ISSN 0026-2617, Microbiology, 2020, Vol. 89, No. 3, pp. 328–336. © Pleiades Publishing, Ltd., 2020.
Russian Text © The Author(s), 2020, published in Mikrobiologiya, 2020, Vol. 89, No. 3, pp. 334–343.

EXPERIMENTAL ARTICLES

Genome Analysis of a Member of the Uncultured Phylum *Riflebacteria* Revealed Pathways of Organotrophic Metabolism and Dissimilatory Iron Reduction

V. V. Kadnikov[a], A. V. Mardanov[a], A. V. Beletsky[a], O. V. Karnachuk[b], and N. V. Ravin[a, *]

[a] *Institute of Bioengineering, Research Center of Biotechnology, Russian Academy of Sciences, Moscow, 119071 Russia*
[b] *Laboratory of Biochemistry and Molecular Biology, Tomsk State University, Tomsk, 634050 Russia*
**e-mail: nravin@biengi.ac.ru*

Received December 2, 2019; revised December 19, 2019; accepted December 20, 2019

Abstract—The candidate phylum *Riflebacteria* was described based on analysis of genomes assembled from the metagenomes of various anaerobic ecosystems; however, to date, no member of *Riflebacteria* has been isolated in a pure culture. In this work, the genome of a new member of *Riflebacteria* was obtained from the metagenome of the microbial community of a deep subsurface thermal aquifer in Western Siberia. Phylogenetic analysis indicates that this bacterium, designated Ch46, represents a new genus of the phylum *Riflebacteria*, distinct from the previously described *Candidatus* Ozemobacter. The genome of Ch46 was found to encode putatively secreted alpha-amylases and various sugar and peptide transporters. Reconstruction of central metabolic pathways suggests that Ch46 is an anaerobic organotroph capable of fermenting sugars and proteinaceous substrates. The bacterium lacks the genes of the known pathways of autotrophic carbon fixation, as well as the aerobic respiratory chain. The transmembrane ion gradient can be generated by an Rnf complex, as well as by a sodium-transporting NADH–quinone oxidoreductase. The presence of multiheme *c*-type cytochromes indicates the possibility of dissimilatory Fe(III) reduction. The pathways of dissimilatory reduction of sulfate, nitrate, nitrite, arsenate, and sulfur were not detected. In the subsurface aquifer, *Riflebacteria* probably utilize starch and similar polysaccharides, as well as low-molecular-weight organic compounds. Based on the results of phylogenetic and genomic analysis, it is proposed to classify the novel bacterium as '*Candidatus* Rifleibacterium amylolyticum.'

Keywords: uncultured microorganisms, *Riflebacteria*, subsurface biosphere, metagenome

DOI: 10.1134/S0026261720030078

The candidate phylum *Riflebacteria* was first described based on the results of phylogenetic analysis of a genome assembled from the metagenome of a 4- to 6 m-deep aquifer adjacent to the Colorado River, near Rifle, United States (Anantharaman et al., 2016). Altogether, this study described 2540 genomes that represented 47 new phylogenetic lineages of the phylum level; one of them, *Candidatus* Riflebacteria bacterium GWC2_50_8, was described as a representative of a novel phylum *Riflebacteria* (Anantharaman et al., 2016). The genomes of two further members of *Riflebacteria*, HGW-Riflebacteria-1 and HGW-Riflebacteria-2, were obtained from the metagenome of groundwater sampled at the depth of several hundred meters in the Horonobe Underground Research Laboratory, Hokkaido, Japan (Hernsdorf et al., 2017). Next, the genome of an uncultured bacterium CG2_30_54_10 was obtained from the metagenome of the Crystal Geyser spring water (Utah, United States) (Probst et al., 2017). Subsequently, the genomes of further *Riflebacteria* members from subsurface ecosystems were described: *Candidatus* Riflebacteria isolate UBA8953 (Parks et al., 2018) and *Candidatus* Ozemobacter sibiricus BY5 (Kadnikov et al., 2018), as well as Rumen uncultured genome RUG334 from a bovine rumen community (Stewart et al., 2018).

Little is known about the metabolic capabilities of *Riflebacteria* and their ecological role. It was reported that genomes of *Riflebacteria* from subsurface ecosystems contained the genes of anaerobic sulfite reductase and [FeFe]-type hydrogenase, inviting the hypothesis that these bacteria might participate in the sulfur cycle (Anantharaman et al., 2018; Hernsdorf et al., 2017). To date, detailed analysis of metabolic pathways has been performed only for *Ca.* Ozemobacter sibiricus BY5, the genome of which was assembled from the metagenome of a Western Siberian subsurface aquifer (Kadnikov et al., 2018). Western Siberian subsurface thermal aquifers lying at the depths of 1 to 3 km in Cretaceous sedimentary rocks are accessible via oil exploration boreholes that enable artesian

water discharge under natural pressure (Frank et al., 2016; Kadnikov et al., 2018). Genome analysis showed that *Ca*. Ozemobacter sibiricus BY5 is apparently an anaerobic organotroph capable of fermenting certain carbohydrates and proteinaceous substrates, as well as of dissimilatory Fe(III) reduction; at the same time, its genome lacked sulfite reductase genes (Kadnikov et al., 2018).

Subsequently we analyzed the composition of the microbial community of an aquifer opened by artesian borehole 5P located near the Chazhemto settlement (Tomsk region, Russia) (Kadnikov et al., 2017a, 2017b) and sequenced its metagenome (Kadnikov et al., 2017c). In this community, approximately a half of all organisms were methanogenic archaea, and the other half were bacteria of diverse uncultured lineages (Kadnikov et al., 2017a). In particular, analysis of 16S rRNA gene sequences showed that the community contained bacteria of the candidate phylum *Riflebacteria* that were phylogenetically distinct from *Ca*. Ozemobacter sibiricus BY5.

