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Methylomonas albis sp. nov. and *Methylomonas fluvii* sp. nov.: Two cold-adapted methanotrophs from the river Elbe and emended description of the species *Methylovulum psychrotolerans* ☆,☆☆,★

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ABSTRACT

Three strains of methanotrophic bacteria (EbA^T, EbB^T and Eb1) were isolated from the River Elbe, Germany. These Gram-negative, rod-shaped or coccoid cells contain intracytoplasmic membranes perpendicular to the cell surface. Colonies and liquid cultures appeared bright-pink. The major cellular fatty acids were 12:0 and 14:0, in addition in Eb1 the FA 16:1ω5t was also dominant. Methane and methanol were utilized as sole carbon sources by EbB^T and Eb1, while EbA^T could not use methanol. All strains oxidize methane using the particulate methane monooxygenase. Both strains contain an additional soluble methane monooxygenase. The strains grew optimally at 15–25 °C and at pH 6 and 8. Based on 16S rRNA gene analysis recovered from the full genome, the phylogenetic position of EbA^T is robustly outside any species clade with its closest relatives being *Methylomonas* sp. MK1 (98.24%) and *Methylomonas* sp. 11b (98.11%). Its closest type strain is *Methylomonas methanica NCIMB11130* (97.91%). The 16S rRNA genes of EbB^T are highly similar to *Methylomonas methanica* strains with *Methylomonas methanica* R-45371 as the closest relative (99.87% sequence identity). However, average nucleotide identity (ANI) and digital DNA-DNA-hybridization (dDDH) values reveal it as distinct species. The DNA G + C contents were 51.07 mol% and 51.5 mol% for EbA^T and EbB^T, and 50.7 mol% for Eb1, respectively. Strains EbA^T and EbB^T are representing two novel species within the genus *Methylomonas*. For strain EbA^T we propose the name *Methylomonas albis* sp. nov (LMG 29958, JCM 32282) and for EbB^T, we propose the name *Methylomonas fluvii* sp. nov (LMG 29959, JCM 32283). Eco-physiological descriptions for both strains are provided. Strain Eb1 (LMG 30323, JCM 32281) is a member of the species *Methylovulum psychrotolerans.* This genus is so far only represented by two isolates but Eb1 is the first isolate from a temperate environment; so, an emended description of the species is given.

Introduction

Methane is an important greenhouse gas. After a period of stability, atmospheric methane concentrations are increasing again, especially in northern latitudes [1]. Several studies have estimated the sources and sinks of methane in different environments [2–6]. Methane oxidation, either under anaerobic conditions by archaeal consortia and some bacteria [7], or under aerobic conditions by methanotrophic *Proteobacteria* is the only biological sink of methane on Earth [8].

Abbreviations: BCCM–LMG, Belgian Co-Ordinated Collections of Micro-Organisms; DSM, German Collection of Microorganisms and Cell Cultures; Elbe A, EbA^T; Elbe B, EbB^T; Elbe 1, Eb1; JCM, Japanese Collection of Microorganisms; LPSN, List of Prokaryotic Names with Standing in Nomenclature; NMS medium, nitrate mineral salts medium; pMMO, particulate methane monooxygenase; PBS, phosphate-buffered saline solution; sMMO, soluble methane monooxygenase; UBCG, up-to-date bacterial core gene.

☆ The NCBI accession number for raw sequencing data, genome assembly and annotation of EbA^T is JACXSS000000000.

☆☆ The NCBI accession number for raw sequencing data, genome assembly and annotation of EbB^T is JACXST000000000.

★ The GenBank accession numbers are for Eb1 KX129798 (16S rRNA), and KX958423 (*pmoA* gene); and KX958427 (*nifH*).

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The aerobic methanotrophic *Proteobacteria* are a unique group of bacteria that use methane as their sole source of energy and carbon. For the conversion of methane to methanol, two forms of methane monooxygenase have been found. One form, the membrane-associated or particulate methane monooxygenase (pMMO), is found in most known methanotrophs and is located in the cytoplasmic membrane. The second form, the soluble methane monooxygenase (sMMO), is found in some methanotrophs and is located in the cytoplasm. Key genes encoding for these enzymes are *pmoA* and *mmoX* [9,10]. For many years, oxygen was thought to be the only electron acceptor for methanotrophic *Proteobacteria*. However, under oxygen limitation they can also use alternative electron acceptors such as nitrate, both in natural environments [11–13] and in pure cultures [14]. Even fermentation has been identified as a possible adaptation to mitigate oxygen starvation [15].

Based on phylogenetic analyses of 16S rRNA gene sequences, methanotrophic *Proteobacteria* were initially divided into three subgroups: type I and type X methanotrophs correspond to the *Gammaproteobacteria*, and type II to the *Alphaproteobacteria* (e.g. [16]). However, the taxonomic structure of the *Gammaproteobacteria* was reconstructed using genome-based phylogeny, and three family lineages were proposed as "*Methylomonadaceae*" (Type Ia) [17,18,13], *Methylococcaceae* (Type Ib) [19], and *Methylothermaceae* (Type Ic) [20]. However, the family "*Methylomonadaceae*" is not validated yet. Among the family "*Methylomonadaceae*" and type Ia methantrophs belong the genera *Methylomonas* with 7 validated species (and 5 proposed) and *Methylovulum* with 2 species [21].

The genus *Methylomonas* forms a relatively tight cluster within the *Gammaproteobacteria*, as indicated by phylogenetic analysis using 16S rRNA sequences [22]. The G + C content of *Methylomonas* DNA covers a relatively broad range of 48–59% mol% (Tm). Species within the genus of *Methylomonas* mostly form red, pink, and orange carotenoid non-water-soluble pigments. The major fatty acids include C14:0 and C16:1 ω8c. They have been isolated from a wide range of habitats, including the sediments of freshwater lakes and rivers, wetland muds, rice fields [23] as well as activated sludge, wastewater, and drainage waters [22]. The type species is *Mm. methanica*, described by Whittenbury & Krieg [24] and emended by Bowman et al. [25]; and its complete genome sequence is available [26]. A more applied aspect of this group is that some of its members can be used for methanol production from methane and for other processes of methane valorization [27].

