycerol (cardiolipin) (10 %) and minor amounts of phosphatidylserine, phosphatidic acid and an unidentified phospholipid. Phosphatidylcholine and lysolecithin were not found.

The enzyme profiles of methanol- or methylamine-grown cells indicated that strain F31^T oxidizes methanol to formaldehyde by means of an inducible pyrroloquinoline quinone (PQQ)-linked methanol dehydrogenase (Table 2). Methylamine dehydrogenase PQQ and amine oxidase were absent in methylamine-grown cells. Alternatively, the isolate possessed an inducible γ -glutamylmethylamide synthetase and *N*-methylglutamate synthase/lyase, the specific enzymes of the *N*-methylglutamate pathway producing formaldehyde, which is further oxidized by glutathione-dependent formaldehyde dehydrogenase to formate. The latter is partly oxidized to CO₂ by NAD-dependent formate dehydrogenase. Formaldehyde assimilation occurs via the RuMP cycle (Entner–Doudoroff variant), as confirmed by the presence of 3-hexulose phosphate synthase and 2-keto-3-deoxy-6-phosphogluconate aldolase. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are active with both NAD⁺ and NADP⁺. Rather high levels of these enzymes indicate the preferential oxidation of formaldehyde to CO₂ via the dissimilatory hexulose phosphate cycle, which provides the methylotroph with the reduced equivalents and energy for biosynthesis. However, we cannot rule out the same potential role of the tetrahydromethanopterin (H₄MPT)-dependent oxidation pathway in formaldehyde dissimilation in our isolate because high activities of methenyl H₄MPT cyclohydrolase and NAD(P)-dependent methylene H₄MPT dehydrogenases were found in *Methylobacillus flagellatus* KT^T (Vorholt *et al.*, 1999).

The absence of the serine pathway-specific enzymes (hydroxypyruvate reductase and serine–glyoxylate transaminase) and the ribulose-bisphosphate pathway enzyme (ribulose-bisphosphate carboxylase) means that their operation can be excluded in the case of the methylotroph studied.

The tricarboxylic acid cycle is deficient in 2-oxoglutarate dehydrogenase. The absence of isocitrate lyase and malate synthase indicates the non-functional glyoxylate shunt in strain F31^T. Oxaloacetate is replenished by phosphoenolpyruvate carboxylase. Primary ammonia assimilation occurs by reductive amination of 2-oxoglutarate to glutamate, since the glutamine synthetase/glutamate synthase pathway (GS/GOGAT) is not operative in this organism.

In the phylogenetic tree derived from 16S rRNA gene sequences (Fig. 2), strain F31^T consistently branched with the β -*Proteobacteria*. The relatively high level of 16S rRNA gene sequence similarity (95–97 %) between the strain and members of the genus *Methylobacillus* indicated a close relationship. The level of DNA relatedness between the novel isolate and reference strains of the genus *Methylobacillus* (*Methylobacillus glycogenes* ATCC 29475^T and *Methylobacillus flagellatus* DSM 6875^T) was in the range 28–34 %. Remarkably, strain F31^T had a very low degree of DNA hybridization (5–10 %) with members of the genera *Methylophilus*, *Methylovorus* and *Methylophaga* (*Methylophilus methylotrophus* NCIMB 10515^T, *Methylovorus glucosotrophus* ATCC 49758^T and *Methylophaga marina* ATCC 35842^T). Consequently, strain F31^T is classified as the type strain of a novel *Methylobacillus* species, for which the name *Methylobacillus pratensis* sp. nov. is proposed (Table 3).

Description of *Methylobacillus pratensis* sp. nov.

Methylobacillus pratensis (pra.ten'sis. L. masc. adj. *pratensis* means growing in a meadow).

Gram-negative rods that are 0.9–1.8 × 0.5–0.7 μ m in size. Multiply by binary fission. Cells are motile by means of a single polar flagellum. Colonies on mineral salts/methanol agar are white and 1–2 mm in diameter. Strictly aerobic. Growth factors not required. Able to grow at 10–37 °C, at pH 5.5–8.5 and optimally at 25–30 °C and pH 6.5–7.5. Urease-, catalase- and oxidase-positive. Nitrate is reduced to nitrite. Produces indole (indole 3-acetic acid) from tryptophan on medium with nitrate as nitrogen source. No growth occurs in the presence of 3 % (w/v) NaCl. Obligate methylotroph that utilizes only methanol and methylamine. Methylamine is oxidized to formaldehyde by the *N*-methylglutamate pathway enzymes, γ -glutamylmethylamide synthetase and *N*-methylglutamate synthase/lyase. Formaldehyde is assimilated via the RuMP pathway (Entner–Doudoroff variant). Ammonia is assimilated by glutamate dehydrogenase. Tricarboxylic acid cycle is incomplete at the level of 2-oxoglutarate dehydrogenase; the glyoxylate shunt enzymes are absent. Nitrates, ammonium salts, methylamine, glutamate and urea serve as nitrogen sources. The prevailing cellular fatty acids are C_{16:0} and C_{16:1}.

