Phosphitispora fastidiosa gen. nov. sp. nov., a new dissimilatory phosphite-oxidizing anaerobic bacterium isolated from anaerobic sewage sludge

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Abstract

A new strictly anaerobic bacterium, strain DYL19^T, was enriched and isolated with phosphite as the sole electron donor and CO₂ as a single carbon source and electron acceptor from anaerobic sewage sludge sampled at a sewage treatment plant in Constance, Germany. It is a Gram-positive, spore-forming, slightly curved, rod-shaped bacterium which oxidizes phosphite to phosphate while reducing CO₂ to biomass and small amounts of acetate. Optimal growth is observed at 30 °C, pH 7.2, with a doubling time of 3 days. Beyond phosphite, no further inorganic or organic electron donor can be used, and no other electron acceptor than CO₂ is reduced. Sulphate inhibits growth with phosphite and CO₂. The G+C content is 45.95 mol%, and dimethylmenaquinone-7 is the only quinone detectable in the cells. On the basis of 16S rRNA gene sequence analysis and other chemotaxonomic properties, strain DYL19^T is described as the type strain of a new genus and species, *Phosphitispora fastidiosa* gen. nov., sp. nov.

Phosphorus is the eleventh most abundant element in Earth's crust and plays a key role in all life forms. The most common phosphorus compound in nature is phosphate, but phosphite, hypophosphite and phosphine also can be detected in small amounts [1, 2]. In living cells, phosphate esters such as ATP, ADP, AMP, DNA, RNA and phospholipids play key roles in energy metabolism and cell matter synthesis. Due to its importance in living cells and the limited availability of dissolved phosphate in natural environments, phosphate is often the limiting factor for plant growth, e.g. for growth of aquatic plants in freshwater lakes [3]. Phosphate fertilisers help to overcome phosphate limitation in agriculture to secure human food production and to support sustainable development, but they also cause environmental pollution and eutrophication of inland waters. More recently, phosphite fertilisers are being developed and may help to replace phosphate fertilisers because phosphites are more soluble than phosphates, and phosphite is more readily absorbed into plant tissues than phosphate [4]. Phosphites as biostimulants can increase the economics of farms [5] and improve nitrogen

fixation efficiency and crop yield, thus reducing leaching and potential damage to the environment.

Geochemical evidence suggests that phosphite was abundant on the primitive Earth [6]. Therefore, the study of microbial phosphite metabolism is of significance to our understanding of the early evolution of life on Earth. Sources of reduced phosphorus compounds include phosphide minerals such as schreibersite (Fe,Ni)₃P, lightning strokes and geothermal fluids. Numerous bacteria can use phosphite as a phosphorus source in the synthesis of cell material, e.g., *Pseudomonas stutzeri* strain WM88 [7]. Oxidation of reduced phosphorus compounds such as phosphite (HPO₃²⁻) to phosphate (HPO₄²⁻) releases electrons at an unusually low redox potential ($E_0' = -650$ mV). Hence, phosphite is a very potent electron source, but so far only very few bacteria can use it in their energy metabolism. *Desulfotignum phosphitoxidans* strain FIPS-3 was described first to oxidize phosphite as an electron donor in its energy metabolism; it was isolated from marine sediment in Venice, Italy [8, 9]. More recently, a phosphite-oxidizing enrichment culture from a wastewater treatment plant was reported [10]. In the present paper, we describe

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Abbreviations: AAI, average amino acid identity; FAME, fatty acid methyl ester.

The GenBank accession numbers are MZ227813 for the 16S rRNA gene sequence. The genome was annotated at the Integrated Microbial Genomes and Microbiomes (IMG/M) platform and is available under IMG Taxon ID 2888950683. The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAHHUL000000000. The version described in this paper is version JAHHUL010000000.

a novel phosphite-oxidizing bacterium, *Phosphitispora fastidiosus* gen. nov., sp. nov.