Until now, the genus *Methylovulum* contains only two species: *Mv. psychrotolerans and Mv. miyakonense* [28,29]. Their cells are described as aerobic, non-motile gram-negative bacteria with coccoid or rod-shape cells. They grow with methane and methanol as carbon sources. Both species have been isolated from cold and terrestrial environments. For *Mv. psychrotolerans,* the genome sequence has been released and described recently [30,31].

The Elbe is a large European river that drains into the North Sea. In recent years, we investigated its role in supplying methane to coastal waters, as well as the methane-consuming capacity of the methanotrophic population under different environmental settings [32–35]. Within the course of these studies, we isolated the new strains described here belonging to the genus *Methylomonas* and *Methylovulum*.

Material and methods

Sample collection

The Elbe River originates from the Krkonoše Mountains in the Czech Republic, flows through the port of Hamburg (Germany) and finally drains into the North Sea. In October 2012, water samples were taken at river km EC-659 (9.51°E, 53.67°N, approx. 50 km north of Hamburg) with the RV Ludwig Prandtl. The location is within the oxygen minimum zone and within the tidal freshwater area [36]. Surface water was sampled with an Uwitec water sampler, transferred to 10 L Nalgene bottles and stored at 4 °C in the dark until further processing in the laboratory.

Enrichment, isolation and cultivation

For EbA^T and EbB^T, we performed pre-enrichment from 10 L of river water. A thin silicone tube (approx. 5 cm long, 2 mm thick) was connected to a reservoir of methane (20% CH_4, 80% N_2 in a volume of approx. 2 L). The river water was running through an open system into which the silicon tubing was inserted. Within 2 weeks at 18 °C, this resulted in a thick biofilm on the silicon tubing, where increased methane concentrations are to be suspected. The biofilm was harvested, homogenized, and diluted in nitrate mineral salts (NMS) medium. For the isolation of strain Eb1, 10 L of river water were left to settle for two days at 4 °C. The sedimented flocs were pipetted off and were diluted in NMS. After visible growth had occurred, the enrichments were diluted up to 10^4, and 100 µl of the diluted enrichments was spread onto agar plates. Further purifications were performed on agar plates. Controls were set up without methane and with agar plates containing 0.05% nutrient broth (Difco) and 0.01% yeast extract (Difco). We used a modified NMS medium [37] containing 0.93 g/L $MgSO_4$ 6 H_2O, 1 g/L KNO_3, 0.12 g/L $CaCl_2$, 0.15 ml/L of 1 M PO_4 solution, 5 ml/L of 2 M HEPES buffer, 1 ml/L trace element solution, and 1 ml/L vitamin solution [38]. The pH was adjusted to 7.2. Agar plates contained additional 1.5% agarose (Biomol GmbH) and 0.01% cycloheximide. Liquid incubations were performed with cell culture bottles (Nunc) or in cell culture plates (Costar, 12 × 2 ml). Agar plates or bottles were incubated in desiccators under an atmosphere of 50% CH_4 (rest air) at 18–20 °C in darkness.

Microscopic analyses

For electron microscopy, cells grown on agar plates were suspended in 50 mM phosphate buffered saline solution (PBS, pH 7.5) and fixed by adding glutardialdehyde (Sigma-Aldrich, 25% v/v stock solution, 2.5% final concentration). Cells were washed by centrifugation and resuspension in phosphate-buffered saline (PBS) solution. The final pellet was resuspended in 1.5% (w/v) molten agar (final concentration). The agar block was cut into small pieces of 1 mm^3 volume, and incubated for 1 h in 1% (w/v) aqueous osmium tetroxide solution (Agar Scientific). Agar pieces were washed several times in distilled water; the pieces were then dehydrated in a graded ethanol series (15%, v/v, 30% for 15 min; 50%, 75%, 95% for 30 min; 100% for 1 h) under concomitant temperature reduction to −20 °C. The samples were infiltrated with LR White resin (66%, v/v, in ethanol for 1 h; 100% for 10 h, 4 °C), then polymerized for 24 h at 50 °C. Resin sections of 80 nm thickness were cut with glass knives or diamond knives in an ultramicrotome (Reichert Ultracut E) and stained with 4% (w/v) uranyl acetate solution (pH 7.0). Electron microscopy was performed in a Jeol JEM 1011 transmission electron microscope at 80 kV and calibrated magnifications. Phase-contrast microscopy of cells grown on agar plates was performed with a Zeiss Axiophot (Zeiss Filter No. 43) equipped with a digital camera (Zeiss AxioCam MRm).

Physiological tests

For the different physiological tests, isolates were grown in liquid medium under standard conditions (as described above) with the described modifications. Turbidity was checked by visual

inspection each week, and final results were obtained after three weeks. These results are compared with references given in Table 1. For the tests on temperature, salt, and pH tolerance, cultures were incubated in cell culture plates and their OD_{600} was determined with a Tecan plate reader after 4–25 days. In these tests, our isolates, as well as the close relatives (*Mm. methanica* DSM No. 25384) and *Mv. psychrotolerans,* LMG 29227) were included.

For alternative carbon sources, we tested several carbohydrates (arabinose, fructose, glucose, lactose, maltose, raffinose, and xylose, all 0.1% w/v), as well as formamide and formate (0.1% w/v). These setups were incubated without methane, but otherwise standard conditions. Methanol and formaldehyde as intermediates of the methane oxidation pathway were also tested as carbon sources (without methane). Methanol was tested at concentrations of 10–50 mM, and formaldehyde at 1 mM and at 10–50 mM.

For alternative nitrogen sources, we tested several amino acids (asparagine, aspartic acid, glutamate, glycine, serine, and valine, all 0.05% w/v), as well as urea and ammonium chloride (0.05% w/v), and complex media (peptone and yeast extract, 0.05% w/v). These setups were incubated without KNO_3, but otherwise standard conditions. In addition, we tested whether the strains could grow in the absence of any nitrogen source, except for the air-borne N_2 under an oxygen-reduced atmosphere (20% air, 50% CH_4, 30% N_2).

The pH tolerance was tested with TRIS, HEPES, and MES buffers (all 10 mM, Tris(hydroxymethyl)-aminomethane, pH range 7.2–9.0; 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid, pH range 6.8–8.2, and 2-(N-Morpholino) ethanesulfonic acid (pH range 5.8–6.5). Thus, the tested pH range was from 4 to 9 in 0.5 steps. Temperature tolerance and optima were tested with liquid cultures incubated under standard conditions for up to 6 weeks at 1, 5, 10, 15, 18, 25, 30, 35, and 40 °C. Salt tolerance was tested by adding NaCl to liquid medium with final concentrations of 2.5, 5, 10, 15, and 20 g/L (i.e., 2.5–20‰).