In the present work, the results of metagenomic analysis were used to obtain the genome sequence of a novel member of *Riflebacteria*, to characterize its metabolic properties, and to estimate its global distribution and possible ecological role.

MATERIALS AND METHODS

Characterization of the borehole, collection of water samples, and isolation of metagenomic DNA. This study concerned a subsurface aquifer lying in Cretaceous sedimentary rocks at the depth of ~2 km and discharging to the surface via oil exploration borehole 5P located near the Chazhemto settlement (Tomsk region, Russia). The water sample was collected in April 2016 (Kadnikov et al., 2017c). The water had a temperature of ~20°C, neutral pH (7.43–7.6), and negative redox potential (Eh from −304 to −338 mV).

Microbial biomass from the 20-L water sample was collected by filtration through 0.22-μm membranes. The filters were homogenized by grinding in liquid nitrogen, and metagenomic DNA was isolated using the Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, United States). The total amount of DNA obtained was ~1 μg.

Sequencing of metagenomic DNA and assembling the genomes of microbial community members. Metagenomic DNA was sequenced using the Illumina HiSeq2500 system (Illumina, United States) as described previously (Kadnikov et al., 2017c). Altogether, sequencing in the paired read format (2 × 250 nt) with subsequent filtering by quality (Q > 33) generated a total of approximately 16.9 Gb. The reads were assembled into contigs using metaSPAdes v. 3.7.1 software (Nurk et al., 2017) as described previously (Kadnikov et al., 2017c). The obtained contigs were grouped into clusters corresponding to individual microbial genomes (metagenome-assembled genome, MAG) using the CONCOCT software (Alneberg et al., 2014). Completeness of the assembled MAGs and the level of their contamination with the contigs that belonged to other microorganisms were assessed using the CheckM software (Parks et al., 2015).

The taxonomic position of the assembled genomes was determined according to the GTDB database using GTDB-Tk v. 0.1.3 software (Parks et al., 2018). One of the genomes, designated Ch46, was classified into the candidate phylum *Riflebacteria*.

Additionally, metagenomic DNA was sequenced on a MinION system (Oxford Nanopore, Great Britain) using the Ligation Sequencing kit 1D as recommended by the manufacturer. The sequencing produced 1418419 reads with a total length of ~1.54 Gb. These long reads were utilized to merge the contigs constituting the genome of Ch46 into longer sequences. For this purpose, BWA v0.7.15 software (Li and Durbin, 2009) was used to select those MinION reads that were homologous to contig sequences of Ch46. Contigs were merged using the npScarf software (Cao, 2017); the gaps between contigs were filled using Illumina reads from the metaSPAdes chart.

The genomic sequence of bacterium Ch46 was deposited into the NCBI GenBank under accession no. JAACJG000000000.

Annotation and analysis of the genome of Ch46. Gene search and annotation were performed using the RAST server; the annotation was subsequently verified by comparing the predicted protein sequences with NCBI databases. N-terminal signal peptides were predicted using Signal P v. 5.0 (http://www.cbs.dtu.dk/services/SignalP/) and PRED-TAT (http://www.compgen.org/tools/PRED-TAT/); transmembrane domains were identified using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The iRep program was used to calculate the replication index of genomic DNA (Brown et al., 2016). Classification of hydrogenases was performed using the HydDB online service (https://services.birc.au.dk/hyddb/) (Søndergaard et al., 2016).

Genome-to-genome distance evaluation and phylogenetic analysis. The values of average amino acid identity (AAI) between bacterial genomes were determined using the aai.rb script from the Enveomics Collection (Rodriguez-R and Konstantinidis, 2016). The levels of DNA–DNA hybridization *in silico* were calculated using the GGDC2 tool available at http://ggdc.dsmz.de/ with the recommended formula (2) (Meier-Kolthoff et al., 2013).

Phylogenetic analysis based on complete genome sequences was performed for a dataset that included the genomes of Ch46, seven other members of *Riflebacteria* (Table 1), and a representative of the candidate phylum Wallbacteria phylogenetically close to *Riflebacteria*, UBP16_UBA6123 (GenBank

Table 1. Main characteristics of *Riflebacteria* genomes

Organism/genome	GenBank	Genome size, bp	Genome completeness, %	Number of contigs	G + C, %
Ca. Riflebacterium amylolyticus Ch46	GCA_009917695.1	5391313	98	35	50.1
GWC2_50_8	GCA_001787855.1	4386086	76	881	49.6
HGW-Riflebacteria-2	GCA_002839435.1	5226436	89	199	50.1
HGW-Riflebacteria-1	GCA_002839445.1	5678741	98	50	49.9
UBA8953 (sp002839445)	GCA_003446455.1	4673206	95	817	50
RUG334 (sp900317935)	GCA_900317935.1	4546479	93	381	34.7
Ca. Ozemobacter sibiricus BY5	GCA_003327425.1	5683244	98	58	65.4
CG2_30_54_10	GCA_001872985.1	2825442	62	574	53.5

GCA_002423385.1). For this sample, 120 conserved single-copy marker genes were identified using GTDB-Tk and multiple alignment of concatenated marker gene sequences was constructed.

The obtained multiple alignment was used to construct a maximum-likelihood phylogenetic tree using PhyML v. 3.3 software with default settings (Guindon et al., 2010).

RESULTS AND DISCUSSION

Assembling the genome of a member of the candidate phylum *Riflebacteria*. The metagenome of the microbial community of the subsurface aquifer opened by borehole 5P was sequenced using the Illumina technology; as a result, approximately 16.9 Gb were obtained and assembled into contigs (Kadnikov et al., 2017c). The contigs were clustered into MAGs using the CONCOCT software. One of the obtained MAGs, designated Ch46, was represented by 49 contigs with a total length of 5.4 Mb and average read coverage of 23. The relative abundance of this genotype in the microbial community, as assessed by the share of MAG Ch46 in the entire metagenome, was 0.83%. Taxonomic classification of MAG Ch46 using the Genome Taxonomy database showed that it belonged to the candidate phylum *Riflebacteria*.

