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EXPERIMENTAL ARTICLES

Benzoate-Degrading Bacteria of the Family *Halomonadaceae* Isolated from a Salt Mining Area: Species Diversity and Analysis of the *benA* Genes

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Abstract—Screening of ability to utilize benzoate as the sole carbon and energy source was carried out for 124 strains of the family *Halomonadaceae* (genera *Halomonas*, *Chromohalobacter*, *Salinicola*, and *Kushneria*) isolated from mining sites of the Upper Kama deposit of potassium and magnesium salts. Active growth on benzoate (in the presence of 30–70 g/L NaCl) was shown for 28 *Halomonas* strains closely related to the species *H. taeanensis*, *H. olivaria*, *H. ventosae*, *H. titanicae*, *H. alkaliantarctica*, *H. neptunia*, *H. radicis*, and *H. sulfidaeris*. Strains of the genera *Chromohalobacter*, *Salinicola*, and *Kushneria* either did not grow on benzoate or carried out its transformation (two *Chromohalobacter* strains). PCR screening for the *benA* gene encoding the α-subunit of benzoate 1,2-dioxygenase (1,2-DO), the key enzyme for benzoate degradation, within the family *Halomonadaceae* revealed its presence in all benzoate-degrading *Halomonas* strains. The sequences of the amplified fragments had the highest similarity (not exceeding 95.50%) with the genes encoding the α-subunits of benzoate 1,2-DO, 2-chlorobenzoate 1,2-DO, and other dioxygenases of *Halomonas* strains containing Rieske-type [2Fe-2S] clusters. New data on the genetic systems regulating benzoate degradation in *Halomonas* isolates are of interest for better understanding of molecular mechanisms of aromatics degradation under salinization conditions. The isolated active benzoate degraders may be used to develop the technologies for bioremediation and monitoring of polluted soils.

Keywords: bacterial degraders, benzoate, *benA*, *Halomonadaceae*

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Bacteria of the family *Halomonadaceae* (class *Gammaproteobacteria*) are gram-negative, weakly or moderately halophilic, non-sporulating aerobic microorganisms. Members of this family, which comprises 14 genera, are widely present in saline environments, such as salty lakes, saline soils, solonchaks, marine ice, seafood, marine invertebrates, waste waters, seawater, and hydrothermal springs (de la Haba et al., 2014). Bacteria of the family *Halomonadaceae* have been attracting researchers' attention due to their ability to synthesize osmoprotectors (ectoine, glycine betaine, and hydroxyectoine), biopolymers (exopolysaccharides and polyhydroxyalkanoates), and biosurfactants, as well as to mediate degradation of aromatic compounds (García et al., 2005; de la Haba et al., 2014; Monzón et al., 2018). These bacteria are considered promising agents for biotechnological purposes, for instance, for remediation of saline soils and water bodies from toxic organic pollutants. A large number of studies have reported degradation of aromatic compounds by members of the family *Halomonadaceae*, either individually or in bacterial associations (Le Borgne et al., 2008; Fathepure, 2014). An important intermediate produced by bacterial oxidative catabolism of many aromatic compounds, including environmental pollutants, such as phenol, toluene, biphenyl, or phthalates, is benzoate (Moreno et al., 2011; Li et al., 2013). Benzoate accumulation in the environment is also related to metabolic activity of plants, as well as to anthropogenic activity, since benzoic acid and its derivatives serve as a source for production of a broad range of chemical compounds; they are also used in food preservation, medicine, and perfumery industry (García et al., 2005; Le Borgne, 2008; Li et al., 2013). Among *Halomonadaceae*, the ability to degrade benzoate has been described in *H. halodurans* ATCC 29686^T (Rosenberg, 1983), *H. organivorans* CECT 5995^T (Moreno et al., 2011), *H. elongata* A15.6, *H. eurihalina* A17.6, A25.2 (Garcia et al., 2005), *H. campisalis* ATCC 700597^T (Oie et al., 2007), *Halomonas* sp. KHS3 (Monzón et al., 2018), *Chromohalobacter salexigens* DSM 3043^T (Csonka et al., 2005), and *Chromohalobacter* sp. HS-2 (Kim et al., 2008). However, the currently available information on the structure and functioning of the genetic systems and the metabolic pathways that mediate the degradation

of aromatic compounds under conditions of saline environments in this microbial group is extremely limited (Fathepure, 2014).