To expand our knowledge of the physiological spectrum of our strains by gene prediction, we searched for the presence of characteristic enzymes on the sequenced genomes using the database of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/), focusing on enzymes responsible for the carbon and nitrogen metabolism.

Fatty acid and G + C analyses

Fatty acid analyses were carried out by the BCCM–LMG (Belgian Co-Ordinated Collections of Micro-Organisms). Our deposited strains, as well as the closest relatives *Mm. methanica* LMG 26612 and *Mv. psychrotolerans* LMG-29227 were grown, harvested, and analyzed under the same conditions at the BCCM–LMG. Cells were taken from cultures grown on LMG medium 391 at 20 °C in a CH_4: air (50:50) atmosphere for five days. Inoculation and harvesting of the cells, and extraction and analysis of the fatty acid methyl esters were performed in accordance with the recommendations of the MIDI commercial identification system (Microbial Identification System, Inc., Delaware USA.), except that cells were harvested from two whole plates (QALL). The whole-cell fatty acid composition was determined via gas chromatography. The peak naming table MIDI TSBA 5.0 was used.

For further comparison literature values were included: *Mm. lenta* [39]; *Mm. koyamae* [40]; *Mm. paludis* [41] *and Mm. miyakonense* [29].

Phylogenetic and genomic analyses

Genomic DNA was isolated using the protocol for small amounts of cells, tissues, or plant leaves for the Easy-DNA kit (Invitrogen/ Life technologies) whereby 200 µl of NMS medium has been used for resuspension. Sequencing the genomic DNA (Eurofins Scientific SE) with PacBio RS II resulted in 120,030 reads (1,483,630,000 bp) for *Mm. albis* EbA^T and 111,368 reads (1,210,017,860 bp) for *Mm. fluvii* EbB^T (Table S1). The genomes were assembled using HGAP3. Completeness, contamination, and strain heterogeneity was calculated using checkM [42]. Genome statistics were calculated using own perl scripts. We compared our genome assemblies to 22 available completed reference assemblies of *Methylomonas* and three outlier assemblies. The

Table 1
Characteristics of the strains EbA^T, EbB^T and Eb1 in comparison with other closely related species of the genus *Methylomonas and Methylovulum*. Species: **1** Strain EbA^T; **2** Strain EbB^T; **3** *Mm. methanica* [22,39,74]; **4** *Mm. lenta* [39]; **5** *Mm. paludis* [41], **6** Strain Eb1, **7** *Mv. psychrotolerans* [28], **8** *Mv. miyakonense* [29]. For *Mm. methanica* and *Mv. psychrotolerans,* data obtained in this study are marked with *. "+" and "–" indicate presence/absence of growth, "±" indicates weak growth or no clear result, "n.a." indicated no information available.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Cell shape	rod shaped	rod shaped	rod	Rod	rods	round/cocoid	Cocci	Cocci, short rods
Cell width–length (µm)	2.5 × 0.8	2.5 × 0.8	0.5–1 × 1.0–4.0	0.6–0.9/ 1.3–2.0	1.0–1.5 × 1.0–4.0	Ø 2.5 µm	3–5	Ø 1–2 µm
Storage granules	+		+	+	n.a.	–	n.d.	±
Intracytoplasmic membranes	perpendicular		type I type	vesicular discs	type I type	perpendicular	Stacks of vesicular disks	Disc shaped vesicles
Pigmentation	pink	pink	pink	white pink	pale pink	pink	Light pink	Pale brown
Temperature range [°C]	1–30	1–30	1–30*	15–28	8–30	1–25	1–25*	5–34
Opt. temperature [°C]	15	15	10*	20	20–25	25	25*	24–32
pH range	6.0–9	5.5–9	6.5–9.0*	6.3–7.8	3.8–7.3	4.5–9	4.5–9*	6.0–7.5
Opt. pH	6.0	6.0	8.0*	6.8–7.3	5.8–6.4	8.0	8.0*	6,5
Max NaCl (g/L)	5	15	20*	1	1	5	5*	0.2
Other C sources	None	None	None	none	none	none	None	n.a.
Growth on CH_3OH	–	+	+	+	+	+	+	+
Growth on HCHO	+	+	–	–	n.a.	+	n.d.	–
Other N sources	0.05% yeast	0.05% yeast	several	several	several	0.05% yeast	Nitrate, ammonium salts, casamino acid	Several
N_2 fixation	±	±	±	–	+	+	+	–
Presence of *nifH*	+	+	+	+	+	+**	–	–
Presence of *pmoA*	+	+	+	+	+	+**	+	+
Presence of *mmoX*	+	+	±	–	–	–**	–	+
DNA G + C content	51.07	51.5	50.6–54.0	47	48.5	50.7	51.3–51.9	49.3

** based on PCR data.

characteristics of our genomes are within typical ranges while a very high quality is given (Table S1) as stated in [43].

Pairwise sequence similarities were calculated using the method recommended by [44] for the 16S rRNA genes available via the GGDC web server [45] at http://ggdc.dsmz.de/ (Fig. 4). Phylogenies were inferred by the GGDC web server at http://ggdc.dsmz.de/ using the DSMZ phylogenomics pipeline [46] adapted to single genes. A multiple sequence alignment was created with MUSCLE [47]. Maximum likelihood (ML) and maximum parsimony (MP) trees were inferred from the alignment with RAxML [48] and TNT [49], respectively. For ML, rapid bootstrapping in conjunction with the autoMRE bootstopping criterion [50] and subsequent search for the best tree was used; for MP, 1000 bootstrapping replicates were used in conjunction with tree-bisection-and-reconnection branch swapping and ten random sequence addition replicates. The sequences were checked for a compositional bias using the X^2 test as implemented in PAUP* [51].

