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Cytobacillus pseudoceanisediminis sp. nov., A Novel Facultative Methylotrophic Bacterium with High Heavy Metal Resistance Isolated from the Deep Underground Saline Spring

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Abstract

In this study, we present the characterization of the BNO1^T bacterial strain isolated from the deep subsurface saline spring at the Baksan Neutrino Observatory INR RAS (Kabardino-Balkaria, Russia). The complete genome sequence of the strain BNO1^T is 5,347,902 bp, with a GC content 41 and 49%. The cell wall peptidoglycan contains *meso*-diaminopimelic acid. The major isoprenoid quinone is MK-7 and the polar lipids are diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine. The major fatty acids are anteiso-$C_{15:0}$ (23.34%), iso-$C_{15:0}$ (20.10%), $C_{16:0}$ (11.96%), iso-$C_{16:0}$ (10.88%), and anteiso-$C_{17:0}$ (10.79%). The 16S rRNA gene sequence clearly demarcated the strain as belonging to *Cytobacillus* genera. Based on the phylogenetic analysis, ANI (average nucleotide identity) and dDDH (digital DNA-DNA hybridization) assessments we propose to assign the strain BNO1^T and other related strains to new species and to name it *Cytobacillus pseudoceanisediminis* sp. nov. (The values of ANI and dDDH between BNO1^T and *Cytobacillus oceanisediminis* CGMCC 1.10115 ^{T} are 80.65% and 24.7%, respectively; values of ANI and dDDH between BNO1^T and *Cytobacillus firmus* NCTC 10335 ^{T} are 89% and 38%, respectively). Genomic analysis of strain BNO1^T revealed pathways for C1 compounds oxidation and two pathways for C1 compounds assimilation: serine and ribulose monophosphate pathways. In addition, strain BNO1^T contains a plasmid (342,541 bp) coding multiple genes involved in heavy metal ion balance. Moreover, heavy metal toxicity testing confirmed the high potential of the strain BNO1^T as a source of metal resistance genes and enzymes. The type strain is BNO1^T (= BIM B-1921 ^{T} = VKM B-3664 ^{T}).

Introduction

Life in remote corners of the Earth with extreme conditions has always aroused great interest among scientists. The harsh living conditions in such places force their inhabitants to create special biological adaptations in order to remain active and stable. A range of adaptive enzymes and their pathways may pave way for the potential applications in biotechnology, for instance, enzymes that are stable at wide ranges of temperature and salinity [1, 2] or mechanisms of adaptation to elevated levels of heavy metals that can be used for the development of biosensors and bioremediation techniques [3]. Furthermore, studies of extremophiles is an important area of research in astrobiology due to the similarity of extremophile habitats on Earth to conditions on other planets [4]. Thus, extremophiles are important models for studies related to the search of extraterrestrial life and the development of life-sustaining mechanisms during spaceflights [4].

Unusual habitat conditions, such as highly mineralized underground hydrothermal springs, are of interest in the search for extremophilic organisms. One of these springs is located in an unused part of Baksan Neutrino Observatory (BNO) INR RAS, used for the conduct of studies in nuclear physics and astrophysics [5–7]. Here we report

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about sequencing and characterization of a bacterial strain BNO1^T isolated from the underground highly mineralized spring of BNO located 2 km beneath the mountain Andyrchy in Elbrus region, Kabardino-Balkaria, Russia. In this work, we present the complete genome sequence and phylogenetic analysis of strain BNO1^T, propose its central carbohydrate metabolism, and identify abundant heavy metal ion resistance genes. Based on phylogenetic data we propose the name *Cytobacillus pseudoceanisediminis* sp. nov. for *Cytobacillus oceanisediminis* 2691-related strains including strain BNO1^T with strain BNO1^T as type strain.

To the best of our knowledge, this and previous work [8] are the first studies in the field of biology carried out at Baksan Neutrino Observatory and our work reveals the unique experimental potential of BNO for biophysical, radiobiological, and astrobiological studies [9].

Materials and Methods

Bacterial Strain, Culture Conditions, and Biochemical Tests

The strain was obtained from samples collected in September 2020 in an unused part of the underground tunnel of Baksan Neutrino Observatory INR RAS (Russia) from a spring (41 °C) at a distance of 4 km from the entrance (43°16′32.5″N 42°41′30.3″E) and 2 km under the mountain Andyrchy surface, Baksan Gorge, Kabardino-Balkaria, Russia (Supplementary Fig. 1). The strain was isolated using dilution-plating technique on LB agar at 37 °C. Biochemical characteristics were screened with API® 20E and API® 50CH microbiological tests (BioMérieux®, France), according to Logan and Berkeley [10]. Nitrate reduction test was performed with sodium nitrate and Griess reagent (Lenreactiv®, Russia), according to Skerman [11]. For biochemical profiling, three independent repeats were performed.

