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# New Representatives of the Class *Ignavibacteria* Inhabiting Subsurface Aquifers of Yessentuki Mineral Water Deposit

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**Abstract:** The Yessentuki mineral water deposit (YMWD) is a well-known source of balneologically valuable drinking mineral water, but it has rarely been investigated in terms of the microbes inhabiting it. In this work, we have studied the microbial communities of the continuously operating production well 9, penetrating the Lower Cretaceous aquifer of the YMWD, and characterized, in detail, two novel representatives of class *Ignavibacteria* (*Bacteroidota*). One representative of the so-called XYB12-FULL-38-5 group within the family *Melioribacteraceae* has been isolated in pure culture, designated strain 09-Me, and physiologically characterized. It is a facultatively anaerobic thermotolerant microorganism capable of fermentation and respiration on simple and complex sugars (lichenan, xanthan gum, glucomannan, curdlan, pachyman). In addition to oxygen, ferric iron, arsenate, and elemental sulfur were also used as electron acceptors. Phylogenomic and physiological analyses reveal this novel isolate to represent a novel genus and species for which the name *Stygiobacter electus* gen. nov., sp. nov. is proposed. The second representative of the family *Melioribacteraceae* described here belonged to the so-called DSXH01 group, which comprises the dominant group (up to 28%) of the microbial community of well 9 water. The organism was characterized through the analysis of its genome, assembled from metagenome of well 9 (Ess09-04 MAG). Genes encoding enzymes of carbohydrate utilization and genes responsible for aerobic and anaerobic respiration have been identified in the genomes of both bacteria. The investigation of the environmental distribution of *Stygiobacter* genus-related bacteria and representatives of the lineage DSXH01 has shown that they all are typical inhabitants of the subsurface biosphere, and are often found in bioreactors. These data significantly expand our knowledge on the microbes of subsurface water basins and pave the way for future studies of the novel members of *Ignavibacteria* class.

**Keywords:** *Ignavibacteria*; *Stygiobacter*; thermotolerant; facultative anaerobe; Yessentuki mineral waters; subsurface aquifers



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## 1. Introduction

In 2010, the first cultivated representative of the deep phylogenetic branch known as ZB1, *Ignavibacterium album*, was described, and a new class *Ignavibacteria* was proposed within the phylum *Chlorobi* [1]. In 2013, a second representative, *Melioribacter roseus*, was isolated, and the entire class was ranked as the phylum *Ignavibacteriae*, which was renamed and validated as *Ignavibacteroidota* in 2021 [2,3]. However, according to the current GTDB classification, these bacteria still belong to the class *Ignavibacteria* (to the families *Ignavibacteraceae* and *Melioribacteraceae*, respectively) within the phylum *Bacteroidota* [4]. Until now, the class consisted of only two cultivated representatives, as mentioned above. Both members of the class are facultatively anaerobic chemoorganotrophic moderately thermophilic bacteria isolated from thermal environments and growing optimally at low

salinity. Cells of *I. album* and *M. roseus* are Gram-negative motile rods with polar flagella. The common features of the class members are the ability to grow with oxygen and arsenate as electron acceptors or by fermentation on carbohydrates. At the same time, they differ in optimal temperature and pH values for growth, salt tolerance, substrate preferences, and electron acceptors spectra. This is probably due to the different conditions at the isolation sites: a sulfide-rich hot spring in the case of *I. album* and a microbial mat under a flow of deep subsurface hydrocarbon-bearing water for *M. roseus*.

Despite the thermophilic lifestyle of both cultivated representatives, other members of *Ignavibacteria* have been detected in a variety of environments: thermal springs in Armenia [5], China [6], USA [7,8] and Russia [9], an anammox membrane bioreactor [10], microbial fuel cells [11], and an upflow anaerobic sludge blanket reactor [12]. Considering the outstanding ability of *M. roseus* to utilize different electron acceptors, it is interesting to note that members of *Ignavibacteria* are often detected in sites enriched with iron [13], nitrous [14,15] or sulfur compounds [12], and selenate [16]. In addition, more recent work has shown that the members of *Ignavibacteria* can play an important role in the degradation of a number of aromatic compounds, such as aniline [17], nitro- and aminophenol [18], or benzoate [19], suggesting their potential involvement in bioremediation. In addition to the ecotopes described above, environmental clones related to *Ignavibacteria* have been found in marine hydrotherms and coral tissues, wetlands, oil reservoirs, mines, and deep groundwater. Notably, the proportion of *Ignavibacteria* in all the discussed ecosystems varies widely, from minor representation [6,13,20] to dominance [8,9,14].

The Yessentuki mineral water deposit (YMWD), with an area of about 200 $km^2$, is a part of the Mineralovodskiy artesian basin. The history of the YMWD formation is connected with the Alpine cycle of geological development of the Caucasus region. The complex structural–tectonic and hydrodynamic conditions cause a violation of the normal hydrogeochemical zoning of this field, which is expressed in the presence of a gas–hydrogeochemical anomaly in the geological section of the YMWD. The Lower Cretaceous aquifer of the deposit, composed of terrigenous sediments represented mainly by sandstones, is generally characterized by high filtration properties, and is connected to the day surface in the elevated northern part of the folded structure of the Caucasus. It contains warm gas-free sulfate–hydrocarbonate calcium–sodium waters. The aquifers located above and below the Lower Cretaceous aquifer (the Upper Cretaceous and the Upper Jurassic) are composed mainly of limestones, characterized by low filtration properties of rocks, and contain balneologically valuable mineral carbonate sparkling thermal waters [21]. It has been reported that microorganisms can play an important role in the formation of the YMWD and other deposits of the Caucasian mineral waters region [22–25]. In their turn, the pronounced hydrochemical zonation and relative separation of the YMWD aquifers from each other are supposed to affect the physiological and phylogenetic diversity of their inhabitants.

The characterization of the phylogenetic diversity of the microbial communities inhabiting different YMWD aquifers was recently initiated [25]. The aim of the current work was to isolate and describe a member of the class *Ignavibacteria*, which constitutes a significant part of the microbial community of fresh carbonless waters extracted from the Lower Cretaceous aquifer of the YMWD through well 9.

## 2. Materials and Methods

### *2.1. Sampling and Enrichment*

Water samples were collected from the well 9 (44°2.30′ N 42°48.10′ E; Yessentuki, Pre-Caucasus, Russia; pH 7.9 and 21.9 °C) twice, in September and November 2020. Prior to the first sampling, the well operated at an average daily flow rate of 23.92 $m^3$/day for 204 days. Thereafter, the well was not operated for two months. Prior to the second sampling in November 2020, 3 wellbore volumes were removed from the well to minimize the impact of microbial processes occurring in the well in the absence of continuous water withdrawal. All the samples were transported to the laboratory in sterile 100 mL vials within 1 day.

To obtain enrichment culture, 20% (*v*/*v*) of water from well 9 was added to the 15 mL Hungate tubes filled with 5 mL of aerobic modified Pfennig medium ($NH_4Cl$—0.16 g $L^{-1}$, KCl—0.16 g $L^{-1}$, $MgCl_2 \cdot 6H_2O$—0.16 g $L^{-1}$, $CaCl_2 \cdot 2H_2O$—0.16 g $L^{-1}$, $KH_2PO_4$—0.16 g $L^{-1}$) supplemented with xanthan gum (0.5 g $L^{-1}$, Kelco, Inc., Woodstock, IL, USA), and the tubes were incubated at 25, 37 and 47 °C for 1 month. A serial 10-fold dilution technique was used to isolate pure cultures.