An additional phylogenetic tree (Fig. S1) was based on 22 nearly complete *Methylomonas* genomes available at NCBI. Assemblies of *Mv. psychrotolerans, Methylocapsa acidiphila* and *Methyloferula stellata* were used as outlier group. 16S rRNA genomic sequences were predicted using infernal 1.1.2 [52] and SSU_rRNA_bacteria models from RFAM 14.1 [53]. The tree was created using MrBayes 3.2 [54] for 16S rRNA sequences (lset nst = 6 rates = invgamma; 1,000,000 iterations) based on alignments created with MAFFT [55]. MrBayes (prset aamodelpr = mixed; 1,000,000 iterations) was also applied to reconstruct a phylogenetic tree based on the amino acid sequences of the p-MMO enzyme (Fig. 5, only with the *pmoA* gene). The p-MMO protein sequences have been predicted based on a RAST reannotation (RastTk using default values, [56] of the published genomes.

The phylogenetic analysis is complemented by a genome related index (OGRI) that has been analyzed by ANI (Fig. S3, Table S3), by dDDH (Table S2), and by concatenated bacterial core genes (Fig. S2).

Isolation of DNA from Eb1 was done using the UltraClean Microbial DNA isolation kit (MoBio Laboratories). The *pmoA* gene was targeted with the primers A189 f (5′-GGNGACTGG GACTTCTGG-3′) and A682 (5′-GAASGCNGAGAAGAASGC-3′) [57] with annealing at 55°. For Eb1 we performed PCRs for the *mmoX* gene which was targeted with the primers mmoX-206 f_(5′-ATCGC BAARGAATAYGCSCG-3′) and mmoX-886 r (5′-ACCCANGGCTCGA CYTTGAA-3′) [58] with annealing at 61 °C. The *nifH* gene of Eb1 was targeted with the primers Pol f (5′-TGCGAYCCSAARGCB GACTC-3′) and Pol r (5′-ATSGCCATCATYTCRCCGGA-3′) [59] with annealing at 55 °C.

Results and discussion

Strain isolation

After incubation for 3 weeks, the enrichments in fluid NMS medium showed strong turbidity and pink pigmentation. They were diluted and spread on NMS-agarose plates. After 6 days distinct colonies appeared, which were further purified. Among them, strains EbA^T, EbB^T and Eb1 were chosen for further characterization. Colonies of strain EbA^T were faintly pink pigmented and their structure also appeared dull and uneven. Colonies of strain EbB^T appeared shiny and raised, pigmentation varied from faint pink to an intense salmon pink [32].

Morphology

Cells of strain EbA^T were rod-shaped (approx. 2.5 × 0.8 μm, Fig. 1A). Electron microscopy revealed the presence of storage granules of which most appeared bright in a stained ultrathin section (Fig. 1B) as is common for polyhydroxyalkanoates [60]. Some granules contained dark-stained, fine-grained inclusions (Fig. 1C), which may account for another storage polymer, e.g., glycogen [61,62]. Due to the high density of storage granules, intracytoplasmic membranes could rarely be observed. If present, they were arranged between storage granules, perpendicular to the cytoplasmic membrane (Fig. 1E). The cell envelope corresponds to gram-negative cells (Fig. 1D). Strain EbB^T also had rod-shaped cells (Fig. 2A). Intracytoplasmic membranes appeared sometimes curved (Fig. 2B), but in most cells perpendicular to the cell surface (Fig. 2D). The structure of the cell envelope corresponded to that of EbA^T. Occasionally, bright storage granules were visible (Fig. 2C). A bacterial chemoreceptor array could be observed in one cross-section (Fig. 2E; [63]). In both *Methylomonas* strains genes for glycogen synthesis were present in their genome (Glycogen phosphorylase, Glycogen branching enzyme, Glycogen synthase) and no genes for PHB synthesis were found, indicating that the storage granules consisted of glycogen. But also, poly-phosphate is possible as the gene for the polyphosphate kinase 2 was found in the genome.

The cell shape of strain Eb1 was round/coccoid, with up to 2.5 μm in diameter (Fig. 3A, B). Electron microscopy revealed no storage granules. Intracytoplasmic membranes (Fig. 3B, C) were arranged perpendicular to the cell surface. The cell envelope was multi-layered, as described for typical gram-negative bacteria (Fig. 3D; [60]). For Eb1 no genome information is available.

Physiological characteristics

None of the investigated multi-carbon sources could be used by the strains for growth (Table 1). The presence of the *ackA* gene for the acetate kinase, sequences for the acetyl-coenzyme A carboxyl transferase in the genomes of strains EbA^T and EbB^T indicates that they could be able to perform C2 metabolism. Even though the presence of these enzymes is not conclusive for acetate metabolism [64]. Strains Eb1 and EbB^T were growing with methanol as sole carbon source over the whole range from 10 to 50 mM. In comparison, strain EbA^T showed no growth at any of the tested concentration. However, the presence of the *mxaF* gene, for the methanol dehydrogenase in the genomes of strain EbA^T and EbB^T indicates that also EbA^T should be able to grow with methanol, but probably at lower concentrations [65]. Both strains EbA^T and EbB^T grew on 1 mM formaldehyde, but did not tolerate 10 mM. Eb1 grew up to a concentration of 30 mM formaldehyde (all in the absence of methane). The ability to use formaldehyde is supported by the fact that the *Fdh1* gene for the formate dehydrogase was present in the genomes of strains EbA^T and EbB^T. In addition, the presence of genes for methylamine utilization (*mauG*) in strains EbA^T and EbB^T indicates further metabolic diversity.

As alternative nitrogen sources, only yeast extract allowed for growth in all strains. The results on the ability to fix molecular nitrogen were ambiguous: In strain EbA^T, we observed only weak growth in the absence of the main N-source (except airborne N_2 and traces of organic nitrogen from the buffers and vitamins). However, the *nifH* gene was located in the genome sequence. This indicates that although the *nifH* gene is present on the genome, the nitrogenase was not (yet) active. In contrast, in strain EbB^T the *nifH* gene was present in the genome sequence. But no growth was observed in the absence of any nitrogen source, indicating that the inability to fix nitrogen was related to other physiological constrains such as too high oxygen level. The presence of the genes for nitrate and nitrite reductase *(NR* and *nirB)* and the absence of other genes involved in the nitrogen metabolism in EbA^T and EbB^T indicate an impaired nitrogen metabolism. In Eb1, the *nifH* gene was