Chemotaxonomic Characterization

To determine 2,6-diaminopimelic acid stereoisomers, the cells were hydrolyzed with 6-M HCl at 100 °C for 18 h, the acid was evaporated in air, and the hydrolysis products were separated in the system methanol/water/HCl/pyridine (80:17.5:2.5:10) using paper chromatography. The *meso*-diaminopimelic acid was detected with the ninhydrin spray reagent [12]. Respiratory quinones were extracted from freeze-dried cells with chloroform/methanol (2:1 v/v) and analyzed by mass spectrometer LCQ Advantage MAX (Thermo Finnigan) [13]. The fatty acid composition was analyzed using a gas chromatography-mass spectrometer 7890B + 5977B (Agilent Technologies, USA). The cell biomass was dried, saponificated (3.75 M NaOH/MeOH, 100 °C, 30 min), and subjected to acidic methanolysis (6 N HCl/MeOH, 80 °C, 10 min). The products of methanolysis were extracted with hexane:methyl tert-butyl ether (1:1 w/w) and processed with alkali (0.3 M NaOH, 5 min). The obtained products were separated on a 5% phenyl methyl silicone capillary column HP-5MS (0.25 mm × 30 m) in the temperature gradient from 45 to 300 °C at 40 °C min^{-1}. Fatty acids and other lipid components were ionized by electron impact and analyzed in the scan mode. The compounds were identified using the NIST17 mass spectrometer library (https://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:nist17). The fatty acid content was determined as a percentage of the total ion current peak area [14]. Polar lipids were extracted from freeze-dried cells with chloroform/methanol (2:1, by vol.) and separated on HPTLC Silica gel 60F plates (Merck, Germany) using the following solvent systems: chloroform/methanol/water (65:25:4, by vol.) in the horizontal dimension and chloroform/methanol/acetic acid/water (80:12:15:5, by vol.) in the vertical dimension for two-dimensional TLC. Polar lipids were identified using spraying reagents for visualizing the spots: molybdenum blue for phospholipids, ninhydrin for aminolipids, and alpha-naphthol solution for glycolipids [15].

DNA isolation, Sequencing, and Genome Assembly

Total DNA was extracted with QIAGEN Genomic-tip 20/G (QIAGEN, Germany), according to manufacturer's protocol. DNA was sequenced without amplification using Oxford Nanopore® MinION with r9.4.1 flow cell. Sequencing data were basecalled using Guppy® 5.0.14. Basecalling ended with 1.39 million of sequences (3.256 billion of bases), N50 of 3.6 kb, quality score of 11.7. Assembly was performed with Canu 2.2 [16]. Genome sequence is available in NCBI databases under accession GCA_023516215.1.

Phylogenetic Analysis and Gene Annotation

Initial classification of the strain BNO1^T was performed using GTDB-Tk v2.0.0 [17]. That included gene calling for 120 bacterial genes [18], multiple sequence alignment to GTDB genomes, trimming of alignment to approximately 5000 positions, and mapping on GTDB preconstructed tree (release 207). A dataset containing all available *C. oceanisediminis* genomes (from NCBI genome database), except for low-quality and incomplete ones (strains DE0166, DE0392, MN36-4, 1T3), as well as representative genomes of all *Cytobacillus* species and also *S. globispora* genome were used for further phylogeny analysis. Same as in the initial classification gene calling, multiple sequence alignment and trimming of alignment were performed using GTDB-Tk v2.0.0 [17]. Based on this alignment tree was inferred in MEGA 11 [19] using maximum-likelihood approach

under WAG model [20], 1000 bootstrap resamplings were performed. Representation of the tree was polished using ETE3 Toolkit v3.1.2 [21] and Treemmer v0.3 [22]. Average nucleotide identity (ANI) values were calculated using webservice (https://www.ezbiocloud.net/tools/ani) [23]. Digital DNA–DNA Hybridization values were calculated using webservice (https://ggdc.dsmz.de/ggdc.php) [24]. The 16S rRNA gene tree was inferred from Clustal Omega sequence alignment [25] in MEGA 11 [19] using the maximum-likelihood approach under Tamura 3-parameter model [26], with 1000 bootstrap resamples.

Gene annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (build 5742) as well as the RAST v2.0 server [27]. Annotations produced by RAST contained 7124 coding sequences and 144 RNAs, and annotations produced by PGAP contained 5830 coding sequences and 150 RNAs. The genes (28%) were assigned to curated subsystems using RAST annotator. Attention has been paid to methylotrophy due to huge number of genes assigned to subsystem "One-carbon Metabolism" (80 genes) as opposed to number of genes from "Central Carbohydrate Metabolism" subsystem (142 genes). Enzymes associated with methylotrophy were added to the metabolic circuit if their genes were annotated with at least one of the two annotation tools.

Evaluation of Culture Growth

The strain BNO1^T was grown at 37 °C in personal bioreactors RTS-1C (Biosan®, Latvia) using Buffered Peptone Water (Bio-Rad, USA) and Buffered Peptone Water containing 4% of methanol. The devices were calibrated on the same set of culture dilutions and ran simultaneously; cell growth kinetics were registered every 30 min. Obtained growth curves were fitted with Gompertz function [28].