To improve the quality of Ch46 genome assembly, 1.4 million long reads (with a total length of approximately 1.5 Gb) were obtained by nanopore sequencing. As a result, 49 contigs were combined into 35 contigs with a total length of 5391313 bp (Table 1). As assessed using the CheckM service, 103 of 105 conserved single-copy genes were present as a single copy and one as two copies in this genome, which indicates 98.31% completeness of Ch46 MAG and the level of potential contamination of 1.69%. Based on the degree of completeness and the lowest contig number, the obtained MAG is the best among the currently sequenced genomes of *Riflebacteria* (Table 1).

Analysis of the Ch46 genome identified the genes of 16S and 23S rRNAs, as well as 45 genes of tRNAs. Genome annotation predicted 4623 putative protein-coding sequences; however, it was only for 1573 of them that their function could be inferred by comparison with NCBI databases. The genome of Ch46 contains two CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) loci comprising 35 and 58 repeat–spacer units, and a set of genes encoding a type 1-C CRISPR system located in vicinity of one of these loci.

The genome of Ch46 was found to contain a set of genes encoding the flagellar apparatus and the chemotaxis, as well as type IV pili proteins responsible for motility in this bacterium.

The rate of DNA replication in bacterium Ch46 in situ, in the subsurface aquifer, was estimated using the iRep software (Brown et al., 2016). The obtained iRep value for the genome of Ch46 was 1.33, which suggests that the growth of these bacteria is relatively slow (~33% cells were replicating at the moment of sample collection), but nevertheless they represent the metabolically active fraction of the microbial community.

Phylogenetic position of Ch46. A search of the Genome Taxonomy database for phylogenetically related microorganisms based on genome similarity showed that the closest relative of Ch46 is *Candidatus* Riflebacteria bacterium HGW-Riflebacteria-2 (Hernsdorf et al., 2017). For these two genomes, the average amino acid sequence identity (AAI) was 85.16%. The AAI values for Ch46 and other representatives of *Riflebacteria* lay within the range from 51 to 78% (Table 2). Considering the threshold AAI proposed for taxonomic classification of uncultured microorganisms: 65–95% for the same genus and 95–100% for the same species (Konstantinidis et al., 2017), Ch46 and HGW-Riflebacteria-2 represent different species of the same genus. The estimated level of DNA–DNA hybridization *in silico* (24%) also indicates that these two microorganisms belong to different species.

To analyze the phylogeny of *Riflebacteria*, we constructed a phylogenetic tree based on concatenated sequences of 43 conserved marker proteins, which included Ch46 and other available genomes of *Rifle-*

Table 2. Average identity of amino acid sequences of *Riflebacteria* members

	Organism/genome	1	2	3	4	5	6	7	8
1	*Ca.* Riflebacterium amylolyticus Ch46	100	85	78	78	78	54	51	51
2	HGW-Riflebacteria-2	85	100	79	78	78	54	51	51
3	HGW-Riflebacteria-1	78	79	100	95	86	54	51	51
4	UBA8953 (sp002839445)	78	78	95	100	86	54	51	51
5	GWC2_50_8	78	78	86	86	100	53	51	50
6	RUG334 (sp900317935)	54	54	54	54	53	100	48	48
7	*Ca.* Ozemobacter sibiricus BY5	51	51	51	51	51	48	100	65
8	CG2_30_54_10	51	51	51	51	50	48	65	100

Numbers in the cells are the values of AAI (%) for the corresponding pairs of genomes.

bacteria. The obtained results confirmed that the closest relative of Ch46 is HGW-Riflebacteria-2 (Fig. 1). The tree shows three clearly distinct candidate genera proposed in the GTDB taxonomy: genus UBA8953, which includes Ch46, UBA8953-sp002839445, HGW-Riflebacteria-1, HGW-Riflebacteria-2, and GWC2_50_8; genus RUG334 with the only species sp900317935; and the previously described genus *Candidatus* Ozemobacter (Kadnikov et al., 2018), which includes *Ca.* Ozemobacter sibiricus BY5 and bacterium CG2_30_54_10 (in the GTDB taxonomy, this genus is denoted CG2-30-54-10).

Among the members of the candidate genus UBA8953, the 16S rRNA gene sequence is known only for HGW-Riflebacteria-1; it has 98.1% identity with the 16S rRNA gene of Ch46. The sequences of the 16S rRNA genes of Ch46 and *Ca.* Ozemobacter sibiricus BY5 have 89.9% identity, which corresponds to genus-level difference, in agreement with the AAI data.

Possible growth substrates and central metabolic pathways of Ch46. A search for genes of glycosyl hydrolases carrying N-terminal signal sequences characteristic for secreted proteins identified only four alpha-amylases (genes GQF41_2102, GQF41_2155, GQF41_3117, and GQF41_3457) and pullulanase (GQF41_4095) that can perform extracellular hydrolysis of starch and similar polysaccharides. Uptake of maltose and maltodextrin can be mediated by ABC-type transporters, and their subsequent utilization, by the corresponding intracellular hydrolases. No genes of extracellular hydrolytic enzymes that might mediate hydrolysis of other polysaccharides were found. Genome analysis revealed the presence of transporters of other sugars, including glucose, mannose, fructose, and N-acetyl-β-glucosamine. Metabolism of imported sugars is apparently linked to the glycolytic pathways. For instance, N-acetyl-β-glucosamine can be phosphorylated by N-acetylglucosamine kinase NagC and then deacetylated by N-acetylglucosamine-6-phosphate deacetylase NagA. Finally, glucosamine-6-phosphate deaminase NagB transforms N-acetyl-D-glucosamine-6-P into fructose-6-P, which enters the pathway of glycolysis.