### *2.2. Phenotypic Characterization*

The cell morphology of strain 09-Me$^T$ was examined under a 1000× phase-contrast light microscope CX41RF (Olympus). Ultrathin sections of whole cells were visualized via transmission electron microscopy, as described by [26]. The effects of temperature and pH on growth were analyzed in the aforementioned mineral medium with yeast extract as a substrate. Growth was monitored in the temperature range of 14–60 °C. The following buffers (10 mM each) were used to determine the optimum pH and pH range: MES (5.2–6.1, Sigma-Aldrich, St. Louis, MO, USA), MOPS (6.5–7.4, Dia-M, Moscow, Russia), and Tricine (7.9–8.8, Sigma-Aldrich). The effect of the NaCl concentration was tested in the range of 0–3.5%. For studying anaerobic growth, the mineral medium was boiled and supplemented with $Na_2S \cdot 9H_2O$ (0.3 g $L^{-1}$). The headspace of the Hungate tubes was filled with 100% $N_2$. All growth experiments were performed in duplicate. The utilization of organic substrates by strain 09-Me$^T$ was tested in the aerobic medium under optimal growth conditions (47 °C, pH 7.0). The following substrates were tested as sole carbon and energy sources: xanthan gum, guar gum, locust bean gum, microcrystalline cellulose, carboxymethyl cellulose (CMC), filter paper, lichenan, xylan, dextrin, starch, xyloglucan, galactan, mannan, glucomannan, curdlan, pachyman. These substrates were added (4 g $L^{-1}$) directly to the mineral medium before autoclaving, while glucose, fructose, xylose, mannose, maltose, arabinose, galactose, cellobiose, sucrose, trehalose were added from filter-sterilized stock solutions after autoclaving to a final concentration of 2 g $L^{-1}$; yeast extract, beef extract, peptone, gelatin, alginate, amorphous chitin, colloidal chitin, mannitol, sorbitol were added to the concentration 1 g $L^{-1}$; formate, acetate, propionate, lactate, ethanol, propanol, butanol—20 mM. Elemental sulfur (1 g $L^{-1}$), thiosulfate, sulfate, nitrate (10 mM), sulfite, nitrite, arsenate (5 mM), antimonate (2 mM), ferric citrate (10 mM) or synthesized ferrihydrite (final content of Fe(III) 50 mM) were tested as possible electron acceptors for anaerobic respiration. Acetate (20 mM), starch, or yeast extract (1 g $L^{-1}$) were used as electron donors in all anaerobic respiration tests. Anaerobic medium without any external electron acceptors was used to verify the ability for fermentative growth. In all the substrate/acceptor experiments, the cultures were consequently transferred to the same medium at least three times to ensure that the inoculum contents were not used as the growth substrates.

For the analysis of cellular fatty acids and quinones, the cells of strain 09-Me$^T$ grown on liquid medium supplemented with starch and yeast extract were harvested in the late exponential growth phase. Cellular fatty acid profiles were determined using GC-MS (Thermo Scientific Trace GC Ultra DSQ II, HP-5MS column, EI70 eV) of methyl ester derivatives prepared from 5 mg of freeze-dried cell material treated with anhydrous HCl/MeOH, based on retention time (using Supelco standards), reference equivalent chain length values, and mass spectra (NIST MS Search 2.0 program, provided with the GC-MS setup) [27]. The cellular fatty acid contents were determined as percentages of the total ion current peak area. Isoprenoid quinones were determined as previously described [2].

### *2.3. 16S rRNA Gene Amplicon and Metagenome Library Preparation, Sequencing and Analysis*

Subsurface water was sampled directly from the wellhead using sterile connections. For 16S rRNA gene profiling, 100 L of water was passed through track membrane filters with 0.2 μm pore size. DNA was extracted directly from the filters using the FastDNA Spin Kit for Soil (MP Biomedicals, Irvine, CA, USA) according to the manufacturer's instructions. The V4 region of the 16S rRNA amplicon libraries' preparation, sequencing,

and analysis were performed as previously described [28]. The preparation and sequencing of a shotgun metagenome library were carried out by BioSpark Ltd., Moscow, Russia using a KAPA HyperPlus Library Preparation Kit (KAPA Biosystems, UK) according to the manufacturer's protocol and a NovaSeq 6000 system (Illumina, San Diego, CA, USA) with a reagent kit, which can read 100 nucleotides from each end. Raw reads were processed with Cutadapt [29]. Reads were processed in MetaWRAP [30] for assembly and binning. Bin completeness and contamination were evaluated using CheckM [31]. Taxonomies were assigned to each bin using GTDBtk [32]. All the sequencing data are deposited in NCBI BioProject PRJNA958107.

### *2.4. Genome Sequencing, Assembly and Phylogenetic Analysis*

Genomic DNA was isolated from the freshly grown cells of strain 09-MeT using a FastDNA™ SPIN Kit (MP Biomedicals, USA) according to the manufacturer's instructions. Shotgun WGS library preparation and sequencing were performed at BioSpark Ltd., Moscow, Russia using a KAPA HyperPlus Library Preparation Kit (KAPA Biosystems, London, UK) according to the manufacturer's protocol and a NovaSeq 6000 system (Illumina, San Diego, CA, USA) with a reagent kit, which is capable of reading 100 nucleotides from each end. Raw reads were processed with Trimmomatic [33] for adapter removal and quality filtering. The assembly of the reads was performed by Unicycler v0.4.8 [34]. This whole genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number JARGDL000000000. The version described in this paper is the JARGDL010000000 version.

A complete 16S rRNA gene and the set of 120 bacterial single-copy conservative marker genes (120bac; [35]) were used for phylogenetic analysis. Phylogenetic trees based on 16S rRNA gene and 120bac set were built using the IQ-TREE 2 program [36] with fast model selection via ModelFinder [37] and ultrafast approximation for phylogenetic bootstrap [38], as well as an approximate likelihood ratio test for branches [39].

### *2.5. Genome Annotation and Analysis*

Whole genome sequences were deposited in GenBank and annotated using the NCBI Prokaryotic Genome Annotation Pipeline 6.6 [40].

Carbohydrate-active enzymes (CAZymes) genes were searched in genomes of strain 09-Me$^T$ and its relatives (*Melioribacter roseus* P3M-2$^T$ and Ess09-04, the genome assembled from the metagenome (MAG) of the source water sample for the isolation of the 09-Me$^T$ strain) using dbCAN2 script v2.0.11 [41] and HMMER v3.3 [42], DIAMOND v2.0.6 [43] with default thresholds as detection tools. The most probable activities of the identified CAZymes (glycoside hydrolases, polysaccharide lyases and carbohydrate esterases) were predicted using a BLAST search against the Swiss-Prot database [44] by comparison with biochemically characterized enzymes.

The genes encoding enzymes involved in energy conservation pathways (fermentation, both aerobic and anaerobic respiration) were identified using a BLAST search with characterized enzyme sequences as queries and *in silico* translated aforementioned genomes as local protein databases (e-value $\leq 1 \times 10^{-5}$). The positive hits were checked using BLAST against the Swiss-Prot and PDB databases with special attention given to the BLAST hits with the "evidence at protein level". The genes determining Fe(III) respiration and Fe(II) oxidation were screened for using a previously described procedure and query sequences [45].