The metabolic pathway of benzoate degradation most commonly occurring in prokaryotes begins with incorporation of hydroxy groups into the chemically stable aromatic ring of the molecule with production of dihydroxybenzoate (catechol); this step is mediated by benzoate 1,2-dioxygenase (benzoate 1,2-DO; EC 1.14.12.10) and dihydroxybenzoate dehydrogenase (EC 1.3.1.25) (https://www.genome.jp/pathway/map00362+C00180). It was shown that the substrate specificity of benzoate 1,2-DO is determined by its α-subunit (Parales and Resnick, 2006); therefore, the *benA* gene encoding this subunit is frequently employed as a genetic marker indicating the possibility of benzoate 1,2-DO induction in bacterial cells.

Previously, individual bacterial strains of the family *Halomonadaceae* and bacterial consortia that included members of this family were isolated at the mining site of the Upper Kama deposit of potassium and magnesium salts, which is characterized by a high salinity level and the presence of various organic pollutants, including mono- and polyaromatic compounds (Anan'ina et al., 2005; Bachurin and Odintsova, 2009; Korsakova et al., 2013; Olsson et al., 2017; Pyankova et al., 2020). Several strains of the genus *Halomonas* were capable of growing on naphthalene, *ortho*-phthalic, protocatechuic, gentisic, and benzoic acids as sole sources of carbon and energy (Yastrebova et al., 2019).

The goal of the present work was to characterize benzoate-degrading bacteria of the family *Halomonadaceae* isolated from various ecotopes of the salt mining area of the Upper Kama deposit and to describe the diversity of key genes of benzoate degradation (*benA*) in these halophilic strains.

MATERIALS AND METHODS

Specimens. The study was conducted in bacteria of the family *Halomonadaceae* (genera *Halomonas*, *Chromohalobacter*, *Kushneria*, *Salinicola*) from the working collection of the Laboratory of Microbiology of Technogenic Ecosystems (Institute of Ecology and Genetics of Microorganisms). These bacterial strains were isolated from various samples collected in the salt mining area of the Upper Kama deposit (near the towns of Solikamsk and Bereziniki, Perm krai): plant rhizosphere, soil, ground, clay bottom sediments of mine brine tanks, production waste (slurry storages, salt tailing piles), and brine ponds (Anan'ina et al., 2005; Korsakova et al., 2013; Olsson et al., 2017; Yastrebova et al., 2019; Pyankova et al., 2020). The study also involved the type strains *Halomonas taeanensis* DSM 16463^T, *Chromohalobacter canadensis* DSM 6769^T, *Chromohalobacter beijerinckii* DSM7218^T, *Chromohalobacter japonicus* CECT7219^T, *Chromohalobacter salarius* CECT5903^T, *Salinicola socius* SMB35^T, and *Salinicola salarius* DSM18044^T.

Benzoate-degrading capacity was assessed by culturing the bacteria in liquid Raymond's mineral medium (RMM) (Raymond, 1961) with different concentrations of NaCl (30–150 g/L), as well as without salt addition. Benzoate (in the form of 10% sodium benzoate solution) was added to the final concentration of 1 g/L. Cultures were grown for 14 days in a thermostated shaker at 28°C and 140 rpm. Growth was evaluated by measuring the optical density (*OD*) of the culture liquid at the wavelength of 600 nm using a UV-Visible BioSpec-mini spectrophotometer (Shimadzu, Japan) and a cell with the optical pathway length of 1 cm.

To assess benzoate utilization, bacterial cells were removed from the culture liquid by centrifugation at 9660 *g* for 3 min in a miniSpin centrifuge (Eppendorf, Germany). The presence of benzoate in the supernatant was determined using HPLC analysis in the acetonitrile–0.1% H_3PO_4 (60 : 40) system on an LC-20AD Prominence chromatograph (Shimadzu) with a C-18 column 150 × 4.6 mm (Sigma-Aldrich, United States) and a SPD-20A UV detector (at 205 nm). The substrate amounts were evaluated based on the height of the chromatogram peaks and the area under the peaks in comparison to the standard curve obtained for 0.01% benzoate solution in water. Experiments were performed in three replicates.