The genome analysis revealed the genes for all enzymes of the Embden–Meyerhof pathway of glycolysis, gluconeogenesis, and the pentose phosphate pathway, including its oxidative and nonoxidative stages (Fig. 2). The known pathways of autotrophic carbon fixation were not found. The storage polysaccharide in Ch46 is probably glycogen, as suggested by the presence of the genes encoding the key enzymes of its synthesis and degradation, such as glucose-1-phosphate adenyltransferase, glycogen synthase, 1,4-alpha glucan branching enzyme, glycogen phosphorylase,

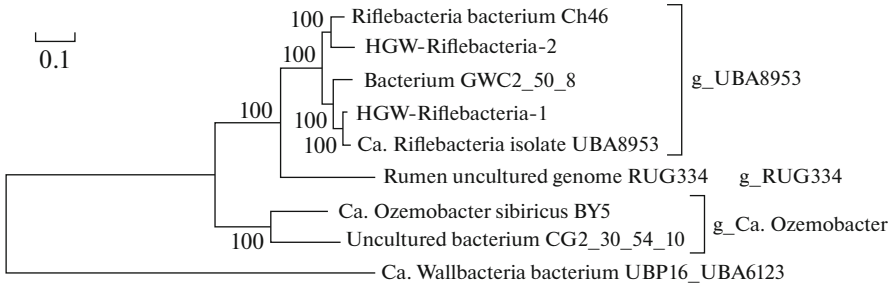

Fig. 1. Phylogeny of the candidate phylum *Riflebacteria* based on analysis of concatenated amino acid sequences of protein products of conserved marker genes. The genome of bacterium UBP16_UBA6123 from the candidate phylum *Wallbacteria* was used as an outgroup. Branch support values were determined using the Bayesian test with PhyML.