Chemical Analysis of Water

Chemical analysis of water was performed in Dubna Eco-analytical Laboratory of Federal State Budgetary Water Management Institution "Centrregionvodhoz."

Heavy Metal Toxicity Tests

Heavy metal toxicity tests were performed on LB agar plates supplemented with heavy metal ion in concentrations: Cu^{2+}−300 µM, 1 mM, 3 mM, and 5 mM; Pb^{2+}−300 µM, 1 mM, 3 mM, 5 mM, and 9 mM; Zn^{2+}−1 mM, 3 mM, 5 mM, 9 mM, and 27 mM; and Cd^{2+}−100 µM, 300 µM, 500 µM, and 1 mM. Stock solutions were prepared from $CuSO_4$, $Pb(NO_3)_2$, $Cd(NO_3)_2{\cdot}4H_2O$, and $Zn(NO_3)_2{\cdot}6H_2O$ (Sigma-Aldrich®). Experiments were done in duplicate. Plates were checked for growth after 24 h of incubation at 37 °C (Supplementary Fig. 2). The lowest heavy metal concentration that inhibited strain growth was recorded as minimum inhibitory concentration (MIC) of Cu, Pb, Zn, and Cd. The highest heavy metal concentration that did not prevent strain growth was recorded as survival limit. The test for polymetallic resistance was performed with a mixture of metals at a concentration of half the survival limit: copper (1.5 mM), lead (2.5 mM), and cadmium (250 µM).

The Sequence Accession Number

Genome sequence is available in NCBI databases under accession number GCA_023516215.1.

Results

Genome Sequence and Phylogenetic Analysis of the Strain BNO1^T

Genome coverage was × 94.9. As a result of the assembly, two circular contigs were formed, representing a chromosome and a plasmid. The chromosome consists of 5.347.902 bp with a G + C content of 41.49% and the plasmid consists of 342.541 bp (Table 1).

In the initial classification using GTDB-Tk, the isolate was placed into the clade of *Cytobacillus* strains and all three indels specific to the genus *Cytobacillus* were identified, namely in genes coding histidinol dehydrogenase, transcription repair conjugation factor, and serine protease (PDZ-domain) [29]. The BLAST search [30] of the 16S rRNA gene sequence through the NCBI nucleotide database revealed the 100% similarity of the 16S rRNA gene sequence of the strain BNO1^T with *Cytobacillus oceanisediminis* 2691 strain (Fig. 1).

The result of subsequent phylogenetic analysis performed on the dataset of *Cytobacillus* genomes (described in the Materials and Methods section) and the ANI and dDDH values between strains of this dataset and the strain BNO1^T are represented in Fig. 2. Surprisingly, phylogenetic analysis revealed that *C. oceanisediminis* strains presented in the NCBI genome database form two separate phylogenetic clades—groups A and B, with the group A more closely related to *Cytobacillus firmus* than the group B. This placement can be explained by the erroneous assignment of isolated from sedimentary layer of warm sea (South Korea) in 2012 strain 2691 to *C. oceanisediminis* [31]. The type strain of *C. oceanisediminis* CGMCC 1.10115 ^{T} was first described in 2010 (according to biochemical analysis and phylogenetic analysis based on 16S rRNA gene sequences the strain CGMCC 1.10115 ^{T} was classified as a novel species *C. oceanisediminis* [32]). However, the complete genome of *C. oceanisediminis* CGMCC 1.10115 ^{T} was not sequenced

Table 1 General characteristics of the strain BNO1^T genome in comparison with genomes of *C. oceanisediminis* 2691 and *C. oceanisediminis* CGMCC 1.10115 ^{T}

Attribute	BNO1 (GCA_023516215.1)		*C. oceanisediminis* CGMCC 1.10115 ^{T} (GCF_007830235.1)	*C. oceanisediminis* 2691 (GCF_000294775.2)	
	Chromosome	Plasmid	Unplaced	Chromosome	Plasmid
Size, bp	5.347.902	342.541	5.705.120	5.461.741	386.866
Coding percentage (%)	84.4	76.4	81	82.9	75.7
G+C content (%)	41.49	35.59	40.84	41.24	35.81
Total genes	5620	360	5570	5428	390
tRNA genes	108	0	77	106	0
rRNA genes	36	0	17	33	0
Protein-coding genes	5470	360	5297	5222	372
Pseudogenes	861*	0	172	67	18

Table reproduced results from Jung et al. [22] with the addition of strains BNO1^T and CGMCC 1.10115 ^{T}. *—pseudogenes in the present study are defined according to the PGAP annotation pipeline as sequences with frame shifts, incomplete sequences, or containing internal stop codon sequences