Identification of benzoate-degrading bacteria. DNA was isolated from pure bacterial cultures using the conventional technique (*Short Protocols in Molecular Biology*, 1995). Bacteria were identified based on analysis of the nucleotide sequences of the 16S rRNA genes (fragment length, 915–1418 bp). Fragments of the 16S rRNA gene were amplified by PCR with the universal bacterial primers 27F (5'-AGAGTTTGATC(A/C)TGGCTCAG-3') and 1492R (5'-ACGG(C/T)TACCTTGTTACGACTT-3') (Lane, 1991) using a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, United States). Electrophoresis, sequencing, and analysis of the obtained 16S rRNA gene sequences were performed as described below.

The key genes of benzoate degradation (*benA*) were studied by amplifying a 521-bp-long fragment of the gene encoding the α-subunit of benzoate 1,2-DO with the primers benA-F (5'-GCCCACGAGAGCCAGATTCCC-3') and benA-R (5'-GGTGGCGGCGTAGTTCCAGTG-3') as described (Baggi et al., 2008) with subsequent sequencing of the amplicons and analysis of their sequences.

PCR products were detected by horizontal electrophoresis in a 1% agarose gel in 1× TBE buffer (Tris, 10.8 g/L, borate, 5.5 g/L, 0.5 M EDTA, 4 mL, distilled water, 79.7 mL/L) at 5–15 V/cm and room temperature for 20–40 min. The gels were stained with 0.5 μg/mL ethidium bromide solution for 10–15 min and photographed in UV light using the BioDocAna-

lyze gel documentation system (Bio-Rad Laboratories). The size of the amplified fragments was determined using the 100+ bp DNA Ladder (Eurogene, Russia).

Nucleotide sequences of 16S rRNA and *benA* genes were determined on an automated Genetic Analyzer 3500XL system (Applied Biosystems, United States) using the Big Dye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems) according to the manufacturer's protocol. Phylogenetic analysis of the 16S rRNA and *benA* gene sequences was conducted using the programs Sequence Scanner v. 2.0. and MEGA 7.0 (http://www.megasoftware.net), as well as the BLAST service (http://www.ncbi.nlm.nih.gov). The search for homologous sequences was performed in the international databases EzBioCloud (http://www.ezbiocloud.net) and GenBank (http://www.ncbi.nlm.nih.gov). Phylogenetic trees were constructed using the Neighbor-Joining algorithm of MEGA 7.0. Statistical significance of the tree branching was assessed by bootstrap analysis based on 1000 alternative trees.

The nucleotide sequences of the 16S rRNA and *benA* genes of the strains studied were deposited in the GenBank database under the accession numbers MW757272–MW757286, MZ359852–MZ359857 (16S rRNA gene), and MW862486–MW862498 (*ben A*).

RESULTS AND DISCUSSION

Screening for the ability to utilize benzoate as growth substrate. One hundred and twenty four strains of the family *Halomonadaceae* (genera *Halomonas*, *Chromohalobacter*, *Salinicola*, and *Kushneria*) isolated previously from various ecotopes of the salt mining area of the Upper Kama deposit, as well as seven type strains of the genera *Halomonas*, *Chromohalobacter*, and *Salinicola*, were tested for their ability to grow on benzoate as the sole carbon and energy source. Among the 63 analyzed strains representing different species of the genus *Halomonas*, 29 strains (including *H. taeanensis* DSM 16463^T) exhibited growth on RMM with benzoate in the presence of 30 g/L NaCl. Growth on benzoate was observed in bacteria closely related to the type strains of the species *H. taeanensis*, *H. olivaria*, *H. ventosae*, *H. titanicae*, *H. alkaliantarctica*, *H. neptunia*, *H. radicis*, and *H. sulfidaeris*, but not in members of *H. venusta*, *H. hydrothermalis*, *H. utahensis*, *H. alimentaria*, *H. meridiana*, *H. piezotolerans*, and *H. songnenensis*. Twenty-two active benzoate degraders of the genus *Halomonas* were next investigated in greater detail (Table 1). Among 52 analyzed strains of the genus *Chromohalobacter* (closely related to *C. beijerinckii*, *C. japonicus*, *C. salarius*, and *C. canadensis*) and four type strains of the genus, three strains of *C. canadensis* (including the type strain *C. canadensis* DSM 6769^T) were capable of degrading (transforming) benzoate, as indicated by substrate depletion and changing color of the medium in the course of cultivation (Table 1). No benzoate-degrading activity was detected in six members of the genus *Salinicola* closely related to the species *S. halophyticus*, *S. socius*, and *S. salarius*, the type strains *S. socius* SMB35^T and *C. salarius* CECT 5903^T, as well as in three strains of the genus *Kushneria* closely related to *K. phyllosphaerae* EAod3^T.