Strain DY119^T was enriched and isolated from digesting anaerobic sewage sludge obtained from the municipal wastewater treatment plant in Constance, Germany. Enrichment cultures contained 10 mM sodium phosphite as the sole electron source in anoxic, bicarbonate-buffered, sulphide-reduced freshwater mineral medium under an oxygen-free mixture of N₂/CO₂ (80/20) [11]. 2.5 g l⁻¹ NaHCO₃, 1 mM Na₂S·9H₂O, 1 ml trace element solution SL-10 [12], 1 ml selenite-tungstate [13] and 1 ml seven-vitamin solution [14] were added from concentrated stock solutions after cooling. The initial pH of the medium was adjusted to pH 7.2. Cultivation was performed in 100 ml and 1 l glass bottles with butyl rubber stoppers under N₂/CO₂ (80:20; v/v) gas atmosphere in the dark at 30 °C. Pure cultures were obtained by repeated agar (1%) shake dilutions [11] and repeated pasteurization. Purity was checked by phase contrast light microscopy (Axiophot, Zeiss; with internal size calibration) after growth with 10 mM phosphite in the presence and absence of a mix of 10 mM glucose plus 10 mM fumarate and 0.05% (w/v) yeast extract. Pure cultures were transferred every 3 weeks and stored in liquid medium at 4 °C. Photographs were taken using the agar slide technique [15]. Gram-staining was performed using a staining kit (Difco Laboratories) according to the manufacturer's instructions, and confirmed by the KOH test [16]. Spore formation was checked microscopically after growth in the presence of 1 mM MnSO₄, and by testing growth after repeated pasteurization at 80 °C for 20 min.

Utilization of various carbon sources at 10 mM concentration was tested in freshwater medium. Reduction of nitrate (10 mM) and sulphate (10 mM) was checked via nitrite and sulphide formation. Nitrate or nitrite were analysed with commercial test strips (Quantofix, Macherey-Nagel). Sulphide was quantified by the method described by Cline [17]. All tests were conducted independently in duplicates.

Growth was monitored by measuring the optical density at 600 nm wavelength in a cuvette photometer (Spectrophotometer Jenway 6300) or in a tube photometer (M107, Camspec Analytical Instruments). Phosphite and phosphate were separated by HPLC on a Rezex RHM-Monosaccharide H+ (8%) column, 300/7.8 mm (Phenomenex) as described previously [18, 19]. Phosphite and phosphate eluted after 9.2 min and 10.4 min, respectively, with 30 mM sulphuric acid as eluent at 0.6 ml per min flow rate at 40 °C, and were detected by optical diffraction (refractive index detector RID-10A, Shimadzu). With the same method, acetate eluted after 16.8 min and sulphate after 7.6 min. Chromatograms were recorded and their analysis performed with Lab Solutions software (Shimadzu).

Hydrogen formation was analysed with a high-sensitivity gas chromatograph Peak Performer 1 (PEAK Laboratories) equipped with a reducing compound photometer, with N₂ as carrier gas.

The mol% G+C content was analysed by HPLC with DNA isolated from 2 g wet cell biomass [20, 21] by the Identification Service of DSMZ (Braunschweig, Germany). Fatty acid methyl esters (FAMES) were analysed with about 40 mg freeze-dried cells grown with 10 mM phosphite at 30 °C and harvested in the late stationary phase. The FAME mixtures were separated using the Sherlock Microbial Identification System (MIDI) consisting of an Agilent model 6890 N gas chromatograph fitted with a 5 % phenyl-methyl silicone capillary column (0.2 mm×25 m), a flame ionization detector, an Agilent model 7683A automatic sampler, and an HP computer with the MIDI database (Hewlett-Packard). Peaks were integrated automatically and fatty acid percentages were calculated by the MIS Standard Software (Microbial ID) [22–24] by the Identification Service of the DSMZ (Braunschweig, Germany) who also analysed the respiratory quinones by thin-layer chromatography.