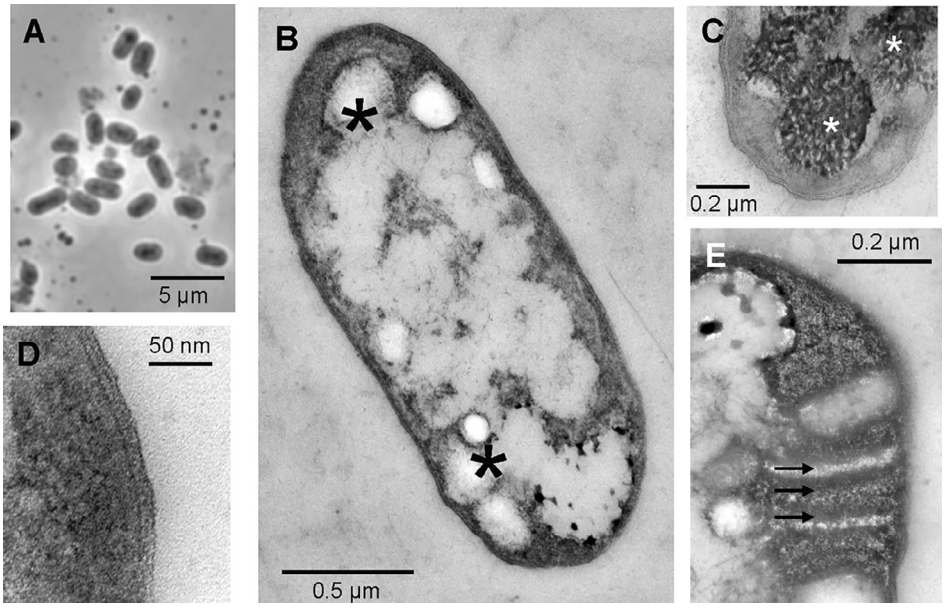

Fig. 1. Structural features of isolate EbA^T. (a) Light micrograph of a cell suspension; (b) Whole cell with storage granules (asterisks); (c) Storage granules filled with dark-stained, granular material (asterisk); (d) Multi-layered cell envelope; (e) Intracytoplasmic membranes (arrows) arranged perpendicular to the cell surface).

detected with PCR and the strain grew well in the absence of any nitrogen source.

Strain EbA^T showed growth within the pH range 6.0–9.0 (Table 1), strain EbB^T tolerated a pH range of 5.5–9.0 and *Mm. methanica* DSM 25384 grew only from pH 6.5–9.0. The optimal pH (highest OD) of the three strains was 6.0, 6.0, and 8.0, respectively. Strain Eb1 and *Mv. psychrotolerans,* LMG 29227 grew within the pH range 4.5–9.0 (Table 1). Their optimal pH was 8.0.

The strains EbA^T, EbB^T and *Mm. methanica* DSM 25384 grew from 1 °C up to 30 °C, but no growth was observed at 35 °C and 40 °C. Their optimal growth (highest OD) was reached at 15 °C, 15 °C and 10 °C, respectively. Eb1 and *Mv. psychrotolerans,* LMG 29227 grew from 1 °C up to 25 °C, and no growth was observed above 30 °C. Optimal growth (highest OD) of both strains was reached at 25 °C.

The strain EbA^T tolerated only up to 5 g/L NaCl while strain EbB^T grew up to 15 g/L NaCl and *Mm. methanica* DSM 25384 revealed the highest salinity tolerance growing up to 20 g/L NaCl (Table 1). The optimal NaCl (highest OD) concentrations for these strains was 2.5; 2.5 and 0 g/L. Strain Eb1 and *Mv. psychrotolerans,* LMG 29227 were more sensitive to NaCl and only grew up to 5 g/L NaCl (Table 1). Their optimal growth occurred without NaCl addition.

Ecology

All strains from this study have been isolated from the Elbe estuary. This environment is highly variable in respect to salinity and temperature with a salinity range of 0.1–5 PSU (approx. 0.1–5 g/L NaCl) and a range of water temperatures from 3.4–19.8 °C [35]. With a fingerprinting method (monooxygenase intergenic

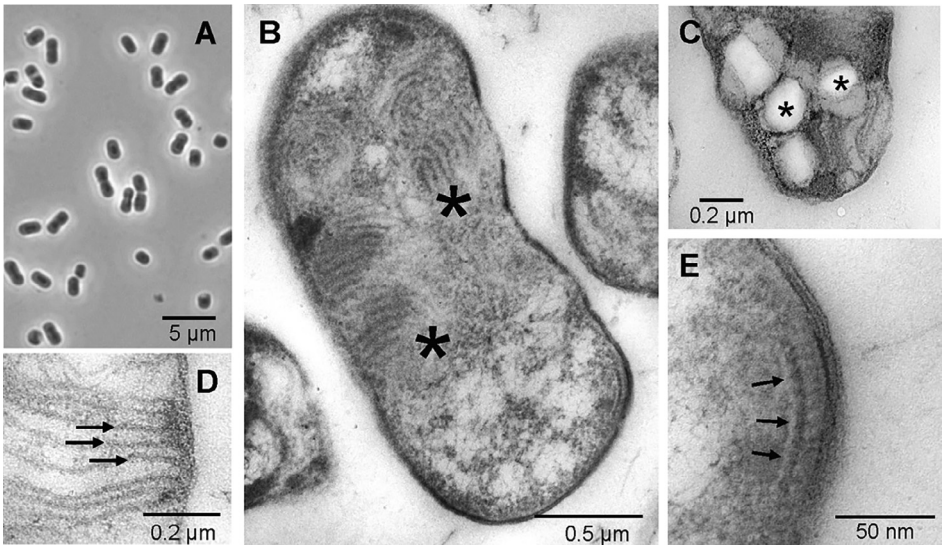

Fig. 2. Structural features of isolate EbB^T. (a) Light micrograph of a cell suspension; (b) Whole cell with intracytoplasmic membranes (asterisks); (c) Bright-stained storage granules (asterisks); (d) Intracytoplasmic membranes (arrows) arranged perpendicular to the cell surface; (e) Multi-layered cell envelope with a chemoreceptor array in side-view (arrows).