### *2.6. Environmental Distribution*

To estimate the abundance of members of the genus-level lineages represented by 09-MeT or Ess09-04 MAG in various ecotopes, 16S rRNA gene sequences from the SILVA database [46] that have a BLAST [47] score higher than 300 and identity higher than 97% with the defined sequences of these genus-level lineages were selected. Then, to avoid

multiple repetitions, sequences with a unique combination of "isolation source" entry and paper title were selected.

## 3. Results and Discussion

### *3.1. Microbial Community of YMWD Well 9*

The microbial community of water from YMWD well No. 9 was analyzed via 16S rRNA gene-based NGS profiling. As a result of reading of ~18,000 sequences, 53 amplicon sequence variants (ASVs) were identified. The community was characterized by relatively low diversity, with a Shannon index of 1.87 and a Simpson index of 0.28. Two phylotypes were the most abundant in the community: one phylotype, representing 43% of the total sequences, belonged to an uncultured microorganism of the *Thermodesulfovibrionia* class (phylum *Nitrospirota*), and the second phylotype, representing 29% of sequences, referred to an uncultured microorganism of the *Melioribacteraceae* family. In addition, there was a significant representation (8%) of "*Candidatus* Desulforudis" [48]. Given the limited number of studies on *Ignavibacteria* (only two described cultivated representatives in the class) and unique combination of metabolic characteristics (degradation of a wide range of biopolymers, reduction of various electron acceptors), we decided to investigate this particular community component in detail. Furthermore, the isolation of a dominant microorganism of a community would shed light on its functioning in the natural subsurface environment.

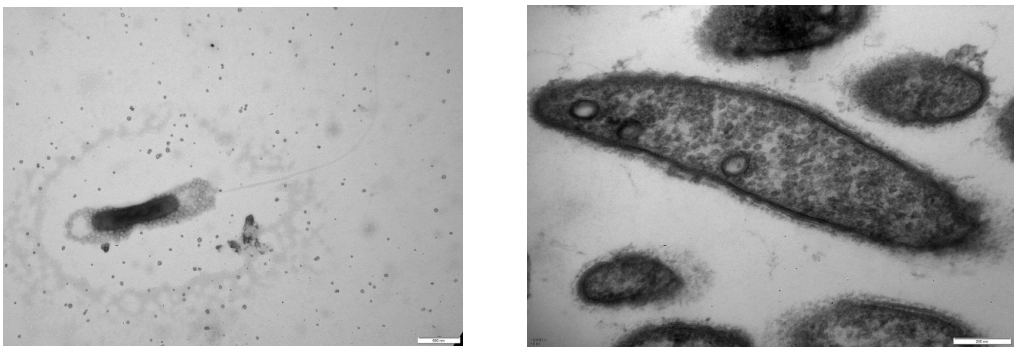
To reconstruct the complete genome of the representative of *Melioribacteraceae*, we performed metagenomic sequencing of the total DNA of the microbial community of well 9. By sequencing the total DNA of the microbial community concentrated on the filter in two replicates, we obtained 25.87 Gb of data. As a result of the assembling, we obtained 13,794 contigs with a total length of 80,772,497 base pairs (N50—18,864 bp; L50—567; largest contig—1,241,385 bp). Sixteen MAGs were recovered from the metagenome, with >85% completeness and <5% contamination. The statistics of the MAGs, including the number of contigs, genome size, the presence of the 16S rRNA gene, and relative abundance calculated based on coverage estimation, are shown in Table 1.

**Table 1.** Overview of all MAGs assembled from the well 9 water metagenome with >85% completeness and <5% contamination.

| Bin ID | Taxon (According to the GTDB) | Abundance, % | Completeness, % | Contamination, % | # Contigs | Genome Size, Mbp | 16S rRNA Gene, bp |
|---|---|---|---|---|---|---|---|
| Ess09-01 | "Edwardsbacteria" (g__UBA2226) | 0.87 | 100 | 1.098 | 24 | 2.62 | 1531 |
| Ess09-02 | *Desulfobacterota* (g__QYQD01) | 4.11 | 100 | 0.595 | 45 | 2.71 | 1220 |
| Ess09-03 | *Humidesulfovibrio* | 1.34 | 99.11 | 0 | 73 | 3.45 | - |
| Ess09-04 | *Melioribacteraceae* | 28.21 | 98.88 | 1.117 | 29 | 4.06 | 1540 |
| Ess09-05 | "Desulforudaceae" | 6.38 | 98.72 | 0.716 | 50 | 2.25 | 273 |
| Ess09-06 | *Desulfovibrionaceae* | 0.69 | 97.32 | 0.297 | 150 | 4.08 | 276 |
| Ess09-07 | *Brevundimonas* | 1.34 | 96.26 | 0.854 | 411 | 2.90 | 390 |
| Ess09-08 | p__CSP1-3 (f__CSP1-3) | 1.04 | 95.83 | 1.111 | 44 | 2.79 | 665 |
| Ess09-09 | *Elusimicrobiales* (g__UBA2231) | 2.51 | 95.5 | 1.123 | 98 | 2.75 | 940 |
| Ess09-10 | *Desulfomonilaceae* | 0.30 | 95.26 | 0.322 | 425 | 5.51 | - |
| Ess09-11 | *Thermodesulfovibrionales* (f__SM23-35) | 37.02 | 95 | 4.545 | 56 | 2.65 | - |
| Ess09-12 | "Eremiobacterota" | 5.03 | 94.87 | 1.282 | 69 | 7.43 | 905 |
| Ess09-13 | "Desulforudaceae" (g__SURF-60) | 0.92 | 91.1 | 3.105 | 246 | 2.00 | - |
| Ess09-14 | *Planctomycetota* | 1.05 | 88.84 | 1.239 | 736 | 4.07 | 929 |
| Ess09-15 | "Firmicutes_E" (f__UBA995) | 2.18 | 87.12 | 2.475 | 34 | 2.34 | - |

### *3.2. Phenotypic Properties of Strain 09-Me$^T$*

Cells of strain 09-Me$^T$ were straight or slightly curved rods, 0.6–3.5 μm in length and 0.25–0.35 μm in width. They were present singly, in pairs, rosettes, or aggregates, and formed biofilms; they were motile in the exponential growth phase by means of flagella (Figure 1a). Ultrathin sections of the cells revealed a Gram-negative cell-wall type (Figure 1b). The pellets of cells grown under aerobic or anaerobic conditions had a pinkish or white color, respectively. Under microaerobic conditions in agarose-solidified modified Pfennig medium, strain 09-Me$^T$ formed small (1–1.5 mm), circular, pinkish-orange, submerged colonies during two weeks of incubation at 47 °C.



(**a**) (**b**)

**Figure 1.** (**a**) Micrographs of cells of strain 09-Me$^T$ demonstrating a single cell with a polar flagellum. Bar, 0.5 μm. Magnification; 30,000; High Voltage: 80 kV; (**b**) transmission electron micrograph of a thin section of the cells, showing the Gram-negative structure of the cell wall. Bar, 0.2 μm. Magnification; 50,000; High Voltage: 80 kV.