Enrichment cultures with 10 mM phosphite as electron donor and 10 mM sulphate plus CO₂ as electron acceptor exhibited turbidity after 4–6 weeks of incubation at 30 °C. After five transfers, cultures were dominated by a slightly curved, rod-shaped, spore-forming bacterium. After repeated pasteurization and dilution in agar-solidified medium, white-yellow lens- to UFO-like shaped colonies were observed in the agar from which pure cultures were obtained. As judged from phase contrast microscopy, these isolates were morphologically identical. Of these, strain DY119^T was further characterized.

Cells of strain DY119^T were slightly curved rods, 5–9 µm long and 1 µm wide, formed terminally to subterminally located oval spores (Fig. 1), and stained Gram-positive. During growth, the optical density (OD₆₀₀) increased from 0.05 to 0.45, while phosphite was oxidized completely to phosphate, and CO₂ was reduced to biomass and small amounts of acetate (about 0.5 mM acetate from 10 mM phosphite, corresponding to ca. 20% of the total electrons supplied). No other organic products (fatty acids, lactate, succinate, alcohols) could be detected by HPLC after 60 min analysis under the conditions described above.

Numerous compounds were tested as possible growth substrates. No growth was observed with acetate, acetone, arabinose, 4-amino benzoate, benzoate, betaine, butyrate, choline, crotonate, ethylamine, fumarate, formate, glucose, glutamate, glycine, glycolate, glyphosate, glutarate, hypophosphite, lactate, malate, maleate, methanol, methyl phosphonate, dimethyl phosphonate, 2-oxoglutarate, phenol, proline, 1,2-propanediol, pyruvate, sarcosine, succinate, xylose, yeast extract (0.1% w/v), aniline (2 mM), H₂ or CO (each about 10% in the headspace) with or without sulphate as electron acceptor. Fumarate, nitrate, sulphate, sulphite, thiosulphate, sulphur and Fe(OH)₃ were not reduced with phosphite as electron donor. All substrate tests were run at least twice. Growth with phosphite was partially inhibited by addition of 10 mM sulphate, and no sulphide was formed.

Strain DY119^T grew well in freshwater or brackish water (with additional 1% w/v salt [11]) medium and very slowly in marine (with additional 2% (w/v) salt [11]) medium. It grew optimally

Fig. 1. Phase-contrast microscopy image of cells of strain DY119^T, after growth with 10 mM sodium phosphite and carbon dioxide as sole electron acceptor, including sporulated cells and free spores. Bars, 5 μ m.

with phosphite as electron donor and CO₂ as electron acceptor at 30 °C and pH 7.2. The doubling time was 3 days and the growth yield after complete oxidation of 10 mM phosphite to phosphate was 10.2 g cell dry mass per mol phosphite.

The DNA G+C content was 45.95 mol%. The only respiratory quinone found was dimethyl menaquinone-7. Polar lipids were mainly phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The major fatty acids were C_{14:0}, C_{16:1} ω 9c, C_{18:1} ω 9c and C_{18:1} ω 11c DMA (Fig. S1, available in the online version of the article).

Genomic DNA was isolated by the JGI-protocol ‘Bacterial genomic DNA isolation using CTAB’ [19]. Genome sequencing raw data, a total of 6903600 paired-end reads obtained with an Illumina NovaSeq 6000 platform, were provided by Eurofins Genomics Europe Sequencing GmbH (Constance, Germany). Trimmomatic version 0.39 [25] was used to remove remaining adapters from the reads with default settings, to filter the reads by quality (LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20) and to discard those shorter than 50 bp. The resulting reads were *de novo* assembled with SPAdes version 3.14.1 [26] using the ‘isolate’ option and kmer sizes of 21, 33, 55 and 77. The assembly procedure generated 255 scaffolds. Bowtie2 version 2.4.1 [27] and Samtools version 1.9 [28] were used to align the trimmed reads back to the assembled scaffolds (default settings) and to calculate the average fold, respectively. Finally, the scaffolds were sorted in descending order according to their length and annotated by Joint Genome Institute Integrated Microbial Genomes and Microbiomes Genomes OnLine Database (GOLD) [29]. The genome annotation is publicly available in IMG under GOLD Analysis Project ID Ga0451573 (IMG Taxon ID 2888950683) and in GenBank under the accession JAHHUL000000000.