Characterization of benzoate-degrading bacteria of the genus *Halomonas*. Bacteria of the genus *Halomonas* are moderately halophilic microorganisms capable of growing both in the absence of salt and in the presence of high sodium chloride concentrations of up to 325 g/L (de la Haba et al., 2014). We found that all the strains isolated from various ecotopes of the salt mining area could grow on Raymond's rich medium (Yastrebova et al., 2019) with 30 to 150 g/L NaCl. Several strains (*Halomonas* spp. PD13-5, PMK3, D2, 610-2, 61g, and *H. taeanensis* DSM 16463^T) did not grow in the absence of salt, and the optimal NaCl concentration in the medium was 70–150 g/L. Furthermore, the strains NDT27, D2, NDT28, NDT31, and *H. taeanensis* DSM 16463^T were able to grow at higher NaCl concentrations (200–300 g/L). The strains were tested for their ability to grow on RMM with benzoate in the presence of various NaCl concentrations (Table 1). For most strains, the highest biomass yield as determined by the optical density of the culture was observed in the medium containing 30 g/L NaCl. A the same time, a number of strains (NDT27, D2, BNL26, BBL18, BBL22, M56-2, M135-4Nt, 61g, and 610-2) exhibited active growth at higher salt concentrations: 50 and 70 g/L NaCl. None of the strains studied could grow on benzoate at salt concentrations of 100 g/L and higher.

In this study, we determined and analyzed nearly complete 16S rRNA gene sequences (1350–1418 bp) of 14 benzoate-degrading strains: BBL18, BNL26, BNL3-2, BFL1, PD13-5, NDT13, BBL22, NDT27, D2, 610-2, 61g, NDT21, NDT31, and NDT28. The strains SMB56, M56-2, M135-4Nt, SMB61, TC193, and TC195 were for the first time identified based on analysis of their 16S rRNA genes. Comparison of the nucleotide sequences of the 16S rRNA genes of the bacteria studied (915–1418 bp long) and the type strains of the genus *Halomonas* (http://www.ezbiocloud.net) showed that active benzoate degraders were phylogenetically close to the species *H. alkaliantarctica*, *H. alkaliphila*, *H. neptunia*, *H. olivaria*, *H. sulfidaeris*, *H. taeanensis*, *H. titanicae*, *H. ventosae*, and *H. radices*. Based on the 16S rRNA gene sequences, six strains had 99.24–100% identity to the marine strain *H. titanicae* BH1^T (Sanchez-Porro et al., 2010), four strains were closely related (99.29–100% identity) to *H. taeanensis* DSM 16463^T (Lee et al., 2005), and five strains had 99.62–99.85% identity to *H. alkaliantarctica* CRSS^T isolated from an Antarctic salt lake

Table 1. Growth of bacterial strains of the family *Halomonadaceae* in the presence of various NaCl concentrations

Strain	Raymond's rich medium, NaCl (g/L)	Raymond's mineral medium with benzoate (1 g/L), NaCl (g/L)			
		no NaCl	30	50	70
Strains closely related to the type strain of *Halomonas taeanensis*					
H. taeanensis DSM 16463^T	30–250	–	+++	++	+
SMB56	0–150	+	++	++	+
NDT27	0–250	+	++	+++	++
D2	30–300	–	+++	+++	++
Strains closely related to the type strain of *H. olivaria*					
NDT31	0–200	++	++	+	+
NDT21	0–150	+	+	–	–
Strains closely related to the type strain of *H. ventosae*					
610-2	30–150	–	+++	+++	+
PMK3	30–150	–	++	++	+
Strains closely related to the type strain of *H. titanicae*					
BNL26	0–150	++	+++	+++	++
BBL18	0–150	++	++	++	++
NDT13	0–150	+	+	–	–
BNL3-2	0–150	+	+	–	–
M125-1	0–150	–	+	+	+
PD13-5	30–150	–	+	+	+
Strains closely related to the type strain of *H. alkaliantarctica*					
BBL22	0–150	+	+++	+++	++
8CN1-1	0–150	+	+	+	+
6CN3-12	0–150	+	+	–	–
5RN1	0–150	+	+	–	–
Strains closely related to other species of the genus *Halomonas*					
M135-4Nt (*H. sulfidaeris*)	0–150	–	+++	+++	+
NDT28 (*H. neptunia*)	0–200	++	++	+	+
M56-2 (*H. alkaliphila*)	0–150	–	+++	+++	+
61g (*H. radicis*)	30–150	–	+	+++	+
Strains closely related to the type strain of *Chromohalobacter canadensis*					
C. canadensis B201	30–300	–	– dcm*	– dcm	–
C. canadensis 55	30–300	–	– dcm*	– dcm	–
C. canadensis DSM 6769^T	30–300	–	– dcm*	–	–