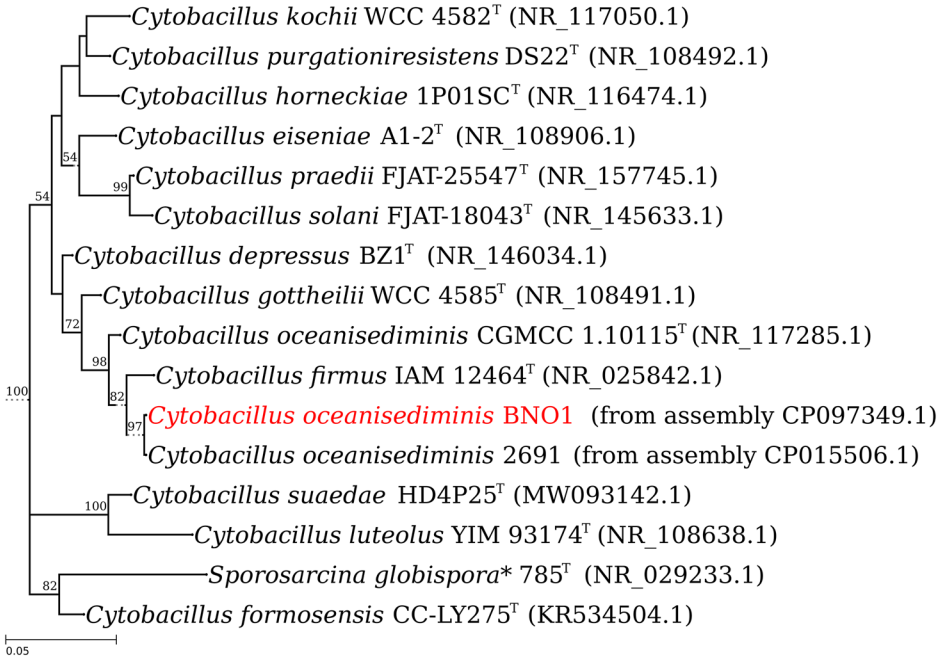

Fig. 1 Phylogenetic tree constructed on 16S rRNA gene sequences of type strains of *Cytobacillus* genera. RefSeq 16S rRNA gene sequences are used when available. *—*S. globispora* genome should be reclassified, as discussed in Fig. 2 description

until 2015 [33]. Therefore, the strain 2691 was assigned to *C. oceanisediminis* in 2012 solely on the basis of the high similarity of 16S rRNA gene sequences between 2691 and CGMCC 1.10115 ^{T} strains, which is not a suitable criterion for identifying members of this family [32, 34].

The DNA–DNA hybridization (DDH) method has been proposed as a reliable way to distinguish between species with high 16S rRNA gene sequences similarity [35] and its complementary digital approaches, Average Nucleotide Identity (ANI) and digital DDH (dDDH) have been shown to correlate with DDH values [36, 37]. In this work, evidence provided by ANI values (using an ANI threshold of 95% to distinguish between species [35]), dDDH values (threshold of 70% to distinguish between species [27]), and phylogenetic analysis suggest that groups A and B belong to different species (Fig. 2). The results of metabolic tests also revealed differences in metabolism of the type strain *C. oceanisediminis* CGMCC 1.10115 ^{T} (group B) and the strain BNO1^T (group A) (Table 2). The presence of methylotrophy genes as well indicates the similarity of strains from group B to each other, in contrast to strain *C. oceanisediminis* CGMCC 1.10115 ^{T} (group A) (Supplementary Table 1).

Thus, we suggest to attribute group A to a different species than group B (that contains the type strain of *C. oceanisediminis* CGMCC 1.10115 ^{T} discovered by Zhang et al. 2010 [32]) and propose to name it *Cytobacillus pseudoceanisediminis* sp. nov. with type strain BNO1^T [38].