For amplifying the 16S rRNA gene by PCR, a crude preparation of genomic DNA was obtained by centrifugation of

10 ml culture of strain DY119^T at 10000 *g* for 10 min. The supernatant was discarded and the cell pellet was washed once by suspending in 1.5 ml PCR quality H₂O (MP Biomedicals) followed by repeated centrifugation. The cell pellet was suspended in 50 μ l PCR H₂O and incubated at 99 °C for 10 min. After chilling on ice, this mixture was centrifuged again and the DNA-containing supernatant was stored at –20 °C until further use. The 16S rRNA gene was amplified by PCR using the primers 8F (5′-AGAGTTTGTATCCTGGCTCAG-3′) [30] or 533F (5′-GTGCCAGCAGCCGCGGTAA-3′) [31] and 1492R (5′-TACGGYTACCTTGTACGACTT-3′) [31] and a ready-to-use reaction mix (Bioline/Meridian Bioscience Mango Mix, BioCat) according to the manufacturer’s instructions. The reaction was performed with an initial denaturation at 96 °C for 2 min followed by 32 cycles of 94 °C for 40 s, 54 °C for 40 s, 72 °C for 60 s, and a final extension at 72 °C for 5 min. The amplified 16S rRNA gene fragment of approximately 1.4 kb was purified using a DNA purification kit (DNA clean and concentrator, Zymo Research) and sequenced at Eurofins Genomics Europe Sequencing GmbH. The 16S rRNA gene sequence was trimmed and assembled with the genious software (Biomatters), and the resulting assembled DNA-sequence (1461 bp) was analysed with a BLASTN-search to identify close phylogenetic relatives [32] (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). This search revealed that strain DY119^T is a member of the family *Peptococcaceae*. The species most similar to strain DY119^T were the Gram-positive bacteria *Thermincola ferriacetica* strain Z-0001 [33] with 91.7% sequence identity and *Thermoanaerobacterium fracticulis* DRI-13 [34], which both are phylogenetically far remote from the Gram-negative *Desulfotignum phosphitoxidans*. The assembled 16S rRNA gene sequence was also used for a BLASTN-search against the genome sequence of strain DY119^T using BLAST integrated in IMG to reveal possible mutations that might have occurred during PCR amplification using a non-proofreading DNA polymerase (<https://img.jgi.doe.gov>).

Fig. 2. Phylogenetic tree based on the 16S rRNA gene sequence comparison. Empty circles indicate a bootstrap support of >70%, full circles >90% derived from 1000 samples. Scale bar indicates one nucleotide substitution per site. Gram-positive strains are marked in bold, Gram-negative strains are marked in grey and uncultured clones in white. The alignment was calculated using the SINA Aligner and the tree was created using IQ-Tree. The tree was visualized using iTOL version 6.

This 16S rRNA gene sequence exhibited 100% identity with the gene with the locus tag Ga0451573_046_206_1739 and carries the annotation 16S rRNA. We therefore considered this sequence as the 16S rRNA gene, which thereafter was used to reconstruct a phylogenetic tree.

The 16S rRNA gene sequence of DYL19^T was also analysed using the identification service of EzBioCloud. An alignment with related strains was calculated using the SINA Aligner online tool (www.arb-silva.de/aligner) removing bases remaining unaligned at the ends. The alignment of marker genes from the genome sequences was calculated using the Genome Taxonomy Database toolkit (gtdb-tk) [35]. Both alignments were used to calculate trees with IQ-Tree [36]. The trees were then visualized in the online tool iTOL version 6 (<https://itol.embl.de/>) [37] (Fig. 2). Phylogenetic analysis of the almost complete 16S rRNA gene identified strain DYL19^T as a member of the family *Peptococcaceae* within the order *Clostridiales*.