–, no growth detected; +, OD_{600} from 0.1 to 0.3 units; ++, OD_{600} from 0.4 to 0.7 units; +++, OD_{600} higher than 0.7 units; dcm, dark coloration of the medium.
* Data on benzoate utilization are provided in the text.

(Poli et al., 2007). Other strains exhibited high (99.02–100%) similarity to the species *H. olivaria* (strains NDT21 and NDT31, 99.93–100%), *H. ventosae* (strains 610-2 and PMK3, 99.06–99.42%), *H. neptunia* (NDT28, 99.57%), *H. sulfidaeris* (M135-4Nt, 99.02%), and *H. alkaliphila* (M56-2, 99.79%). It should be mentioned that the 16S rRNA gene sequence (1408 bp) of the strain 61g isolated from the bottom sediment of the brine collector at the mining site had low similarity (98.41%) to the closest type

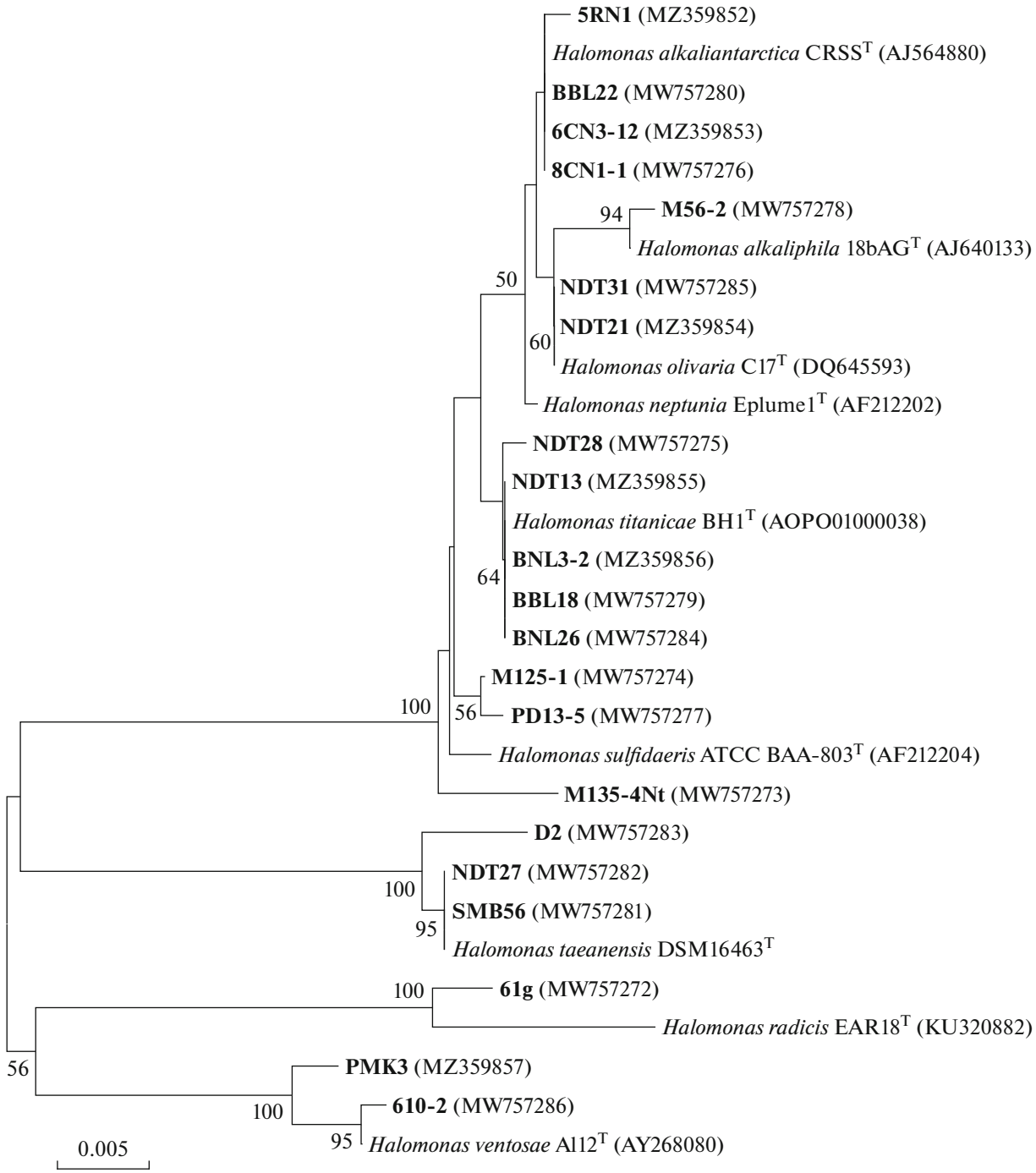

Fig. 1. Positions of the studied strains of the genus *Halomonas* in the phylogenetic tree constructed using the neighbor-joining approach based on comparison of the nucleotide sequences of their 16S rRNA genes. The evolutionary distances were calculated using the Jukes-Cantor method. Numbers indicate the statistical significance of the branching order determined by bootstrap analysis of 1000 alternative trees (only the values higher than 50% are shown). The scale bar corresponds to 5 substitutions per 1000 nucleotides. The GenBank accession numbers are given in parentheses.

strain *H. radicis* EAR18^T (Navarro-Torre et al., 2020), suggesting that this strain may represent a novel taxon of the family *Halomonodaceae*. The positions of the strains studied and the closely related type strains of the genus *Halomonas* in the phylogenetic tree constructed using the neighbor-joining method are shown in Fig. 1.

Diversity of *benA* genes in bacteria of the genus *Halomonas*. PCR amplification of the *benA* genes encoding the α-subunit of benzoate 1,2-DO showed