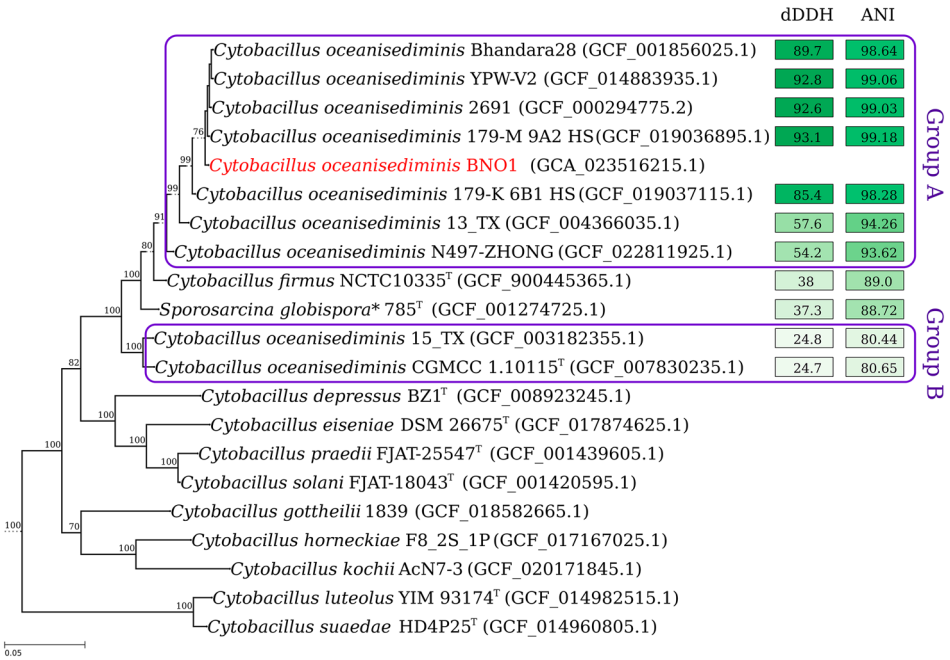

Fig. 2 Phylogenetic tree of NCBI representative species for the genera *Cytobacillus* along with all strains of *Cytobacillus oceanisediminis* and the strain BNO1^T. Four strains of *C. oceanisediminis* (DE0166, DE0392, MN36-4, 1T3) are not represented due to the low quality of assembly. *Cytobacillus formosensis* and *Cytobacillus purgationiresistens* are not represented due to the absence of their genome assemblies in NCBI database. NCBI IDs are shown in brackets, and branch support numbers greater than 50% are shown on nodes. The ANI values of the strains compared to the strain BNO1^T are represented on the right. *—deposited genome of *Sporosarcina globispora* (GCF_001274725.1) apparently belongs to the genera *Cytobacillus*. It contains three indels specific to the genera *Cytobacillus* [23] and does not contain *Sporosarcina*-specific indels [32]. In addition, the genome size of *S. globispora* is significantly larger than that of other *Sporosarcina* species (by 1–3 Mb more) and shares less than 70% of ANI with them. Thus, as suggested by Gupta and Patel [32], *S. globispora* should be reclassified

Chemotaxonomy Characteristics of the Strain BNO1^T

Analysis of 2,6-diaminopimelic acid showed the presence of *meso*-stereoisomer. The major menaquinone of strain BNO1—MK-7. The major fatty acids are anteiso-$C_{15:0}$ (23.34%), iso-$C_{15:0}$ (20.10%), $C_{16:0}$ (11.96%), iso-$C_{16:0}$ (10.88%), and anteiso-$C_{17:0}$ (10.79%) (Supplementary table 2). The major polar lipids were identified as diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine. All chemotaxonomic properties are typical of the genus *Cytobacillus* [32, 39–42].

One-Carbon Metabolism of the Strain BNO1

Genome annotation using Prokaryotic Genome Annotation Pipeline (PGAP) [43] revealed that the strain BNO1^T contains 5470 protein-coding genes (Table 1). Annotation using RAST [27] resulted in the categorization of genes to curated subsystem categories with a huge number of genes assigned to subsystem "One-carbon Metabolism" (80 genes) as opposed to the number of genes from "Central Carbohydrate Metabolism" subsystem (142 genes). Thus, methylotrophy has been proposed as the strain BNO1^T central carbon metabolism. Throughout the characterization, we followed the approach proposed by Chistoserdova [44] where she considered the phenomenon of methylotrophy as the sum of moduli for each step of substrate oxidation and carbon fixation. The scheme of methylotrophic metabolism of the strain BNO1^T is depicted in Fig. 3. Numbers for enzymes in the text are given in parentheses corresponding Fig. 3.

Primary Oxidation

Genome analysis of the strain BNO1^T revealed that it does not contain any known methane-oxidizing genes. For methanol oxidation, the strain BNO1^T has three copies of the gene encoding Fe-containing NAD(P)-dependent alcohol dehydrogenases. Although these genes demonstrated quite low similarity to *Bacillus methanolicus* genes encoding methanol dehydrogenases (about 25%), they have 40% similarity to Gram-negative *Cupriavidus necator* gene coding methanol dehydrogenase (NAD(P)-dependent) [45]. Moreover, the strain BNO1^T also possesses NUDIX hydrolase gene, which is 73% identical to *B. methanolicus* methanol dehydrogenase activator protein ACP. It is also important to note that alcohol dehydrogenases are able to oxidize a number

Table 2 Comparison of physiological properties of the strain BNO1^T with *Cytobacillus oceanisediminis* CGMCC 1.10115 ^{T} and other closely related strains

Characteristic	1	2	3	4	5
Isolation source	Underground spring	Marine sediment	Soil	Soil	Host
Reduction of nitrate	+	+	−	+	−
H_2S production	−	ND	ND	−	−
Oxidase	+	+	−	+	−
Catalase	ND	+	+	+	+
Indole production	−	+	ND	−	−
Acetoin production	−	ND	−	−	+
Enzymes					
Arginine dihydrolase	+	+	−	−	−
Lysine decarboxylase	−	ND	ND	ND	−
Ornithine decarboxylase	−	ND	ND	ND	−
Urease	−	−	−	+	−
Tryptophane deaminase	−	ND	ND	ND	−
Growth at/with					
Temperature range	ND	4–45	10–40	6–40	15–37
Temperature optimum	ND	37	37	32	30
NaCl range	ND	0–13	0–10	0–5.5	0–9
Hydrolysis of					
Gelatin	+	+	−	+	−
Starch	+	+	+	+	−
Glycogen	−	+	ND	+	−
Utilization of citrate	+	−	v	−	−
Acid produced from					
Galactose	−	+	−	−	−
Glycerol	−	w	−	−	−
Inulin	−	+	−	+	−
myo-Inositol	−	+	−	−	−
D-Lactose	−	+	−	−	−
D-Maltose	+	w	−	−	+
D-Mannitol	+	+	v	−	+
D-Mannose	−	−	+	+	−
Sorbitol	−	+	−	−	−
Trehalose	+	w	−	−	−
Xylitol	−	+	−	−	−
DNA G+C content (mol%)	41.5	44.8	43.7	44.5	38.5