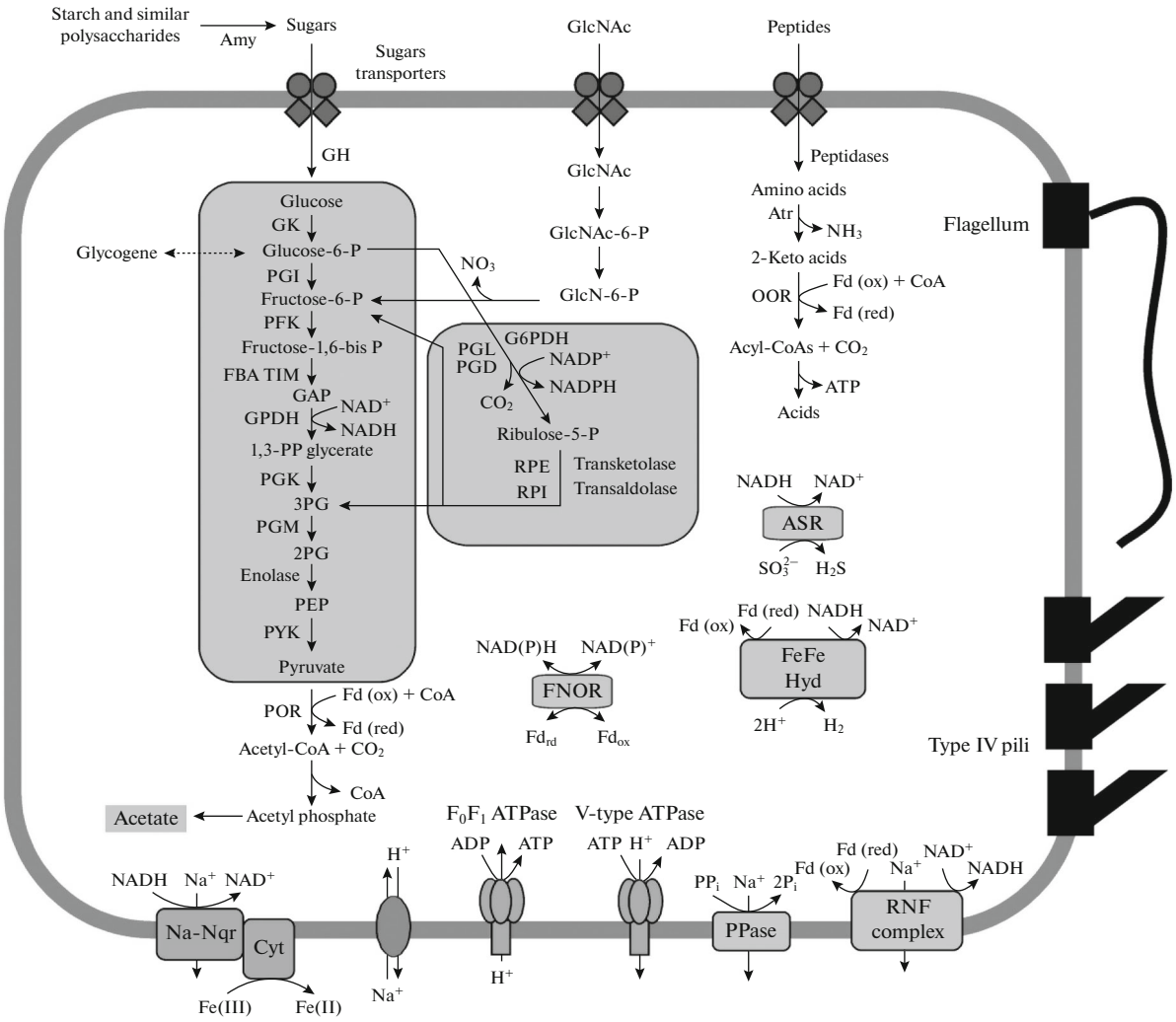

Fig. 2. Major metabolic pathways in *Ca*. Riflebacterium amylolyticus Ch46. Enzyme abbreviations: Amy, alpha amylase; GH, glycosyl hydrolase; GK, glucokinase; PGI, glucose-6-phosphate isomerase; PFK, 6-phosphofructokinase; FBA, fructose-biphosphate aldolase; TIM, triosephosphate isomerase; GPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; PYK, pyruvate kinase; G6PDH, glucose-6-phosphate 1-dehydrogenase; PGL, 6-phosphogluconolactonase; PGD, 6-phosphogluconate dehydrogenase; RPE, ribulose-phosphate 3-epimerase; RPI, ribose 5-phosphate isomerase; POR, pyruvate ferredoxin (flavodoxin) oxidoreductase; Atr, aminotransferase; OOR, ferredoxin-dependent 2-ketoacid oxidoreductases; PPase, pyrophosphatase; Na-Nqr, Na^+-transporting NADH–quinone oxidoreductase; [FeFe] Hyd, [FeFe]-type hydrogenase; FNOR, ferredoxin : $NADP^+$ oxidoreductase; ASR, anaerobic sulfite reductase. Other abbreviations: 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; GAP, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; Fd, ferredoxin; ox/red, oxidized and reduced form; cyt, cytochrome *c*; CoA, coenzyme A; PP, pyrophosphate; P, phosphate.

and glycogen debranching enzyme. The synthesis of trehalose, another storage polysaccharide and an osmolyte, is mediated by trehalose synthetase, as well by two other enzymes: maltooligosyl trehalose synthase and maltooligosyl trehalose hydrolase.

Pyruvate generated in the course of glycolysis can be converted into acetyl-CoA by pyruvate–ferredoxin oxidoreductase. Acetyl-CoA can be oxidized to produce acetate and generate ATP in a two-stage reaction mediated by phosphate-acetyltransferase and acetate kinase; at the same time, the gene of acetyl-CoA synthetase was not detected. The presence of aldehyde and alcohol dehydrogenases suggests that, apart from acetate, fermentation may also produce alcohols. Acetyl-CoA generated from pyruvate can enter the tricarboxylic acid cycle. However, this cycle is incomplete, lacking succinyl-CoA synthetase, succinate dehydrogenase, and malate dehydrogenase, and probably serves only for biosynthetic purposes.

A further possible carbon and energy source for Ch46 are amino acids and short peptides, which might be imported by means of ABC-type transporters. Amino acids, either imported or produced by intracellular peptide hydrolysis, can be deaminated and con-

verted into 2-ketoacids (e.g., pyruvate or 2-oxoglutarate). Next, 2-ketoacids can be oxidized to acyl-CoA derivatives by ferredoxin-dependent oxidoreductases of different specificity and further cleaved to produce the corresponding acids and generate ATP (Fig. 2).

The reduced products generated in the course of fermentation, NADH and reduced ferredoxin, can be reoxidized by [FeFe] hydrogenases (Greening et al., 2016). All hydrogenases lack N-terminal signal peptides or transmembrane domains and are probably located in the cytoplasm. The genome of Ch46 contains two clusters of hydrogenase genes. One of them (genes GQF41_0195–GQF41_0204) encodes two three-subunit group A3 hydrogenases that can mediate electron bifurcation between H_2, ferredoxin, and NADH, which involves ferredoxin oxidation coupled with NADH oxidation and production of molecular hydrogen (Schut and Adams, 2009; Greening et al., 2016). The other cluster (GQF41_3791–GQF41_3797) encodes a three-subunit hydrogenase of group A3, as well as a group B [FeFe] hydrogenase that can oxidize reduced ferredoxin with H_2 production (Greening et al., 2016). On the whole, activity of these hydrogenases can ensure reoxidation of NADH and reduced ferredoxin generated in the course of fermentation.

A further possible mechanism of NAD^+ regeneration is NADH-dependent sulfite reduction mediated by cytoplasmic anaerobic sulfite reductase Asr. In Ch46, this enzyme is encoded by the *asrABC* operon (GQF41_2511–GQF41_2509) similar to the corresponding operons of *Clostridium* and *Salmonella* (Hallenbeck et al., 1989). The genes encoding anaerobic sulfite reductase are also present in the genomes of three other members of the candidate genus UBA8953: GWC2_50_8, HGW-Riflebacteria-1, and HGW-Riflebacteria-2, and it was supposed that these bacteria may perform sulfite reduction using the Asr complex (Anantharaman et al., 2018).

Generation of transmembrane ion gradient. Analysis of the Ch46 genome did not reveal the genes of the principal components of the aerobic respiratory chain, specifically, NADH dehydrogenase, membrane-bound succinate dehydrogenase, cytochrome-*bc*1 complex or alternative complex III, and cytochrome oxidases. The known pathways of dissimilatory reduction of sulfate, nitrate, nitrite, arsenate, and sulfur were also not detected.

However, Ch46 bacteria nevertheless possess several mechanisms of generating transmembrane ion gradient, which can be employed by membrane-bound F_0F_1 ATP synthase for ATP production. First, the genome of Ch46 contains a complete set of genes that encode the membrane-bound ion-transporting complex Rnf (GQF41_3883–GQF41_3888). Rnf activity can be either anabolic, with ferredoxin reduction and NADH oxidation, or catabolic, which involves ferredoxin oxidation, NAD^+ reduction, and transfer of protons or sodium ions across the cytoplasmic membrane (Biegel et al., 2011). Based on the gene order, *rnfCDGEAB*, the Rnf operon of Ch46 belongs to type 3 usually found in *Firmicutes*, where it performs the catabolic function as an energy conserving ferredoxin : NAD^+ oxidoreductase. Two further enzymes can also contribute to the generation of transmembrane ion gradient: V-type ATPase, which utilizes hydrolysis of ATP produced during fermentation for proton transport, and membrane-bound pyrophosphatase GQF41_0857, which performs translocation of protons or sodium ions using pyrophosphate hydrolysis. The lack of lysine in the signature sequence GNXX (K/A) (Malinen et al., 2007) indicates that this pyrophosphatase transfers sodium ions and not protons. The balance between H^+ and Na^+ concentrations may be maintained by H^+/Na^+ antiporters encoded in the genome.