Illumina sequencing and assembly of the genome of strain DYL19^T yielded a total of 4094493 bp, with a DNA-coding number of basepairs of 3612790 bp on 255 scaffolds with 45.95 mol% G+C content. The genome contains 15 genes annotated as 16S rRNA, of which 14 are most likely misannotated 250 to 350 bp fragments, while only one of these genes (Ga0451573_046_206_1739) has a size of 1534 bp as expected for a complete 16S rRNA gene. The genome harbours 3056 protein-coding genes with function prediction and of these 924 are connected to KEGG pathways and 760 are connected to MetaCyc pathways. Of the latter, 171 are predicted to be involved in energy metabolism, among them genes for oxidative phosphorylation (NADH: quinone oxidoreductase subunits A to N) and various genes whose proteins are associated with C1-metabolism and CO₂ fixation via the Wood-Ljungdahl pathway (carbon monoxide dehydrogenase, formate dehydrogenase, [methyl-Co(III)] methanol-specific corrinoid protein]:coenzyme M methyltransferase, methylamine-corrinoid protein Co-methyltransferase and

Fig. 3. Phylogenetic tree based on the genome sequence comparison. Empty circles indicate a bootstrap support of >70 %, full circles >90% derived from 1000 samples. Scale bar indicates one nucleotide substitution per site. Gram-positive strains are marked in black, Gram-negative strains are marked in grey and uncultured clones in white. The alignment was calculated using gtdb-tk and the tree was created using IQ-Tree. The tree was visualized using iTOL version 6.

trimethylamine-corrinoid protein Co-methyltransferase). It is therefore surprising that strain DYL19^T apparently has no other growth mode available than anaerobic growth with phosphite as electron donor and CO₂ as electron acceptor.

The alignment of the genome tree was calculated using gtdb-tk, and the alignment of the 16S rRNA gene tree was calculated using the SINA Aligner. The genome tree is shown in Fig. 3. Average amino acid identity (AAI) values of strain DYL19^T with all strains used in the trees were pairwise calculated using the software toolkit CompareM and are listed in Table 1.

We propose that DYL19^T represents a novel bacterial genus and species within the family *Peptococcaceae*. For the closest described relative *Thermincola ferriacetica*, a 16S rRNA gene sequence identity of 91.7% was calculated. As the currently recommended thresholds for the classification of new species and genera by 16S rRNA gene similarity are 98.7% [38] and 94.5% [39] respectively, a classification as a novel genus is

indicated. Analysis of AAI of the genome of strain DYL19^T with other closely related strains confirms this classification as a new genus as the AAI threshold for the same genus is 65% [40] and the highest values calculated for the reference type strain observed in this study is 62.8%.

Strain DYL19^T differs from its closest relatives, *Thermincola ferriacetica* and *Thermoanaerosceptrum fracticalcis*, by being mesophilic. Moreover, neither one of these two species contains genes in its published genome that are indicative of dissimilatory oxidation of phosphite, which is the only substrate that our strain DYL19^T can use. Based on these results, we suggest assign strain DYL19^T to a novel genus and species, *Phosphitispora fastidiosa*.

Desulfotignum phosphitoxidans, so far the only described pure culture of a bacterium growing by dissimilatory phosphite oxidation, was isolated from marine sediment [8, 9]. The new dissimilatory phosphite oxidizer we present here, strain

Table 1. Pairwise amino acid identity (%) values of genomes used in this study compared to the genome of strain DYL19^T

Values were calculated using the software toolkit CompareM.