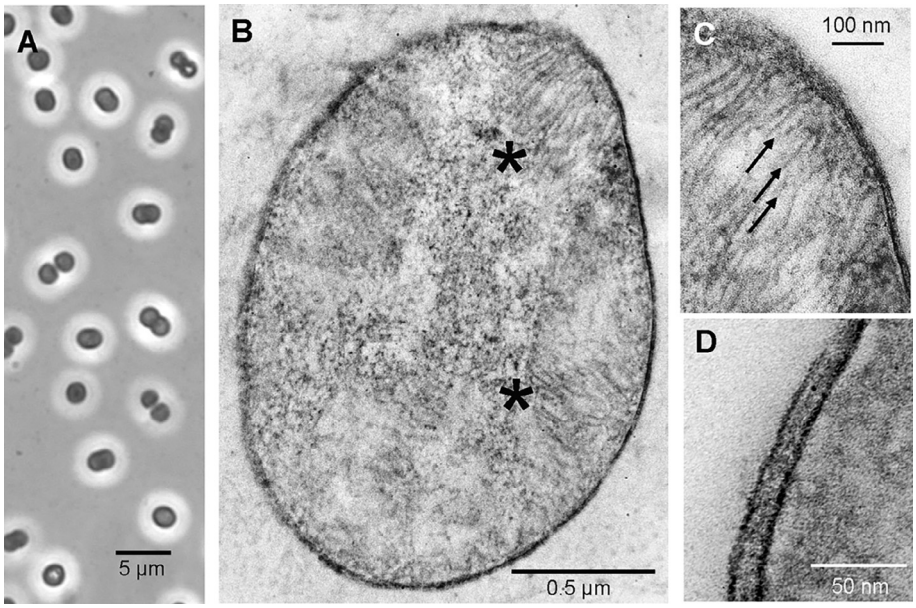

Fig. 3. Structural features of strain Eb1. (a) Light micrograph (phase contrast) of a cell suspension; (b) Whole cell with intracytoplasmic membranes (asterisks); (c) Intracytoplasmic membranes (arrows) arranged approximately perpendicular to the cell surface, (d) Multi-layered cell envelope.

spacer analysis), it could be shown that the OTUs from our strains were widely present in the Elbe Estuary and salinity has been demonstrated to be the major driver in shaping the methanotrophic community [35]. Here, both *Methylomonas* strains, EbA^T and EbB^T, could grow with NaCl concentrations up to 5 and 15 g/L, respectively. Osudar et al. also described the two strains as resistant to changes in salinity and anticipates them to be active and possibly also growing within the lower estuary [66]. Type 1a methanotrophs which include *Methylomonas* are also reported to be resistant to high NaCl concentrations [67,68]. In contrast, Eb1 tolerated up to 5 g/L NaCl, but optimal growth occurred without any NaCl addition. This supports the description of *Methylovulum* as very sensitive to brackish or marine conditions [66,67].

The recorded range of in situ temperatures (3–20 °C) is smaller than the observed physiological temperature range (1–30 °C/25 °C) for growth of EbA^T, EbB^T and Eb1. Several members of the type 1a methanotrophs are reported as psychrotolerant and have been isolated from cold habitats, and the lowest reported (and probably also tested) temperatures for growth were 5, 4, 10, and 4 °C, respectively [29,69–71]. In contrast, all strains of this study were able to grow at 1 °C and 4° (Table 3). The type strain, Sp1^T, was isolated from a cold methane seep in West Siberia [28], other strains have been isolated from Svalbard (strain TFB [72]), and Norway (strain HV10-M2 [31]). Here, we report the first isolation from a temperate environment, the river Elbe.

Chemotaxonomy

Strains EbA^T and EbB^T revealed similar fatty acid (FA) patterns with the dominant FAs being 14:0 at 46% and 52%, respectively (Table 2). FA 14:0 is also dominant in *Mm. methanica* LMG 26612, *Mm. methanica* NCIB11130**5** as well as in *Mm. koyamae* but only at 19–25%, respectively. The fatty acid 12:0 was the second dominant in strains EbA^T and EbB^T, at 19% and 27%, but is reported only with low or no numbers from *Mm. methanica*; *Mm. lenta, Mm. koyamae, Mm. paludis,* (Table 2). In contrast to the other *Methylomonas* strains, EbA^T and EbB^T did not contain any branched 16C FA. Only the unbranched FA 16:0 was detected (8–9%) at slightly higher levels than in the other strains (*Mm. methanica*; *Mm. lenta, Mm. koyamae, Mm. paludis,* 5–8%). The short-chained FA (12:0, 13:0, 14:0) added up to 70 and 82%, which is much more than in the other strains (20–36% in *Mm. methanica* and 8–23% in

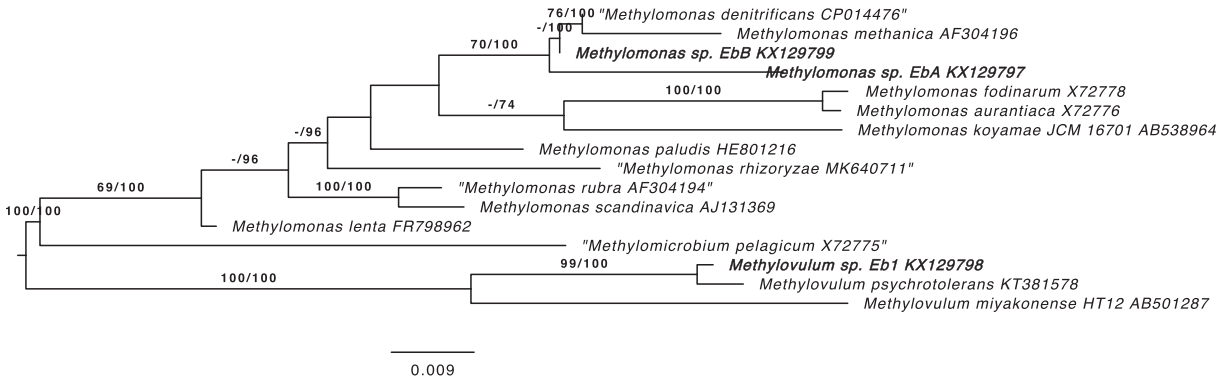

Fig. 4. Phylogenetic tree based on the 16S rRNA genes of our species and other *Methylomonas* and *Methylovulum* gene information available at the GGDC webserver. ML tree inferred under the GTR + GAMMA model and rooted by midpoint-rooting. The branches are scaled in terms of the expected number of substitutions per site. The numbers above the branches are support values when larger than 60% from ML (left) and MP (right) bootstrapping. The gene AF304196 is from the type strain *Mm methanica.*

Fig. 5. Phylogenetic tree of amino acid sequences of genes predicted to be *pmoA*.