Another complex that can store energy in the form of transmembrane ion gradient is Na^+-transporting NADH–quinone oxidoreductase, Na-NQR. In various pathogenic and free-living bacteria, this enzyme complex acts as the main ion pump and the site of electron entry into the respiratory chain (Reyes-Prieto et al., 2014). Na-NQR is a sodium-specific ion pump that has a common evolutionary origin with Rnf. As a rule, Na-NQR comprises six subunits (NqrA–F). The genome of Ch46 was found to contain two *nqr* operons (GQF41_2620–GQF41_2616 and GQF41_4465–GQF41_4461) that encode subunits NqrB, C, D, E, and F, whereas the *nqrA* genes were absent in both operons. In contrast to other subunits, NqrA is located in the cytosol, does not possess cofactor binding sites, and its function is unclear. It is possible that *nqrA* was not detected, or this subunit is unnecessary for Na-NQR functioning in Ch46.

Since Na-NQR oxidoreductase transfers electrons into the quinone pool of the cytoplasmic membrane, Ch46 bacterium must possess a terminal reductase utilizing these electrons for reduction of a terminal acceptor. One of the *nrqBCDEF* clusters includes a gene (GQF41_2615) that encodes cytochrome *c* with eight heme-binding motifs (CxxCH). The genome also contains two genes (GQF41_1560 and GQF41_1756) encoding cytochromes *c* with ten heme-binding motifs. All three cytochromes carry N-terminal signal peptides and possess transmembrane domains, which suggests that they are exported from the cell and located on the outer surface; thus, they can come in contact with an insoluble electron acceptor. Such multiheme *c*-type cytochromes play a central role in electron transfer to an extracellular electron acceptor in gram-negative bacteria *Shewanella* and *Geobacter* that perform dissimilatory Fe(III) reduction (Shi et al., 2007; Richter et al., 2012). One of the decaheme cytochromes (GQF41_1560) contains the (Ser/Thr)-Pro-(Ser/Thr) motif, which can serve for its binding to hematite, an iron oxide mineral (Fe_2O_3), as it was

shown for Fe(III)-reductase of *Shewanella oneidensis* (Lower et al., 2008). Adhesion to Fe(III) minerals can also be mediated by type IV pili; a set of the corresponding genes was identified in the genome of Ch46. Thus, it seems likely that multiheme cytochromes may accept electrons from the quinone pool and mediate extracellular Fe(III) reduction. The predicted pathways of Ch46 metabolism are shown in Fig. 2.

General characteristics of metabolism in *Riflebacteria*. Available information on the metabolic capabilities of other members of *Riflebacteria* is very limited. Although, in addition to Ch46, seven other genomes of *Riflebacteria* have been obtained, and four of them are more than 90% complete, analysis of the metabolic pathways based on the genome sequence data has been performed only for *Ca*. Ozemobacter sibiricus BY5, which belongs to another genus of this phylum (Kadnikov et al., 2018). Similarly to Ch46, *Ca*. Ozemobacter sibiricus BY5 can utilize starch and similar polysacchraides as substrates, as well as simple sugars (including N-acetyl-β-glucosamine), amino acids, and peptides. Both genomes encode the Embden–Meyerhof pathway of glycolysis and the complete pentose phosphate pathway, both possess a partial set of genes of the tricarboxylic acid cycle, and both lack the aerobic respiratory chain. *Ca*. Ozemobacter sibiricus BY5 also possesses the genes that encode cytoplasmic [FeFe] hydrogenases and the Rnf complex, as well as two clusters of Na-NQR oxidoreductase genes with the same set of subunits. However, members of the genera *Ca*. Ozemobacter and UBA8953 have different genes of putative Fe(III) reductases. Specifically, in Ch46, UBA8953-sp002839445, HGW-Riflebacteria-1, HGW-Riflebacteria-2, and GWC2_50_8, one of the *nrqBCDEF* clusters includes only one gene of octaheme cytochrome *c*, while *Ca*. Ozemobacter sibiricus BY5 and CG2_30_54_10 possess instead four genes of multiheme cytochromes *c* and a ferritin gene. Another difference is that *Ca*. Ozemobacter sibiricus BY5 and CG2_30_54_10 do not have the genes of anaerobic sulfite reductase present in the genomes of UBA8953 members.

Global distribution of bacteria related to Ch46 and their ecological role. A search in the GenBank database revealed 45 nearly complete sequences (>1400 bp) of 16S rRNA genes that had over 95% identity to the 16S rRNA gene of Ch46, including 40 sequences with an identity above 97%, i.e., belonging to the same species. All of them were detected in anoxic habitats in various geographic locations: anaerobic biodigesters for organic waste and wastewater treatment (31 sequences), subsurface waters and springs (10 sequences), or lake and river sediments (3 sequences).

The microbial community of the subsurface aquifer from which Ch46 was retrieved is composed by approximately a half of methanogenic archaea, while the other half is represented by bacteria of the phyla *Firmicutes*, *Chloroflexi*, *Proteobacteria*, *Bacteroidetes*, and *Ignavibacteriae* (Kadnikov et al., 2017a). It is known that these phyla include bacteria capable of hydrolyzing complex polymers. For instance, members of *Ignavibacteriae* and *Chloroflexi* (*Anaerolineaceae*) occurring in subsurface waters of Western Siberia can hydrolyze complex polysaccharides and proteinaceous substrates (Kadnikov et al., 2018). Apparently, *Riflebacteria* present in these ecosystems may specialize on hydrolysis of starch and similar polysaccharides preserved in Cretaceous sedimentary rocks since the aquifer formation or generated by autotrophic microorganisms, as well as utilize low-molecular-weight organic compounds produced by hydrolytic microorganisms (e.g., sugars, peptides, or amino acids), generating hydrogen and acetate that serve as substrates for methanogenic archaea. Most likely, *Riflebacteria* grow within biofilms on the surface of mineral rocks, in environments rich in organic compounds and in contact with insoluble electron acceptors. Since subsurface aquifers of Western Siberia are not completely isolated from the surface and have been receiving meteoric waters for millions of years (Kadnikov et al., 2018), *Riflebacteria* were probably introduced into this habitat from the surface and have successfully colonized the novel ecological niche.