Table 2. Enzyme activities in cell extracts of strain F31^T when grown on different substrates

Activities are given as nmol min⁻¹ (mg protein)⁻¹. Abbreviations: PMS, phenazine methosulfate; GSH, reduced glutathione; PEP, phosphoenolpyruvate. There was no activity with any of the following enzymes and cofactors: methylamine dehydrogenase (PMS), amine oxidase, γ -glutamylmethylamide lyase, hydroxypyruvate reductase NAD(P)H, serine-glyoxylate transaminase NAD(P)H, ribulose-bisphosphate carboxylase, isocitrate lyase, malate synthase, fructose-bisphosphate aldolase, 6-phosphofructokinase (ATP), pyruvate kinase, 2-oxoglutarate dehydrogenase, glutamate synthase, glutamine synthetase (Mn²⁺, ATP).

Enzyme	Cofactor(s)	Methanol	Methylamine
Methanol dehydrogenase	PMS	215	0
γ -Glutamylmethylamide synthetase	ATP, Mn ²⁺	0	27
N-Methylglutamate synthase		0	5
N-Methylglutamate lyase		0	3
Formaldehyde dehydrogenase	PMS	0	0
	NAD ⁺ , GSH	61	60
Formate dehydrogenase	PMS	0	0
	NAD ⁺	36	24
3-Hexulosephosphate synthase		1400	1200
Phosphoriboisomerase		560	520
Glucose-6-phosphate dehydrogenase	NAD ⁺	325	410
	NADP ⁺	1090	1120
6-Phosphogluconate dehydrogenase	NAD ⁺	70	70
	NADP ⁺	210	200
Hexokinase	ATP	2	2
Transaldolase		40	40
Transketolase		174	162
2-Keto-3-deoxy-6-phosphogluconate aldolase		125	130
Pyruvate dehydrogenase	NAD ⁺	9	10
Citrate synthase		20	20
Isocitrate dehydrogenase	NAD ⁺	94	92
	NADP ⁺	81	79
Glutamate dehydrogenase	NADH	130	110
	NADPH	45	45
Pyruvate carboxylase	Mg ²⁺	9	7
PEP carboxylase	Acetyl CoA, Mg ²⁺	2	2
PEP carboxykinase	ADP	4	4

Fig. 2. Neighbour-joining tree showing the phylogenetic position of *Methylobacillus pratensis* F31^T among methylotrophs of the β -Proteobacteria. The numbers at the branch points are bootstrap values from 100 replicates. Bar, 2% Jukes–Cantor distance (2 nucleotide substitutions per 100 nucleotides; Jukes & Cantor, 1969).

Table 3. Major characteristics that allow differentiation among members of the genus *Methylobacillus*

Strain: 1, *Methylobacillus pratensis* F31^T; 2, *Methylobacillus glyco-genes* ATCC 29475^T; 3, *Methylobacillus flagellatus* DSM 6875^T. All data were obtained in this study.

Characteristic	1	2	3
Flagellation	1	—	1–4
Methylamine dehydrogenase	—	+	+
N-Methylglutamate pathway	+	—	—
Isocitrate dehydrogenase NAD ⁺	+	+	+
Isocitrate dehydrogenase NADP ⁺	+	+	—
Growth temp. (°C):			
Range	10–37	10–35	10–52
Optimum	25–30	30–33	42
DNA G+C content (mol%)	61.5	53.2	55.5

The major ubiquinone is Q-8. Dominant phospholipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol (cardiolipin). Produces exopolysaccharide containing glucose, galactose and xylose. DNA G+C content is 61.5 mol% (T_m).

The type strain is F31^T (=VKM B-2247^T=NCIMB 13994^T), which was isolated from meadow grass (*Poa trivialis* L.) growing in Helsinki (Finland).

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References

- Doronina, N. V., Trotsenko, Y. A., Krausova, V. I., Boulygina, E. S. & Tourova, T. P. (1998). *Methylopila capsulata* gen. nov., sp. nov., a novel non-pigmented aerobic facultatively methylotrophic bacterium. *Int J Syst Bacteriol* **48**, 1313–1321.
- Doronina, N. V., Darmaeva, T. D. & Trotsenko, Y. A. (2003). *Methylophaga alcalica* sp. nov., a novel alkaliphilic and moderately halophilic, obligately methylotrophic bacterium from an East Mongolian saline soda lake. *Int J Syst Evol Microbiol* **53**, 223–229.
- Govorukhina, N. I. & Trotsenko, Y. A. (1991). *Methylovorus*, a new genus of restricted facultatively methylotrophic bacteria. *Int J Syst Bacteriol* **41**, 158–162.
- Jenkins, O., Byrom, D. & Jones, D. (1987). *Methylophilus*: a new genus of methanol-utilizing bacteria. *Int J Syst Bacteriol* **37**, 446–448.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Urakami, T. & Komagata, K. (1986). Emendation of *Methylobacillus* Yordy and Weaver 1977, a genus for methanol-utilizing bacteria. *Int J Syst Bacteriol* **36**, 502–511.
- Urakami, T. & Komagata, K. (1987). Characterization of species of marine methylotrophs of the genus *Methylophaga*. *Int J Syst Bacteriol* **37**, 402–406.
- Van de Peer, Y. & De Wachter, R. (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosci* **10**, 569–570.
- Vorholt, J. A., Chistoserdova, L., Stolyar, S. M., Thauer, R. K. & Lidstrom, M. E. (1999). Distribution of tetrahydromethanopterin-dependent enzymes in methylotrophic bacteria and phylogeny of methenyl tetrahydromethanopterin cyclohydrolases. *J Bacteriol* **181**, 5750–5757.
- Yordy, J. R. & Weaver, T. Y. (1977). *Methylobacillus*: a new genus of obligate methylotrophic bacteria. *Int J Syst Bacteriol* **27**, 247–255.