Mm. lenta, Mm. koyamae, Mm. paludis). The cis-branched FAs (sum of 15:1ω6c, 15:1ω8c, 16:1ω8c, 16:1ω7c, 16:1ω6c, 16:1ω5c, 17:1ω6c) occurred in EbA^T and only in low quantities (8%) relative to *Mm. methanica* (52–56%) and 38–60% in *Mm. methanica*; *Mm. lenta,* Mm. *koyamae, Mm. paludis*.

The dominant FA of strain Eb1 were 12:0, 14:0, and the branched FA 16:1ω5t with 12, 24 and 32% respectively (Table 2). In contrast, in *Mv. psychrotolerans* FA 12:0 was not dominant (5%) while FA 16:1ω7c was the most dominant FA (55%). The short-chained FA (12:0, 13:0, 14:0) added up to 41%, which is more than in the other strains (34% in *Mm. miyakonense* and 18% in *Mv. psychrotolerans*). The cis-branched FAs occurred in Eb1 only in low quantities (9%) compared to *Mv. psychrotolerans* (55%).

Phylogenetic affiliation

Based on the phylogenetic analysis of the 16S rRNA gene and *pmoA* amplicon sequences, strains EbA^T and EbB^T were subjected to full genome sequencing and analysis as described below.

We reconstructed a phylogenetic tree based on the 16S rRNA gene sequences (Fig. 4) retrieved from the published *Methylomonas* genes, and including *Methylovulum*. In addition, in Fig. S1 we constructed a phylogenetic tree based on 20 nearly complete *Methylomonas* genomes available at NCBI. A phylogenetic tree of amino acid sequences of genes predicted to be *pmoA* is shown in Fig. 5.

The phylogenetic position of *Mm. albis* EbA^T is robustly outside any species clade within the reconstructed 16S RNA phylogeny

Table 2
Cellular fatty acid composition of strains EbA^T, EbB^T and Eb1 in comparison with other closely related species of the genus *Methylomonas and Methylovulum*. Species: **1** Strain EbA^T; **2** Strain EbB^T; **3** *Mm. methanica* LMG 26612; 4 *Mm methanica* NCIB11130**5** [74] *Mm. lenta* [39]; **6** *Mm. koyamae* [40]; **7** *Mm. paludis* [41], **8** Strain Eb1, **9** *Mv. psychrotolerans* LMG 29227, **10** *Mv. miyakonense* [29]. Data for strains 1, 2, 3 and 8, 9 were all investigated in the same lab under the same laboratory conditions, data for other strains are obtained from literature. Compounds at a percentage of 0.2 or higher are listed.

Fatty acid	1	2	3	4	5	6	7	8	9	10
12:0	19.2	26.6	10.1		0.6			12.1	4.5	
13:1 at 12–13	4.8	3.7	1.8							
13:0	5.1	3.7	1.2		0.6			4.3	1.1	
14:0	45.9	51.6	24.9	19.9	6.4	22.9	11.8	24.3	11.9	34.2
15:1ω6c	7.5		0.6		0.5					
15:1ω8c					1.0					
15:1 iso G		4.3	0.4							
15:0 3OH								1.8	1.6	
15:0					5.3	1.2	0.5			3.0
16:1ω8c				41.3	40.8	39.4	22.1			
16:1ω7c				9.8	9.1	4.35	13.9	8.8*	55.2*	
16:1ω6c				5.0	–	–				
16:1ω5c			51.1*			16.7	1.8			
16:1ω5t				14.7		–	34.8	31.7	15.2	
16:0	8.6	7.9	6.1	5.0	5.0	7.7	5.6	8.9	5.9	46.9
16:0 3OH	9.0	5.1	3.8							8.0
17:1ω6c										6.4
17:1ω7c/17:1ω7t				0.7/0.3						
18:1ω7c/18:0/cy19:0				1/0.1/0.2						

* together with 15:0 iso 2OH.

Table 3
Descriptions of *Methylomonas albis* sp. nov., *Methylomonas fluvii* sp. nov. and emended description of the species *Methylovulum psychrotolerans* Oshkin et al. 2016, the emended additions are in bold, previous description in brackets.

Genus name	*Methylomonas*	*Methylomonas*	*Methylovulum*
Species name	*Methylomonas albis*	*Methylomonas fluvii*	*Methylovulum psychrotolerans Eb1*
Specific epithet	*albis*	*fluvii*	
Species status	sp. nov.	sp. nov.	
Species etymology	al'bis. L. gen. n. albis, of the river Albis (Labe - Elbe)	flu'vi.i. L. gen. n. fluvii of a river	
Description of the new taxon and diagnostic traits	Rod-shaped cells up to 0.8 × 2.5 μm, with a high density of storage granules, non-motile. Cells are bright-pink, no cysts were observed, not resistant to heat or desiccation. The strain uses only formate as alternative carbon source. The genes *mmoX* and *nifH* were both detected, but the strain did grow with only airborne N_2 as nitrogen source. Optimal temperature is 15 °C and optimal pH is 6.0. The major fatty acid is 14:0, followed by 12:0.	Rod-shaped cells up to 0.8 × 2.5 μm, with a high density of storage granules, non-motile. Cells are bright-pink, no cysts are observed, not resistant to heat or desiccation. The strain uses only methanol and formate as alternative carbon source. It does possess the genes *mmoX* and *nifH*, but no growth with air-borne N_2 was observed. Optimal temperature is 15 °C and optimal pH is 6.0. The major fatty acid is 14:0, followed by 12:0.	non-motile cocci**, 2.5** (3)–5 μm Ø, stacks of ICMs typical of type I methanotrophs, no storage granules, The temperature range for growth is **1**°(2)–36 °C with an optimum at 20–25 °C, optimal pH is 6.0–7.0, ranging from 4.0 and 8.9. Methane, methanol and formate are the only growth substrates. Methane is oxidized by pMMO; sMMO is absent. Growth factors are not required. NaCl inhibits growth at concentrations above **0.5%** and [72] (0.1%),). The predominant fatty acids are **C16 : 1 ω5c** (and [72]), and C16 : 1 ω6c, C16 : 1 ω8c, C16 : 1 ω7c, in addition to **C14 : 0** (and [72]) and to a lesser extend C16 : 0.
Country of origin	Germany	Germany	Germany
Region of origin	River Elbe	River Elbe	River Elbe
Source of isolation	River water	River water	River water
Sampling date	October 2012	October 2012	December 2012
Latitude	53.67°N	53.67°N	53.67°N
Longitude	9.51°E	9.51°E	9.51°E
Altitude (meters above sea level)	0	0	0
16S rRNA gene accession nr.			KX129798
Genome accession number	NCBI JACXSS000000000	NCBI JACXST000000000	
Genome status	complete	complete	
Genome size	5.40 Mbp	5.59 Mbp	
GC mol%	51.07	51.5	**50.7** (51.4–51.9) 52.2 [72]
Number of strains in study	1	1	1
Designation of the Type Strain	EbA^T	EbB^T	
Strain Collection Numbers	LMG 29958 = JCM 32282	LMG 29959 = JCM 32283	LMG 30323 = JCM 32281