Genome	Accession no.	% AAI with DYL19 ^T
<i>Enterorhabdus mucosicola</i> DSM 19490 ^T	ASM42262v	46.7
<i>Desulfatibacillum alkenivorans</i> DSM 16219 ^T		46.6
<i>Desulfovibrio</i> sp. IOR2 ^T	ASM800123	45.8
<i>Desulfotomaculum profundum</i> strain Bs107 ^T	ASM260785	55.9
<i>Desulfotignum phosphitoxidans</i> DSM 13687 ^T		45.9
<i>Thermincola ferriacetica</i> strain Z-0001 ^T	ASM126341	62.9
<i>Geothermobacter ehrlichii</i> strain SS015 ^T	ASM812461	48.4
<i>Pelotomaculum schinkii</i> strain HH ^T	ASM436920	55.6
<i>Desulfotomaculum arcticum</i> DSM 17038 ^T		55.0
<i>Hydrogenispora ethanolica</i> strain LX-B ^T	ASM434068	50.8
<i>Desulfotomaculum aeronauticum</i> DSM 10349 ^T		55.3
<i>Streptomyces orinoci</i> strain NRRL B-3379 ^T	ASM312129	45.0
<i>Desulfotomaculum sulfoxidireducens</i> DSM 107105 ^T	ASM133764	45.9
Unclassified Deltaproteobacterium clone Phox-21 ^T		46.8
<i>Desulfotomaculum gibsoniae</i> DSM 7213 ^T	ASM23371v	55.3
<i>Desulfovibrio hydrothermalis</i> AM13 DSM 14728 ^T	ASM42524v	45.7
<i>Desulfohalovibrio reitneri</i> strain L21-Syr-AB ^T	ASM71129v	45.7
<i>Thermoanaerobacterium fracticalcis</i> strain DRI-13 ^T	ASM74602v	54.3
<i>Pelotomaculum thermopropionicum</i> DSM 13744 ^T	ASM1056v1	56.4
<i>Ruminiclostridium hungatei</i> DSM 14427 ^T	ASM205158	50.2

DYL19^T, is a Gram-positive spore-former and was isolated from a freshwater sewage treatment plant. Comparison of the 16S rRNA gene sequences (Fig. 2) indicates that strain DYL19^T has very little in common with the formerly described Gram-negative *D. phosphitoxidans* or with the phosphite-oxidizing enrichment culture Phox-21 described more recently [10]. Different from the metabolically versatile *D. phosphitoxidans*, strain DYL19^T has no sulphate reduction genes and oxidizes only phosphite to phosphate while reducing CO₂ to small amounts of acetate plus cell material. Obviously, the capacity for dissimilatory phosphite oxidation is found in different branches of the phylogenetic tree of bacteria, even though we do not yet understand the biochemical concept of this unusual type of energy metabolism.

DESCRIPTION OF *PHOSPHITISPORA* GEN. NOV.

Phosphitispora (Phos.phi.ti.spo'ra. N.L. neut. n. *phosphitum*, phosphite; Gr. fem. n. *spora*, seed or spore; N.L. fem. n. *Phosphitispora*, a phosphite-oxidizing spore-former).

Cells are Gram-positive, rod-shaped, strictly anaerobic and form thermoresistant spores. Growth is mesophilic; grows optimally in freshwater medium. A member of the family Peptococcaceae in the order Eubacteriales. The type species is *Phosphitispora fastidiosa* sp. nov.

DESCRIPTION OF *PHOSPHITISPORA FASTIDIOSA* SP. NOV.

Phosphitispora fastidiosa (fas.ti.di.o'sa. L. fem. adj. *fastidiosa*, fastidious, picky, referring to its very small substrate range).

Gram-positive, rod-shaped bacterium forming terminal to subterminal oval spores. Oxidizes phosphite to phosphate by reducing CO₂ to acetate and cell mass. No other organic or inorganic electron donors used; no reduction of nitrate, nitrite, sulphate, sulphite, thiosulphate, sulphur or iron (III) hydroxide. Growth occurs at 20–35 °C and pH 7.0–8.0. Optimal growth occurs in freshwater and brackish water media with 0.1–1.0 % (w/v) NaCl, at 30 °C, pH 7.2 and 0.1% (w/v) NaCl. Dimethylmenaquinone-7 is the only respiratory quinone. The predominant polar lipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. Major fatty acids are C_{14:0}, C_{16:1} ω9c, C_{18:1} ω9c and C_{18:1} ω11c DMA.

The type strain is DYL19^T (KCTC 25291^T=GDMCC 1.2680^T), which was isolated from anaerobic sewage sludge of a wastewater treatment plant in Constance, Germany. The DNA G+C content of the type strain is 45.95 mol%.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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