Additional substrates for the BNO1^T strain are listed in Supplementary Table 7. Strains: 1, Strain BNO1^T; 2, *Cytobacillus oceanisediminis* 1.10115 ^{T}; 3, *Cytobacillus firmus* ATCC 14575 ^{T}; 4, *Cytobacillus depressus* BZ1^T; 5, *Cytobacillus eiseniae* A1-2 ^{T} [26, 34–36]

+, Positive; *W*, weakly positive; *V*, variable; −, negative; *ND*, no data available

of alcohols, but with different specificities. For example, even classic methanol dehydrogenases of Gram-positive *B. methanolicus* have a higher specificity for oxidation of ethanol rather than methanol [46]. Thus, these data suggest that the strain BNO1^T can have an enzyme with methanol dehydrogenase activity.

The resistance of the strain BNO1^T to methanol was tested by growing the strain in peptone water alone and in peptone water containing 4% methanol. Fitting the growth data with the Gompertz model curve $\log\{ N(t)/N(0) \} = C * \exp(-\exp\{-B * [t - M]\})$ produced B-factor values were 1.22 h^{-1} for peptone water and 1.25 h^{-1} for peptone water with 4% methanol (Fig. 4). Hence, it can be concluded that the strain BNO1^T tolerates a 4% concentration of methanol with slightly decreased value of the optical density at the stationary phase of growth. It is very likely that such resistance of strain BNO1^T is associated with the ability to utilize methanol. In addition, during