(Fig. 4). Its closest type strain is *Mm. methanica* NCIMB 11130 (97.91%, Table S4). Based on the 16S rRNA identity, its closest relatives are *Mm.* sp. MK1 (98.24%) and *Mm.* sp. 11b (98.11%, Table S4). The p-MMO phylogeny also groups *Mm. albis* EbA^T with "*Mm. denitrificans*" and *Mm. methanica* (Fig. 5).

The phylogenetic tree reconstruction based on the 16S rRNA gene sequences places *Mm. fluvii* EbB^T close to the type strain *Mm. methanica* NCIMB 11130 (Fig. 4). While EbB^T is not directly grouping with strains assigned to other *Mm. methanica* strains and associating a high uncertainty with its position (56.92%, Fig. S1). The 16S rRNA genes are highly similar to other *Mm. methanica* strains where the closest strain is *Mm. methanica* R-45371 (99.87% sequence identity, Table S4). The phylogenetic reconstruction for *pmoA* (Fig. 5), which is known to reconstruct phylogenies similar to the 16S rRNA gene [16], shows that the strain diverged and places it near the *Mm. koyamae* to which it has a smaller 16S rRNA sequence identity (96.15%, Table S4).

Genome features

Draft genome sequencing of *Mm. albis* EbA^T yielded a genome of 5.40 Mbp distributed across two scaffolds with a coverage of 130x. The G + C content is 51.07% and 5308 coding genes (of which 3042 have an assigned function) and 45 tRNA sequences have been predicted. The genome can be considered complete (99.77%) with very low contamination (0.52%, Table S1). The genome assembly of *Mm. fluvii* EbB^T resulted in a genome of 5.59 Mbp that has been assembled in 4 scaffolds with an average sequencing depth of 115x. The G + C content is 51.5% and 5524 coding genes (of which 3288 have an assigned function) and 46 tRNA sequences have been predicted. This genome can also be considered complete (99.91%) with very low contamination (0.76%) whereof fractions may result from strain heterogeneity (Table S1). Raw sequencing data, genome assembly, and annotation of EbA^T and EbB^T has been deposited at NCBI with the accession numbers JACXSS000000000 and JACXST000000000, respectively.

The dDDH values for *Mm. albis* EbA^T and *Mm. fluvii* EbB^T are in the range of 19.3–31.8% and 19.4–44.2%, thus, not exceeding the recommended species threshold of 70% [73] to any other published *Methylomonas* genome assembly (Table S2). A comparison of the dDDHs with the type strain *Mm. methanica* NCIMB 11130T revealed a similarity of 31.7 and 31.6 for EbA^T and EbB^T, respectively (Tab. S2). The ANI values for *Mm. albis* EbA^T and *Mm. fluvii* EbB^T are in the range of 82.48–87.65% and 82.97–91.65%, thus, also not exceeding the recommended species threshold of 96% to any other published *Methylomonas* genome assembly (Table S3). A comparison of the ANIs with the type strain *Mm. methanica* NCIMB 11130T revealed a similarity of 87.6% and 87.5% for EbA^T and EbB^T, respectively. Thus, for EbA^T and EbB^T, ANI-values as well as dDDH-values suggest that these strains represent distinct species.

Phylogenomic tree reconstructions, based on ANI values (Fig. S3) and UBCG bacterial marker gene alignments (Fig. S2),

group *Mm. fluvii* EbB^T with the genomes of the currently undescribed species *Mm.* sp. LW13 and *Mm.* sp. Kb3. The phylogenetic position of *Mm. albis* EbA^T is robustly outside any species clade (Fig. S3). The distances of these genomes are above the recommended thresholds for the definition of an own species.

Direct DNA–DNA hybridization between Eb1 and *Mv. psychrotolerans* LMG 29227 showed an ANI value of 97.4% (performed by BCCM-LMG). The DNA G + C content of Eb1 was 50.7 mol%. Thus, Eb1 can only be described as an extension of *Mv. psychrotolerans*, strain Eb1. In the most recent draft genome sequence of *Mv. psychrotolerans* strain HV10-M2, the phylogenetic tree shows the position of Eb1 within the other members of the *Methylovulum* group [31].

The presence of the soluble MMO based on the *mmoX* gene was checked with the genome sequence. In EbA^T and EbB^T its presence was located within the genome. Also the *nifH* gene, was located in the genome of in EbA^T and EbB^T. Based on the sequence of the *nifH* gene of Eb1, the next closest relative to Eb1 is *Mv. psychrotolerans* strain HV10_M2 (97% accordance).

In summary, cell morphology, physiology, 16S rRNA, *pmoA* gene phylogenies, genome comparison as well as DNA G + C content characterize EbA^T and EbB^T as members of the genus *Methylomonas*. However, our strains differ from other *Methylomonas* strains by their distinct fatty acid composition, their lower temperature optima, the low 16S rRNA gene sequence similarity with other methanotrophic isolates in the case of EbA^T, and the low ANI and dDDH values between EbB^T and its next relatives. Thus, we propose classifying the strains as novel, cold-adapted species of the genus *Methylomonas*: *Mm. albis* sp. nov. (EbA^T) and *Mm. fluvii* sp. nov (EbB^T) (Table 3).

Author statements

Ethical statement

No experimental work with animals or humans has been carried out in this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2021.126248.

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