Description of "*Candidatus* Rifleibacterium amylolyticum" Ch46

The genome of bacterium Ch46 meets the criteria required for description of novel taxa of uncultured microorganisms (Konstantinidis et al., 2017); therefore, we propose to describe the new species as "*Candidatus* Rifleibacterium amylolyticus." The name of the candidate genus corresponds to the previously proposed name of the phylum *Riflebacteria* (Anantharaman et al., 2016), modified taking into account the prokaryote nomenclature guidelines, and the species name reflects its presumed ability to hydrolyze starch.

"*Candidatus* Rifleibacterium" gen. nov. (Rif.le.i.bac.te'ri.um. N.L. neut. n. bacterium a rod; N.L. neut. n. *Rifleibacterium* a rod named after Rifle, Colorado).

"*Candidatus* Rifleibacterium amylolyticum" sp. nov. (a.my.lo.ly'ti,cum. Gr. neut. n. *amylon*, starch; N.L. masc. adj. *lyticus* (from Gr. masc. adj. *lytikos*), able to dissolve; N.L. neut. adj. *amylolyticum*, degrading starch). Uncultured. Discovered in a subsurface aquifer in Western Siberia. Presumably, an anaerobic organotroph that obtains energy by fermentation and anaerobic respiration; its possible substrates are starch, as well as low-molecular-weight carbohydrates, amino acids, and peptides. The G + C content in DNA is 50.1 mol %. Represented by the genome (GenBank JAACJG000000000) assembled from the metagenome of the subsurface thermal aquifer opened by borehole 5P (Chazhemto, Tomsk region, Russia).

ACKNOWLEDGMENTS

This work was performed using the the scientific equipment of the Core Research Facility "Bioengineering" (Research Center of Biotechnology, Russian Academy of Sciences).

FUNDING

This work was financially supported by the Russian Science Foundation (project no. 19-14-00245).

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. This article does not contain any studies involving animals or human subjects performed by any of the authors.

THE AUTHORS' CONTRIBUTION.

V.V. Kadnikov and A.V. Mardanov obtained and analyzed the genomic data; A.V. Beletsky performed phylogenetic analysis; O.V. Karnachuk performed sample collection and genome analysis; N.V. Ravin performed genome analysis, prepared the manuscript, and was in charge of the general supervision of the project.

REFERENCES

Alneberg, J., Bjarnason, B.S., De Bruijn, I., Schirmer, M., Quick, J., Ijaz, U.Z., Lahti, L., Loman, N.J., Andersson, A.F., and Quince, C., Binning metagenomic contigs by coverage and composition, *Nat. Methods,* 2014, vol. 11, pp. 1144–1146.

Anantharaman, K., Brown, C.T., Hug, L.A., Sharon, I., Castelle, C.J., Probst, A.J., Thomas, B.C., Singh, A., Wilkins, M.J., Karaoz, U., Brodie, E.L., Williams, K.H., Hubbard, S.S., and Banfield, J.F., Thousands of microbial genomes shed light on interconnected biogeochemical processes in an aquifer system, *Nat. Commun.*, 2016, vol. 7, p. 13219.

Anantharaman, K., Hausmann, B., Jungbluth, S.P., Kantor, R.S., Lavy, A., Warren, L.A, Rappé, M.S., Pester, M., Loy, A., Thomas, B.C., and Banfield, J.F., Expanded diversity of microbial groups that shape the dissimilatory sulfur cycle, *ISME J.*, 2018, vol. 12, pp. 1715–1728.

Biegel, E., Schmidt, S., Gonzalez, J.M., and Müller, V., Biochemistry, evolution and physiological function of the Rnf complex, a novel ion-motive electron transport complex in prokaryotes, *Cell. Mol. Life Sci.*, 2011, vol. 68, pp. 613–634.

Brown, C.T., Olm, M.R., Thomas, B.C., and Banfield, J.F., Measurement of bacterial replication rates in microbial communities, *Nat. Biotechnol.*, 2016, vol. 34, pp. 1256–1263.

Cao, M.D., Nguyen, S.H., Ganesamoorthy, D., Elliott, A.G., Cooper, M.A., and Coin, L.J., Scaffolding and completing genome assemblies in real-time with nanopore sequencing, *Nat. Commun.*, 2017, vol. 8, 14515.

Frank, Y.A., Kadnikov, V.V., Gavrilov, S.N., Banks, D., Gerasimchuk, A.L., Podosokorskaya, O.A., Merkel, A.Y., Chernyh, N.A., Mardanov, A.V., Ravin, N.V., Karnachuk, O.V., and Bonch-Osmolovskaya, E.A., Stable and variable parts of microbial community in Siberian deep subsurface thermal aquifer system revealed in a long-term monitoring study, *Front. Microbiol.*, 2016, vol. 7, p. 2101.

Greening, C., Biswas, A., Carere, C.R., Jackson, C.J., Taylor, M.C., Stott, M.B., Cook, G.M., and Morales, S.E., Genomic and metagenomic surveys of hydrogenase distribution indicate H_2 is a widely utilised energy source for microbial growth and survival, *ISME J.*, 2016, vol. 10, pp. 761–777.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O., New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0, *Syst. Biol.*, 2010, vol. 59, pp. 307–321.

Hallenbeck, P.C., Clark, M.A., and Barrett, E.L., Characterization of anaerobic sulfite reduction by *Salmonella typhimurium* and purification of the anaerobically induced sulfite reductase, *J. Bacteriol.*, 1989, vol. 171, pp. 3008–3015.