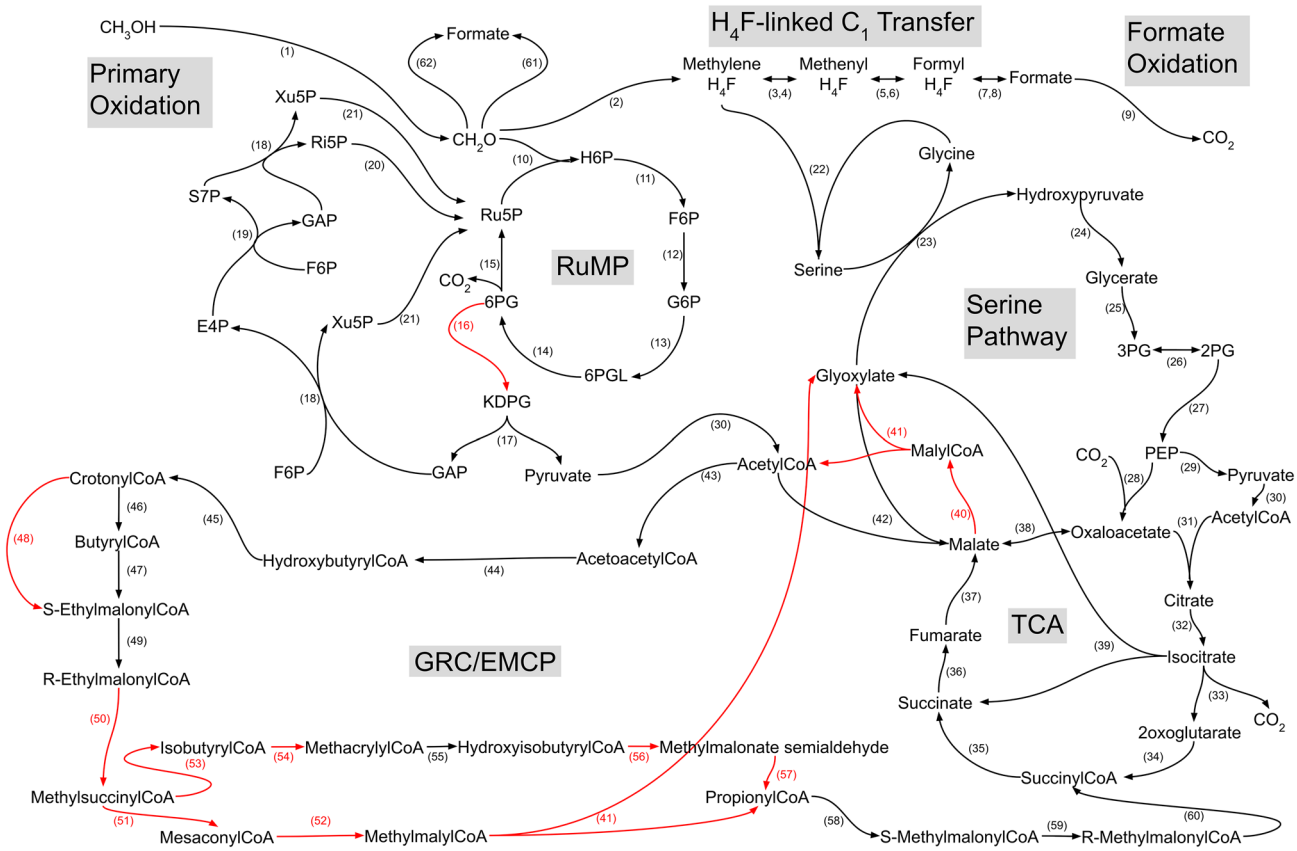

Fig. 3 Scheme of the strain BNO1^T methylotrophy-related pathways. Only methylotrophy pathways with a considerable number of annotated enzymes are shown. Reactions for which enzyme genes were not annotated are shown in red. The names of enzymes and metabolites are listed in Supplementary Tables 5 and 6. *RuMP* ribulose monophosphate pathway, *H4F* tetrahydrofolate pathway, *GRC/EMCP* glyoxylate regeneration cycle/ethylmalonyl-CoA pathway, *TCA* tricarbonic acid cycle (Color figure online)

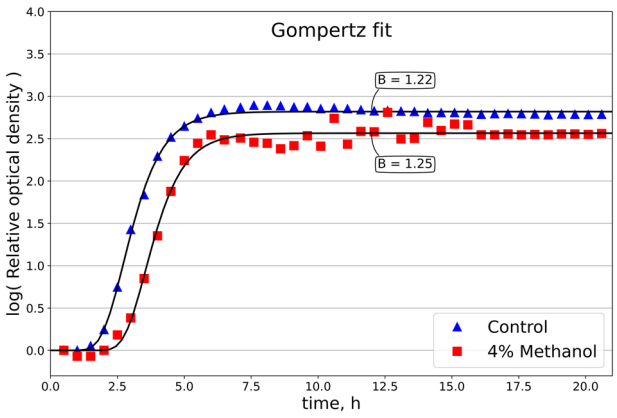

Fig. 4 Growth curves of the strain BNO1^T in peptone water (blue triangles) and in peptone water with 4% methanol (red squares) (Color figure online)

genome analysis the presence of genes coding oxidases of other primary methylated substrates (methylamines, dimethylsulfide, dimethylsulfoniopropionate, methanesulfonic acid) was not detected.

Formaldehyde Oxidation

The genome of the strain BNO1^T contains genes involved in four different pathways associated with the formaldehyde oxidation module (enzyme codes corresponding to Fig. 3 are given in parentheses): NAD-dependent formaldehyde dehydrogenase gene (62), glutathione-dependent formaldehyde dehydrogenase gene (61), dissimilatory ribulose monophosphate pathway genes (13–15), and tetrahydrofolate oxidation pathway genes (3–8). In addition, there are two genes coding two formyl-H4F oxidation enzymes: formyl-tetrahydrofolate cycloligase (*Ftfl*) and formyl-tetrahydrofolate deformylase (*PurU*), the first acts in both directions, while the second only in oxidative direction [44]. NAD-dependent formate dehydrogenase gene was present. Thus, the strain BNO1^T possesses genes for all necessary enzymes for formaldehyde oxidation to carbon dioxide. No trace of

tetrahydromethanopterin formate oxidation pathway genes was found.

Carbon Assimilation

The genome of the strain BNO1^T contains genes involved in two pathways of carbon assimilation (enzyme codes corresponding to Fig. 3 are given in parentheses): Ribulose Monophosphate Pathway (key enzymes: hexulose-phosphate synthase (10) and hexulose-phosphate isomerase (11) and Serine Pathway. Only one gene in assimilatory RuMP, the gene encoding phosphogluconate dehydratase, has not been found (16). It is possible that the activity of phosphogluconate dehydratase can be compensated by enzymes of the same protein family, such as dihydroxy-acid dehydratase (gene encoding dihydroxy-acid dehydratase has been detected). Glyoxylate regeneration for serine pathway is represented via isocitrate-lyase gene of Tricarboxylic Acid Cycle (39) and a number of genes encoding enzymes participating in Ethyl-Malonyl-CoA and Glyoxylate Regeneration pathways was also identified (genes of enzymes of TCA (33–37), genes of enzymes converting acetyl-CoA to ethylmalonyl-CoA (43–49), and all genes of enzymes facilitating conversion of propionyl-CoA to succinyl-CoA (58–60), suggesting that another glyoxylate regeneration pathway except for glyoxylate shunt may be present.

Metabolic tests indicated that the strain BNO1^T can utilize a number of multicarbon substrates (fructose, N-acetylglucosamine, aesculin, maltose, sucrose, trehalose, starch, 5-ketogluconate, L-arginine, citrate, gelatin, D-mannitol) (Table 2). Thus, we can conclude that the strain BNO1^T is a facultative methylotroph and C1 compounds used by the strain BNO1^T likely can be produced by other members of the BNO spring microbe community.