Strain 09-Me$^T$ grew at 30–50 °C and pH 6.0–7.9, with the optimal growth at 47 °C and pH 7.0. Growth optimum was registered without NaCl in the medium. No growth was observed at temperatures ≤25 °C and ≥55 °C, at pH 5.6 and below and 8.0 and above, and at ≥1% of NaCl.

Strain 09-Me$^T$ was a facultative anaerobe. Growth on anaerobic medium containing sodium sulfide was stable, but the growth rate was significantly lower. The strain showed negative oxidase and catalase reactions.

The isolate did not require yeast extract for growth, but the addition of small amounts of yeast extract (0.02 g $L^{-1}$ under aerobic or 0.1 g $L^{-1}$ under anaerobic conditions) stimulated it. The following substrates were utilized: yeast extract, beef extract, peptone, gelatin, glucose, mannose, maltose, cellobiose, xylose, fructose, galactose, dextrin, starch, lichenan, xanthan gum, guar gum or locust bean gum, glucomannan, curdlan, and pachyman. Microcrystalline cellulose (Avicel) or carboxymethyl cellulose, filter paper, xylan, xyloglucan, galactan, mannan, alginate, amorphous chitin, colloidal chitin, arabinose, sucrose, trehalose, mannitol, sorbitol, formate, acetate, propionate, lactate, ethanol, propanol, and butanol were not utilized under either aerobic or anaerobic conditions without electron acceptors. Strain 09-Me$^T$ did not grow lithoautotrophically under a $H_2/CO_2$ atmosphere. Anaerobic respiration was observed on acetate, starch, or yeast extract using elemental sulfur, thiosulfate, arsenate, ferrihydrite, and Fe(III)-citrate. Sulfate, nitrate, and antimonate did not influence the growth, while nitrite and sulfite completely inhibited the growth. The major products of glucose fermentation were acetate and $H_2$, traces of propionate, isobutyrate, and isovalerate were also observed.

The major cellular fatty acids (>5%) of strain 09-Me$^T$ were $C_{15:0}$ (38.9%), iso-$C_{15:0}$ (33.5%), and $C_{16:0}$ (5.6%), while other straight-chain and branched saturated fatty acids were present in small amounts (Table S1, available in the online Supplementary Materials). The only isoprenoid quinone was identified as MK-7.

Thus, as a representative of the class *Ignavibacteria*, strain 09-Me$^T$ has several features common to its two members: a Gram-negative cell wall structure, pink motile rod-shaped

cells occurring singly, in pairs or aggregates, high versatility of energy production processes (aerobic and anaerobic respiration and fermentation), utilization of simple and complex sugars via fermentation or respiration with oxygen or arsenate, and MK-7 as the only detected quinone (Table 2). On the other hand, some features distinguish 09-Me$^T$ from the two other cultured members of *Ignavibacteria*: it is capable of growing without yeast extract as a source of growth factors, respiring with elemental sulfur, and growing on galactose. In addition, colony formation; a lower temperature and pH optima; intolerance to high NaCl concentrations; negative catalase and oxidase reactions; an inability to utilize arabinose, trehalose, microcrystalline and carboxymethyl cellulose, alginate, xyloglucan, galactan, mannan, and xylan; and to reduce nitrate or nitrite make the novel isolate different from its closest cultivated relative, *M. roseus*. Finally, the presence of $C_{15:0}$, and absence of anteiso-$C_{15:0}$ as the major fatty acids also distinguish strain 09-Me$^T$ from *M. roseus*.

**Table 2.** Comparison of the novel genus *Stygiobacter* and other representatives of class *Ignavibacteria*. 1—strain 09-Me$^T$ (this work); 2—*Melioribacter roseus* strain P3M-2$^T$ [2]; 3—*Ignavibacterium album* strain Mat9-16$^T$ [1]. All three strains are Gram-negative, flagellated rods that are pink in aerobic and white in anaerobic conditions and characterized by a facultatively anaerobic lifestyle; the ability to grow on glucose, cellobiose, maltose, mannose, fructose, yeast extract, and beef extract, and to reduce oxygen and arsenate; an inability to utilize sucrose, filter paper, mannitol, sorbitol, lactate, propionate, ethanol, propanol, and butanol as a substrate and sulfite as an electron acceptor; and the presence of MK-7 as a sole quinone. ND, no data available; +, positive growth; -, no growth.

| **Characteristic** | **1** | **2** | **3** |
|---|---|---|---|
| Origin | Subsurface water from a well at Yessentuki, Pre-Caucasus, Russia | Microbial mat at deep subsurface water outflow, Tomsk region, Russia | Hydrothermal spring, Yumata, Nagano, Japan |
| Rosette and biofilm formation | + | + | - |
| Colony formation | + | - | + |
| min/opt/max T, °C | 30/47/50 | 35/52–55/60 | 30/45/55 |
| min/opt/max pH | 6.0/7.0/7.9 | 6.0/7.5/8.7 | 6.5/7.0–7.5/8.0 |
| min/opt/max NaCl (%) | 0/0–0.25/0.5 | 0/0.6/6 | 0/1.0/3.0 |
| Catalase reaction | - | + | - (+) * |
| Oxidase reaction | - | + | - |
| YE-requirement | not required | 0.05 g/L minimal | 0.1 g/L minimal |
| Substrates: | | | |
| Xylose | + | + | - |
| Galactose | + | - | - |
| Trehalose | - | + | - |
| Arabinose | - | + | - |
| Xylan | - | + | ND |
| Alginate | - | + ** | ND |
| Galactan | - | + ** | ND |
| Xyloglucan | - | + ** | ND |
| Mannan | - | + ** | ND |
| MCC | - | + | - |
| CMC | - | + | - |
| Acetate | + | + | - |
| Reduction of: | | | |
| Elemental sulfur | + | - | - |
| Thiosulphate | + | + | - |
| Sulphite | - (inhibition) | - (inhibition) | - |
| Nitrate | - | + | - |
| Nitrite | - (inhibition) | + | - |
| Arsenate | + | + | - (+) * |
| Fe (III) | + | + | - |
| Fe-citrate | + | + ** | - |
| Major fatty acids | $C_{15:0}$, iso-$C_{15:0}$, $C_{16:0}$ | iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{16:0}$ | iso-$C_{15:0}$, anteiso-$C_{15:0}$; iso-$C_{17:0}$, $C_{16:0}$ |
| G + C% | 29.9 | 39.3 | 33.5 |
| Genome size, Mb | 3.18 | 3.34 | 3.66 |

Note: * Data from [2]; ** Data from this study.

### 3.3. Phylogenetic Analysis

To determine the phylogenetic position of the strain 09-Me$^T$ and MAG Ess09-04, we performed a reconstruction based on the concatenated amino acid sequences of 120 bacterial single-copy conservative marker genes (Figure 2a). According to the results obtained, strain 09-Me$^T$ and MAG Ess09-04 represent two separate genera within the family *Melioribacteraceae*. These results are in good agreement with the results of the phylogenetic analysis based on the 16S rRNA gene (Figure 2b). Strain 09-Me$^T$ and MAG Ess09-04 are part of genus-level lineages named XYB12-FULL-38-5 and DSXH01, respectively, in the GTDB. In addition to strain 09-Me$^T$, the genus XYB12-FULL-38-5 includes two other species represented by high-quality MAGs, both of which were assembled from the DNA of subsurface water microbial communities [49,50]. The AAI values between strain 09-Me$^T$ and both these MAGs are close to 70%. MAG Ess09-04 also has two related high-quality MAGs belonging to the same genus (DSXH01). The AAI values between Ess09-04 and both these MAGs are close to 71%. In general, the AAI values between the representative genomes of the genus-level phylogenetic lineages delineated in the GTDB 207 within the *Melioribacteraceae* family ranged from 59 to 67% with a median value of 61%. The AAI value between strain 09-Me$^T$ and *Melioribacter roseus* P3M-2$^T$ is 65.4%.