Hernsdorf, A.W., Amano, Y., Miyakawa, K., Ise, K., Suzuki, Y., Anantharaman, K., Probst, A., Burstein, D., Thomas, B.C., and Banfield, J.F., Potential for microbial H_2 and metal transformations associated with novel bacteria and archaea in deep terrestrial subsurface sediments, *ISME J.*, 2017, vol. 11, pp. 1915–1929.

Kadnikov, V.V., Frank, Y.A., Mardanov, A.V., Beletskii, A.V., Ivasenko, D.A., Pimenov, N.V., Karnachuk, O.V., and Ravin, N.V., Uncultured bacteria and methanogenic archaea predominate in the microbial community of Western Siberian deep subsurface aquifer, *Microbiology* (Moscow), 2017a, vol. 86, pp. 412–415.

Kadnikov, V.V., Frank, Y.A., Mardanov, A.V., Beletsky, A.V., Ivasenko, D.A., Pimenov, N.V., Karnachuk, O.V., and Ravin, N.V., Variability of the composition of the microbial community of the deep subsurface thermal aquifer in Western Siberia, *Microbiology* (Moscow), 2017b, vol. 86, pp. 765–772.

Kadnikov, V.V., Frank, Y.A., Mardanov, A.V., Beletsky, A.V., Karnachuk, O.V., and Ravin, N.V., Metagenome of the Siberian underground water reservoir, *Genome Announc.*, 2017c, vol. 5, e01317-17.

Kadnikov, V.V., Mardanov, A.V., Beletsky, A.V., Banks, D., Pimenov, N.V., Frank, Y.A., Karnachuk, O.V., and Ravin, N.V., A metagenomic window into the 2-km-deep terrestrial subsurface aquifer revealed multiple pathways of organic matter decomposition, *FEMS Microbiol. Ecol.*, 2018, vol. 94, fiy152.

Konstantinidis, K.T., Rosselló-Móra, R., and Amann, R., Uncultivated microbes in need of their own taxonomy, *ISME J.*, 2017, vol. 11, pp. 2399–2406.

Li, H. and Durbin, R., Fast and accurate short read alignment with Burrows–Wheeler transform, *Bioinformatics,* 2009, vol. 25, pp. 1754–1760.

Lower, B.H., Lins, R.D., Oestreicher, Z., Straatsma, T.P., Hochella, M.F., Jr., Shi, L., and Lower, S.K., In vitro evolution of a peptide with a hematite binding motif that may constitute a natural metal-oxide binding archetype, *Environ. Sci. Technol.*, 2008, vol. 42, pp. 3821–3827.

Malinen, A.M., Belogurov, G.A., Baykov, A.A., and Lahti, R., Na^+-pyrophosphatase: a novel primary sodium pump, *Biochemistry,* 2007, vol. 46, pp. 8872–8878.

Meier-Kolthoff, J.P., Auch, A.F., Klenk, H.-P., and Göker, M., Genome sequence-based species delimitation with confidence intervals and improved distance functions, *BMC Bioinform.*, 2013, vol. 14, p. 60.

Nurk, S., Meleshko, D., Korobeynikov, A., and Pevzner, P.A., metaSPAdes: a new versatile metagenomic assembler, *Genome Res.*, 2017, vol. 27, pp. 824–834.

Parks, D.H., Chuvochina, M., Waite, D.W., Rinke, C., Skarshewski, A., Chaumeil, P.A., and Hugenholtz, P., A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life, *Nat. Biotechnol.*, 2018, vol. 36, pp. 996–1004.

Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., and Tyson, G.W., CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes, *Genome Res.*, 2015, vol. 25, pp. 1043–1055.

Probst, A.J., Castelle, C.J., Singh, A., Brown, C.T., Anantharaman, K., Sharon, I., Hug, L.A., Burstein, D., Emerson, J.B., Thomas, B.C., and Banfield, J.F., Genomic resolution of a cold subsurface aquifer community provides metabolic insights for novel microbes adapted to high CO_2 concentrations, *Environ. Microbiol.*, 2017, vol. 19, pp. 459–474.

Reyes-Prieto, A., Barquera, B., and Juarez, O., Origin and evolution of the sodium-pumping NADH: ubiquinone oxidoreductase, *PLoS One*, 2014, vol. 9, e96696.

Richter, K., Schicklberger, M., and Gescher, J., Dissimilatory reduction of extracellular electron acceptors in anaerobic respiration, *Appl. Environ. Microbiol.*, 2012, vol. 78, pp. 913–921.

Rodriguez-R, L.M. and Konstantinidis, K.T., The enveomics collection: a toolbox for specialized analyses of microbial genomes and metagenomes, *PeerJ Preprints*, 2016, vol. 4, e1900v1.

Schut, G.J. and Adams, M.W., The iron-hydrogenase of *Thermotoga maritima* utilizes ferredoxin and NADH synergistically: a new perspective on anaerobic hydrogen production, *J. Bacteriol.*, 2009, vol. 191, pp. 4451–4457.

Shi, L., Squier, T.C., Zachara, J.M., and Fredrickson, J.K., Respiration of metal(hydr)oxides by *Shewanella* and *Geobacter*: a key role for multihaem *c*-type cytochromes, *Mol. Microbiol.*, 2007, vol. 65, pp. 12–20.

Søndergaard, D., Pedersen, C.N., and Greening, C., HydDB: A web tool for hydrogenase classification and analysis, *Sci Rep.*, 2016, vol. 6, p. 34212.

Stewart, R.D., Auffret, M.D., Warr, A., Wiser, A.H., Press, M.O., Langford, K.W., Liachko, I., Snelling, T.J., Dewhurst, R.J., Walker, A.W., Roehe, R., and Watson, M., Assembly of 913 microbial genomes from metagenomic sequencing of the cow rumen, *Nat. Commun.*, 2018, vol. 9, p. 870.

Translated by D. Timchenko