Metal Resistance

The studied strain was found in the underground spring of the Baksan Neutrino Observatory INR RAS and chemical analysis of water from the BNO spring demonstrated that it is highly mineralized (7.3 g/L), and the most abundant ions are calcium and magnesium (represented in Supplementary Table 3 as hardiness) and chloride. The water also contains a certain amount of lead, cadmium, zinc, copper, manganese, and iron ions (Supplementary Table 3).

In the genome of the strain BNO1^T, there are many genes that enable the strain to maintain metal homeostasis. Also about 20% of all plasmid-encoded proteins (that had proper assignments, no "hypothetical protein" assignments) were identified as involved in regulation of concentration of heavy metals; and such a high diversity of genes associated with the homeostasis of various metals (Supplementary table 4) in the strain BNO1^T can serve as a potential source for the creation of genetically engineered constructs for the purpose of bioremediation and biosensors development [47]. For instance, *CadC-T7* genes from closely related 2691 strain have already been successfully used to construct highly specific and sensitive cadmium and lead biosensors for the heavy metals detection [3].

Toxicity tests performed with copper, lead, cadmium, and zinc ions showed that the strain BNO1^T tolerates high concentrations of cadmium, copper, and lead, but completely lacks tolerance of zinc ions (Table 3, Supplementary Fig. 2). The lead survival limit of at least 5 mM places the strain BNO1^T among the most lead-tolerant microorganisms [48–50] and the light coloration of the strain BNO1^T colonies on Petri dishes containing lead indicates no accumulation of lead on the surface of the cells, because otherwise the colonies would be brown [48]. In addition, the strain BNO1^T survives on media containing a combination of copper, cadmium, and lead at half the survival limit concentration and thus the strain also has multi-metal tolerance.

The strain BNO1^T also contains a number of genes providing resistances to various antibiotics: fosfomycin, vancomycin, bleomycin, fosmidomycin, puromycin, tosufloxacin, norfloxacin, penicillin, and streptogramin B. As supposed in work of Jianwen et al. [51], antibiotic resistance genes, along with heavy metal resistance genes, often co-occur as an adaptive mechanism in areas that have been contaminated with various heavy metals for a long time. Since the area near Barsan Neutrino Observatory is contaminated with heavy metals [52], our data support this theory.

Description of *Cytobacillus pseudoceanisediminis* sp. nov.

Cytobacillus pseudoceanisediminis (pseud.o.ce.a.ni.se.di'mi.nis. Gr. masc. adj. *pseudes*, false; N.L. gen. n. *oceanisediminis*, a specific epithet; and N.L. gen. n. *pseudoceanisediminis*, a false (*Cytobacillus*) *oceanisediminis*).

Gram-stain-positive rod-shaped aerobic *Firmicutes* bacterium isolated from deep underground highly mineralized spring of Baksan Neutrino Observatory (North Caucasus). After 24 h at 37 °C on LB agar, colonies are circular, opaque, mud colored, and 2–3 mm in diameter. No diffusible

Table 3 Heavy metal ion survival limits and minimum inhibitory concentrations of the strain BNO1^T

Heavy metal ion	Survival limit, mM	Minimum inhibitory concentration, mM
Cu^{2+}	3	5
Pb^{2+}	5	9
Zn^{2+}	0	1
Cd^{2+}	0.5	1

pigment is observed. The result for the presence of oxidase and arginine dihydrolase is positive. H_2S production, acetoin production, and indole production are negative. Nitrate is reduced. *C. pseudoceanisediminis* can hydrolyze gelatin and starch, but not glycogen and urea and can utilize D-maltose, D-mannitol, trehalose, D-fructose, N-acetylglucosamine, aesculin, D-sucrose, 5-ketogluconate, citrate, sucrose, and L-arginine. The genomic DNA G + C content of the *C. pseudoceanisediminis* is 41.49%. The diagnostic diamino acid of the peptidoglycan is *meso*-diaminopimelic acid. The major menaquinone is MK-7. The phospholipid profile consists of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The major fatty acids are anteiso-$C_{15:0}$ (23.34%), iso-$C_{15:0}$ (20.10%), $C_{16:0}$ (11.96%), iso-$C_{16:0}$ (10.88%), and anteiso-$C_{17:0}$ (10.79%). Bacterium contains several methylotrophy-related pathways, namely serine, ribulose monophosphate, and tetrahydrofolate pathways, and extensive heavy metal ion resistance genes.

The type strain of *C. pseudoceanisediminis* is BNO1^T (= BIM B-1921^T = VKM B-3664^T).

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Author Contributions EK coordinated the overall study, performed the experiments, and prepared and analyzed the data; AY, MZ, and AG collected the samples; KT performed the experiments and bioinformatic analysis; AY performed the experiments; NP and AA performed the chemotaxonomic characterization; EK, KT, AY, and MZ wrote the first draft; EK and KT edited the final version. All authors have read and agreed to the published version of the manuscript.

Declarations

Conflict of interest The authors declare that they do not have any conflict of interest to report.

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