Taken together, the data of our phylogenomic analysis and phenotypic characteristics indicate that strain 09-Me$^T$ represents a novel genus and species within *Melioribacteraceae*, for which we propose the name *Stygiobacter electus* gen. nov., sp. nov.

### 3.4. Genome Analysis

The general genome properties of *Stygiobacter electus* 09-Me$^T$ and MAG Ess09-04 are summarized in Table 3.

**Table 3.** General genome properties of *Stygiobacter electus* 09-Me$^T$ and MAG Ess09-04.

| | ***Stygiobacter electus* 09-Me$^T$** | **Ess09-04** |
|---|---|---|
| Total length, bp | 3,183,904 | 4,057,892 |
| number of contigs | 60 | 29 |
| GC, % | 29.9 | 35.8 |
| N50, bp | 116,791 | 401,998 |
| Completeness *, % | 100 | 98.88 |
| Contamination *, % | 0.56 | 1.117 |
| Genes (total) | 2872 | 3600 |
| Genes (protein coding) | 2814 | 3536 |
| Genes (RNA) | 49 | 57 |
| Pseudo genes (total) | 9 | 7 |
| Complete rRNAs | 1, 1, 1 (5S, 16S, 23S) | 1, 1, 1 (5S, 16S, 23S) |

Note: * Determined by using CheckM [31].

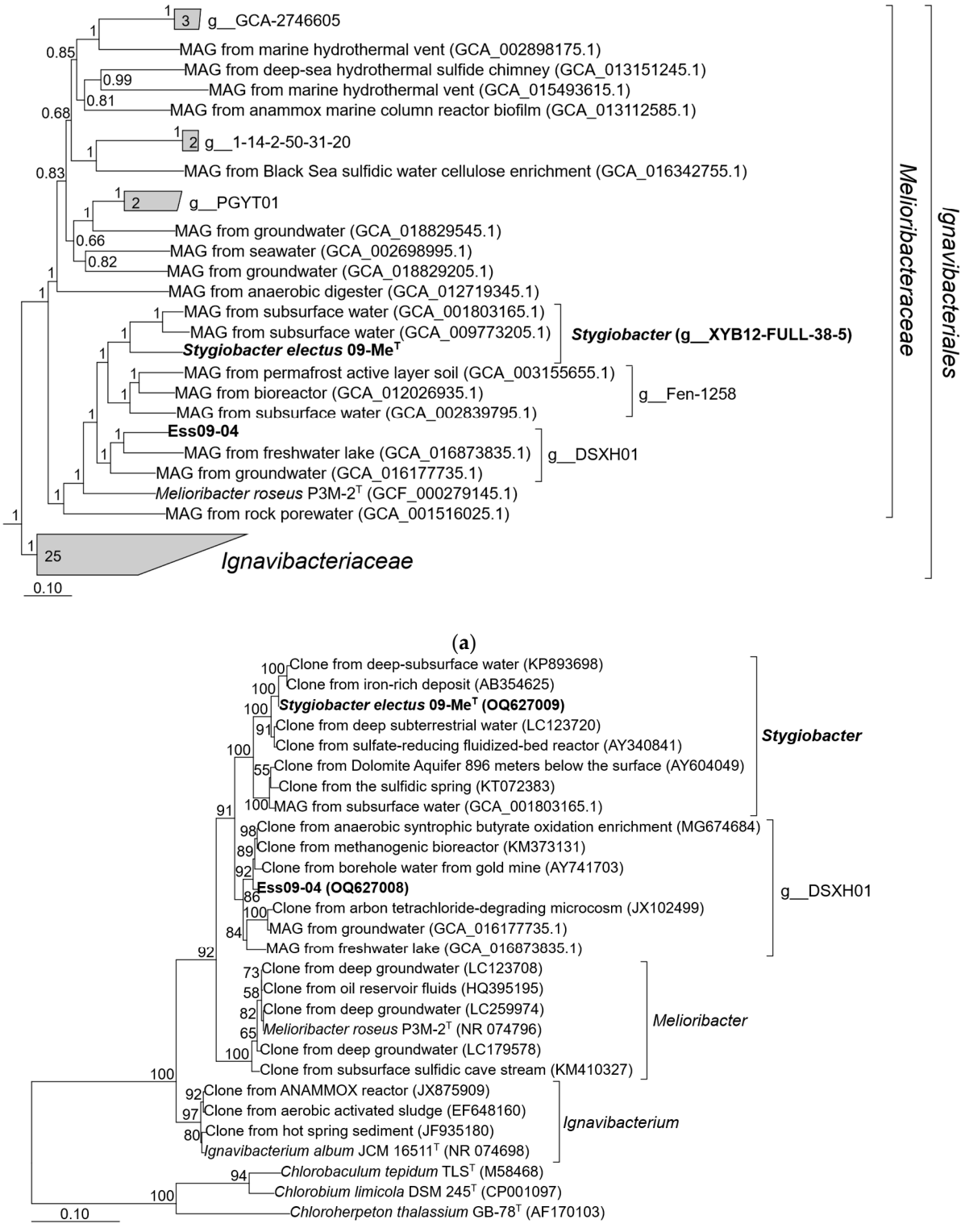



**Figure 2.** Phylogenomic placement of 09-Me$^T$ and MAG Ess09-04 based on (**a**) concatenated partial amino acid sequences of 120 bacterial single-copy conserved marker genes with taxonomic designations according to the GTDB [32]; (**b**) on 16S rRNA gene sequences. The trees were built using the IQ-TREE 2 program [36] with fast model selection via ModelFinder [37] and ultrafast bootstrap approximation [51], as well as an approximate likelihood-ratio test for branches [39]. A bootstrap consensus tree is shown with values placed at the nodes. Bar, 0.1 changes per position.

The first cultivated representative of the *Melioribacteraceae* family, *Melioribacter roseus*, utilizes a wide spectrum of polysaccharides [2] and possesses a large number of glycosidases encoded in its genome [52]. However, the novel strain 09-Me$^T$, which belongs to *Melioribacteraceae*, shows weaker hydrolytic capabilities. The comparison of the CAZymes' repertoires allowed to decipher the differences in the substrates used for growth (Table S2, Figure 3b). Alpha-glucans such as starch and dextrin are hydrolyzed by three alpha-amylases, three neopullulanases and pullulanase of the GH13 family, and alpha-glucosidase of the GH31 family (Figure 3a). Strain 09-Me$^T$ utilizes lichenan through the action of four beta-glucosidases (GH3) and three beta-glucanases (GH16). Depolymerization of curdlan and pachyman can occur under the action of the same enzymes together with a beta-1,3-glucanase (GH148). Locust bean gum and guar gum, both chemically galactomannans, are degraded under the action of endo-1,4-beta-mannosidase of the GH5 family, hydrolyzing the mannan backbone and alpha-galactosidase of GH36, which is active against side chains. On the other hand, these endo-1,4-beta-mannosidases may also hydrolyze glucomannan, but not mannan. Depolymerization of xanthan gum in strain 09-Me$^T$ probably occurs under the action of two endoglucanases (GH5 and GH9 families), three alpha-mannosidases (GH92 and GH125 families), and three beta-glucuronidases (GH2); however, beta-mannosidases or xanthan lyases genes are absent. Despite an inability to grow on chitin, a putative endochitinase from the GH18 family and four beta-hexosaminidases (GH3 and GH20 families) are encoded in the strain 09-Me$^T$ genome. Putative xyloglucanase of the GH74 family has unknown function because strain 09-Me$^T$ does not grow on xyloglucan.

At the same time, the *M. roseus* P3M-2$^T$ genome contains genes encoding endoxylanases (GH10 and GH30 families) and cellobiose phosphorylase (GH94), as well as much more endoglucanases (GH5 and GH9 families) and alginate lyases (PL6, PL17, PL38 families), which provide the ability to utilize xylan, cellulose, and alginate, respectively. Moreover, the genes responsible for utilization of pectin (GH28, PL1, PL9, PL10, CE8), rhamnogalacturonan (GH105, PL11, PL26, PL42), mannan (GH2, GH5. GH26, GH130), galactan (GH2, GH35, GH50, GH53, GH147), lichenan and curdlan (GH3, GH16, GH55, GH148) as well as alpha-glucans (GH13, GH31, GH77, GH97) were found. The growth of *M. roseus* P3M-2$^T$ on alginate, galactan, and mannan was experimentally confirmed in this work (Table 2). Strains 09-Me$^T$ and Ess09-04 MAG have similar CAZymes sets, but differ in their details: in Ess09-04 MAG, additional endo-beta-mannosidase (GH113), beta-1,4-mannooligosaccharide phosphorylase (GH130), pectate lyases (PL9, PL10), ulvan lyase (PL37) and a number of putative beta-galactosidases (GH165) are encoded.

Under aerobic conditions, strain 09-Me$^T$ can respire using oxygen as the electron acceptor (Table S3). The electron transfer chain (ETC) of strain 09-Me$^T$ includes NADH-quinone oxidoreductase, succinate dehydrogenase, alternative complex III, *bd*-type quinol oxidase, and two types of cytochrome oxidases (*caa3*- and *cbb3*-type). In anaerobic conditions, either fermentation or anaerobic respiration occurs (Table S3). During fermentation, acetate can be produced under the action of a phosphate acetyltransferase and an acetate kinase. Two [FeFe]-hydrogenases and one [NiFe]-hydrogenase are responsible for hydrogen generation. Despite the presence of acetaldehyde dehydrogenase and alcohol dehydrogenase genes, strain 09-Me$^T$ does not produce ethanol. There is complete pathway for butyrate formation including acetyl-CoA acetyltransferase, 3-hydroxybutyryl-CoA dehydrogenase, short-chain-enoyl-CoA hydratase, electron transfer flavoprotein and butyryl-CoA:acetate CoA-transferase. Different enzyme complexes provide the capability for anaerobic respiration with several external electron acceptors. There are several enzymes involved in the nitrogen cycle: a two-subunit NrfAH ammonifying nitrite reductase; an octaheme hydroxylamine oxidoreductase, which shares homology with an a εHao group protein of *Caldithrix abyssi*; and a nitrous oxide reductase NosZ (Table S3). The εHao protein is thought to function as an alternative ammonifying nitrite reductase in *C. abyssi* [53]. This set of enzymes would allow strain 09-Me$^T$ to perform nitrite respiration and to gain energy from the reduction of nitrous oxide to $N_2$. However, the growth inhibition by nitrite casts doubts on the functionality of the identified proteins in this organism. The Ess09-04 MAG

also possesses the determinants of nitrite and NO reduction, but it encodes a NorB nitric oxide reductase instead of NosZ, encoded in the genomes of strain 09-Me$^{T}$ and *M. roseus*, and lacks any strong homologs of the NrfH subunit of nitrate reductase, suggesting an alternative protein, such as εHao, is involved in nitrite respiration.

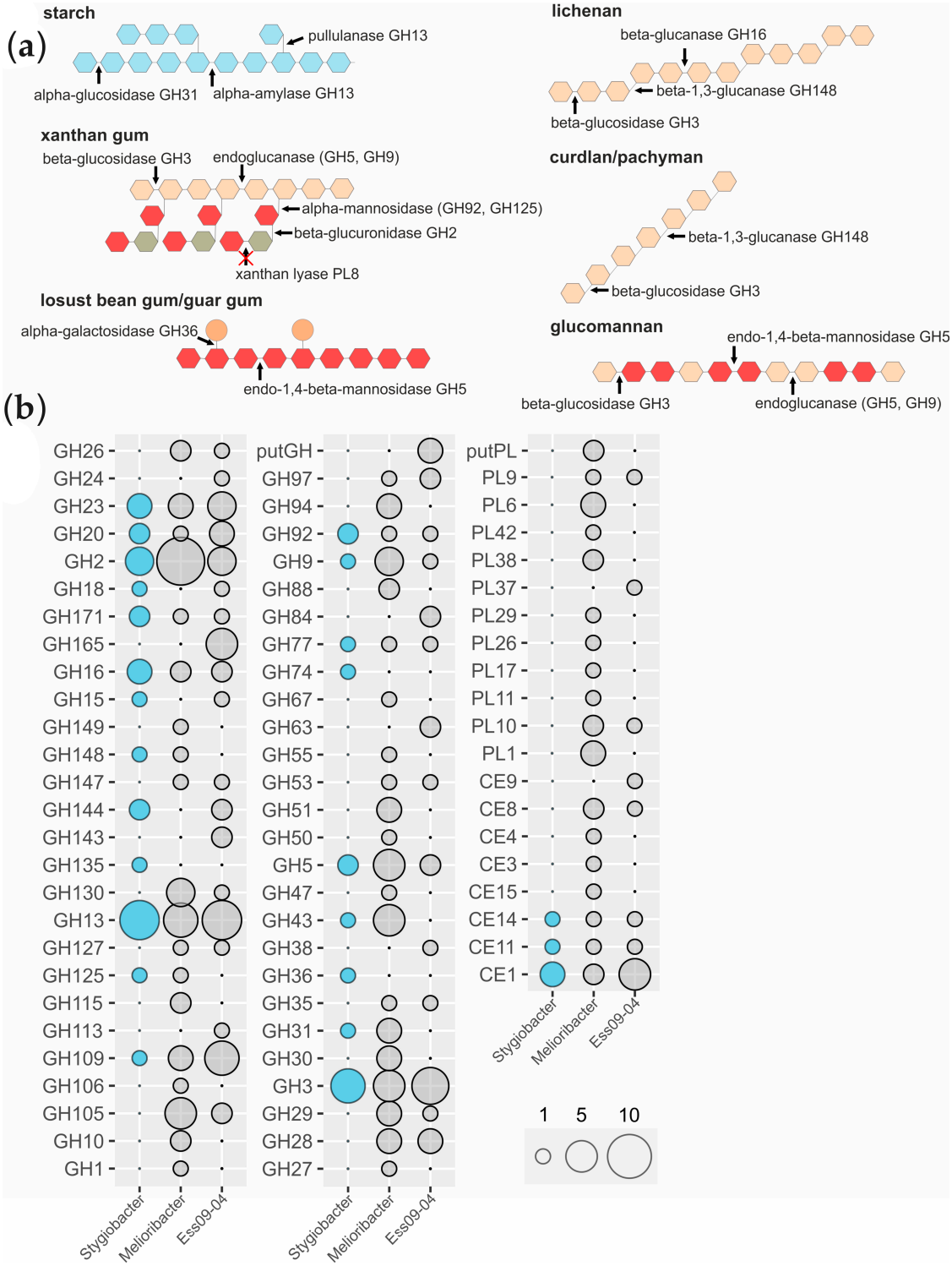



**Figure 3.** (**a**) Carbohydrate decomposition pathways of strain 09-Me$^{T}$: blue/beige hexagons indicate alpha-linked/beta-linked D-glucose, respectively, red hexagons—D-mannose, dark green hexagons—D-glucuronic acid, orange circles—D-galactose; (**b**) Comparison of CAZymes' sets of strain 09-Me$^{T}$, *Melioribacter roseus* P3M-2$^{T}$ and Ess09-04 MAG.

Genome analysis of strain 09-Me$^T$ revealed 12 genes encoding putative determinants of extracellular electron transfer (EET), which possess 5–17 heme *c*-binding motifs within one or more conserved domains of multiheme cytochrome families. The encoded proteins share homology with several cytochromes involved in the reduction of insoluble Fe(III) compounds or in direct interspecies electron transfer (DIET). In total, strain 09-Me$^T$ possesses a complete set of cytochromes required for electron transfer to extracellular insoluble acceptors. These are quinone-reducing cytochromes, periplasmic electron shuttles, and putative cell surface-associated multiheme cytochromes responsible for the interaction with an extracellular electron acceptor. Interestingly, the putative periplasmic electron shuttling cytochrome of strain 09-Me$^T$ shares the highest homology with *M. roseus* cytochrome Mros_1570, which has been proposed to provide for the electron transfer from cytoplasmic redox systems to iron-reducing multiheme *c*-type cytochromes associated with the outer membrane in this organism [2]. This protein in strain 09-Me$^T$ is encoded in the cluster with four other multihemes, including quinol-reducing and surfaceous cytochromes, thus indicating the presence of a membrane-associated complex for EET analogous to that proposed in *M. roseus* and identified in *Geobacteriaceae* representatives [54]. A peculiar feature of this cytochrome gene cluster in 09-Me$^T$ is that the most likely surfaceous terminal oxidoreductase encoded in it shares considerable homology with an external cytochrome C4B56_01400 from an archaeon of the *Methanophagales* ANME-1 group, which is upregulated under enhanced DIET conditions [55]. The strain 09-Me$^T$ also possesses alternative determinants of EET, such as complex iron sulfur molybdopterin (CISM) complexes homologous to the archaeal ones from *Pyrodictium delaneyi* [56].

The Ess09-04 MAG encodes two loci of the homologs of EET-related surfaceous cytochromes similar to those described in *M. roseus* [2]. A strong homolog of the putative periplasmic electron-shuttling multiheme Mros_1570 is also encoded in this genome.

The ability of strain 09-Me$^T$ to use sulfur, thiosulfate, or arsenate as the electron acceptors is most probably provided by the CISM enzyme complexes encoded in its genome. Gene clusters of the same complexes were also identified in the Ess09-04 MAG. Notably, the Ess09-04 MAG also contains the homologs of the genes *sseA*, *glpE*, *tsdA*. These genes together with the genes of a PhsAB CISM complex determine the ability for thiosulfate disproportionation to thiocyanate and sulfite [57].

In general, *M. roseus*, 09-Me$^T$, and Ess09-04 MAG encode similar complexes of the ETC, aerobic respiratory system, as well as similar sets of enzymes responsible for nitrogen, iron, and sulfur respiration. However, the putative nitric oxide reductase NorB is encoded in Ess09-04 MAG instead of NosZ encoded in the genomes of strain 09-Me$^T$ and *M. roseus*. There are also several differences in their fermentation pathways: *M. roseus* and Ess09-04 lack [NiFe]-hydrogenase, but possess lactate dehydrogenase, which can convert pyruvate to lactate. The alcohol dehydrognase gene is absent in Ess09-04 MAG.

### *3.5. Ecology*

To estimate the abundance of representatives of the genera *Stygiobacter* and DSXH01 of the family *Melioribacteraceae* from the information available in public databases, we selected 31 and 22 unique representative 16S rRNA gene sequences, respectively (see Methods). The environmental distribution of the representatives of these two genera looks very similar. In both cases, the majority of 16S rRNA gene sequences were detected either in the ecotopes associated with the subsurface biosphere (mostly subsurface waters) or in various bioreactors. In fact, 52% of *Stygiobacter*-related sequences were recovered from the subsurface, and 32% from bioreactors. For the DSXH01 group, these proportions were 50 and 41%, respectively. Other ecotopes, such as soil, sediments, or freshwater, were only singularly encountered.

The analysis of the microbial community of the Lower Cretaceous aquifer penetrated by the well 9 of the YMWD showed the high stability of its phylogenetic composition over time. However, the representation of Ess09-04 MAG and of a phylotype belonging to the uncultured *Desulfobacterota* showed significant dependence on the operating modes

of the well 9 (Figure 4). Prior to sampling in September 2020, the well underwent a reassessment of groundwater resources, as a result of which water was pumped from the well at maximum load for several months (see the Methods section). In September 2020, the YMWD groundwater resource reassessment was completed, and operation of well 9 was discontinued. The representation of Ess09-04 belonging to DSXH01 was 4% of the community in September 2020, but it increased to 26–28% in November of the same year. Therefore, the abundance of this group depends on physicochemical conditions of the environment, which are influenced by the operation mode of the production well. In this particular case, water stagnation seems to be the most important factor facilitating the increase of Ess09-04 abundance. According to the prediction of optimal growth temperature based on the amino acid composition of the proteins encoded in this MAG [58], this organism is a mesophile. The thermophilic lifestyle of *Stygiobacter* representatives and their association with deeper and warmer aquifers correlate with their detection in the Upper Cretaceous aquifer of the YMWD penetrated by the well 71 (our data), wherein they make up 0.6% of the community. This section of the YMWD contains gaseous carbonaceous thermal and highly mineralized waters [21]. Well 71 is located about 20 km southeast of well 9. Considering that the YMWD is characterized by a highly complex geological structure with a hydrogeological anomaly in this section, the warm, oxygenated freshwater of the Lower Cretaceous aquifer is surrounded by thermal, mineral, carbonaceous, gaseous waters of the Upper Cretaceous and Upper Jurassic aquifers. Natural discharge of YMWD groundwater occurs through numerous vertical faults that locally connect the stratified aquifers [21]. Thus, the introduction of a thermophilic bacterium into the mesophilic microbial community of the Lower Cretaceous aquifer from the overlying Upper Cretaceous one is not surprising. In fact, the lack of wells, penetrating the Upper Cretaceous aquifer in close proximity to well 9 does not allow us to assert high representation of *Stygiobacter* in the overlying section of this particular spot of the YMWD. In any case, the detection of *Stygiobacter* representatives in well 71 allows us to argue that this genus is authentic for the YMWD.

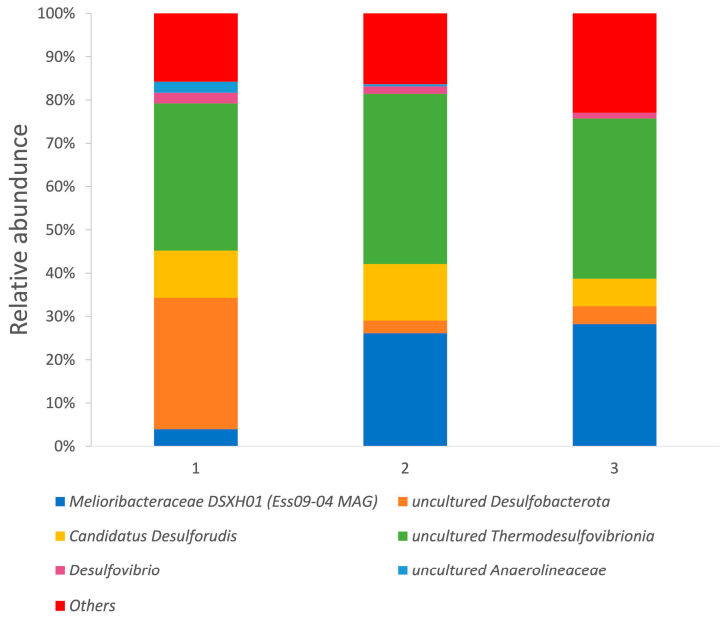



**Figure 4.** The relative abundance of prokaryotes at the genus level in water samples collected from well 9 of the YMWD. (1): Based on 16S rRNA gene profiling of the sample collected in September 2020 at maximal water flow through the well; (2): based on 16S rRNA gene profiling of the sample collected in November 2020, after 2 months of water stagnation in the well; (3): based on the relative abundance of taxa according to metagenomic analysis of the sample collected in November 2020, using the same sample as (2).

## 4. Conclusions

*Ignavibacteria* is a class of *Bacteroidota* whose representatives have been detected in a wide range of habitats, often associated with the water of the thermal or subterranean ecotopes, where they can play the role of organic-matter-degrading bacteria via fermentation or respiratory processes. Unfortunately, this class is rather poorly studied, because only two of its representatives have been obtained in pure cultures so far. In this work, we have described the representatives of two novel genera of this class, both belonging to the family *Melioribacteraceae*. One of these genera, *Stygiobacter* has been described in a pure culture study, while the second genus, DSXH01, has been studied within a genome analysis of Ess09-04 MAG, which represents the dominant microorganism in a community of waters of the Lower Cretaceous aquifer of the YMWD.

The newly isolated microorganism of the class *Ignavibacteria*, *Stygiobacter electus* strain 09-Me$^T$, is a facultatively anaerobic thermotolerant bacterium capable of utilizing a wide range of mono-, di- and polysaccharides and electron acceptors such as sulfur, thiosulfate, arsenate, ferric citrate, and ferrihydrite. The ability of 09-Me$^T$ to utilize a wide range of saccharides is also confirmed by genomic analysis. In this respect, the genomes of the two microorganisms studied were quite similar, and differed only in details. Interestingly, both microorganisms share a number of genetic determinants for EET. 09-Me$^T$ and Ess09-04 MAG encode similar gene complexes of the ETC, aerobic respiration, as well as similar sets of enzymes responsible for iron, sulfur, thiosulfate, and arsenate respiration. The environmental distribution of the representatives of these two genera is also very similar. The majority of related 16S rRNA gene sequences were detected in subsurface waters. Detailed analysis of the physiology, phylogeny and ecology of two new representatives of the family *Melioribacteraceae* allows for a more accurate interpretation of the role of these microorganisms in microbial communities, and opens up opportunities for the cultivation and characterization of new representatives of this group.

### 4.1. Descriptions of *Stygiobacter* gen. nov.

*Stygiobacter* (Sty.gi.o.bac'.ter. L. masc. adj. *stygius*, of the Styx, Stygian, of lower world; L. masc. n. *bacter,* rod; N.L. masc. n. *Stygiobacter,* subterranean rod).

A facultative anaerobic, thermotolerant, neutrophilic bacterium. Rods are motile. Gram-negative. Chemoorganoheterotrophic metabolism. The major fatty acids are $C_{15:0}$, iso-$C_{15:0}$ and $C_{16:0}$. The respiratory system possesses quinones (MK-7). The type species is *Stygiobacter electus*.

### 4.2. Descriptions of *Stygiobacter electus* sp. nov.

*Stygiobacter electus* (e.lec'tus. L. masc. part. adj. *electus*, chosen).

Rods are 0.6–3.5 μm in length and 0.25–0.35 μm in width. Gram-negative and motile. Pink-pigmented cells occur singly, in pairs, rosettes, aggregates, and biofilm. Small pinkish colonies form submerged in microaerobic conditions. Facultative anaerobe. Thermotolerant with a growth temperature range of 30–50 °C and an optimum at 47 °C. The pH range for growth is 6.0–7.9, with growth being optimum at 7.0. Sodium chloride and yeast extract are not required for growth, but addition of 0.02–0.1 g/L of yeast extract stimulates it. Growth occurs with yeast extract, beef extract, peptone, gelatin, glucose, mannose, maltose, cellobiose, xylose, fructose, galactose, dextrin, starch, lichenan, xanthan gum, guar gum or locust bean gum, glucomannan, curdlan, and pachyman. No growth is observed on microcrystalline cellulose (Avicel) or carboxymethyl cellulose, filter paper, xylan, xyloglucan, mannan, galactan, alginate, arabinose, sucrose, trehalose, mannitol, sorbitol, formate, acetate, propionate, lactate, ethanol, propanol, or butanol. Sulfur, thiosulfate, arsenate, Fe-citrate, or ferrihydrite are used as electron acceptors. Sulfate, nitrate, and antimonate are not utilized as the electron acceptor; sulfite and nitrite inhibit growth. Acetate and $H_2$ are the main products of glucose fermentation. The major fatty acids are $C_{15:0}$, iso-$C_{15:0}$ and $C_{16:0}$. MK-7 is the only measured quinone.

The type strain is 09-Me$^T$ (KCTC 92116$^T$ = JCM 39242$^T$ = VKM B-3554$^T$ = UQM 41464$^T$), isolated from a deep subsurface water deposit (Yessentuki, Pre-Caucasus, Russia). The genome size of the type strain is 3.18 Mb, DNA G + C content is 29.9% (genome sequence).

EMBL/GenBank accession (16S rRNA gene): OQ627009.

EMBL/GenBank accession (genome assembly): GCA_029210465.1.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15193451/s1, Table S1: Cellular fatty acids of strain 09-Me$^T$; Table S2: CAZymes genes identified in genome of strain 09-Me and its relatives; Table S3: Enzymes involved in fermentation and respiration pathways.

**Author Contributions:** O.A.P.: conceptualization, methodology, investigation, validation, writing—original draft; A.G.E.: methodology, investigation, software, visualization, writing—review and editing; S.N.G.: methodology, investigation, software, writing—review and editing, project administration, funding acquisition; N.F.P.: investigation, review; A.A.K.: methodology, investigation, formal analysis, visualization, review; D.G.Z.: conceptualization, supervision, data curation, writing—original draft; A.Y.M.: conceptualization, methodology, investigation, software, visualization, writing—original draft. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**Conflicts of Interest:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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