that all 22 benzoate-degrading strains of the genus *Halomonas*, including *H. taeanensis* DSM 16463^T, possessed *benA* genes. Twelve strains of different species affiliation (Table 2) were selected for further sequencing and sequence analysis of their *benA* gene amplicons. The sequences of these *benA* amplicons were compared to homologous sequences from the GenBank database, and it was found that the sequences in question had most similarity (no higher than 97.28%) to genes encoding the α-subunits of benzoate 1,2-DO, 2-chlorobenzoate 1,2-DO, and a Rieske cluster-containing protein of bacteria of the genus *Halomonas* (Table 2). The sequences of the *benA* genes of the strains BBL18, M135-4Nt, and *H. taeanensis* DSM 16463^T exhibited similarity to genes of a strain representing the genus *Chromohalobacter* (*C. salexigens* 40a_TX): benzoate/toluate 1,2-DO (83.82–82.04%) and benzoate 1,2-DO (80.87 and 82.25% for BBL18 and *H. taeanensis* DSM 16463^T, respectively). The *benA* and homologous genes of other members of *Gammaproteobacteria*, as well as of strains representing *Betaproteobacteria*, *Alphaproteobacteria*, and *Actinobacteria*, had lower level of identity to the *benA* genes studied: the highest value of 80.54% was observed for *Marinobacter hydrocarbonoclasticus* strain ATCC 49840^T, class *Gammaproteobacteria* (Table 3).

The phylogenetic tree in Fig. 2 was constructed for the translated amino acid sequences (TASs) encoded by the *benA* genes of the benzoate-degrading strains of the genus *Halomonas*, as well as by the closest homologous genes of *Halomonas* members and of species representing other taxa of proteobacteria (classes *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*) and actinobacteria. In this tree, *benA* TASs of *Halomonadaceae* strains (genera *Halomonas* and *Chromohalobacter*) formed a separate phylogenetic group clearly divided in several clusters (Fig. 2). In particular, the largest cluster included TASs of eight strains: NDT28, M56-2, M125-1, M135-4Nt, PD13-5, 8CN1-1, BBL18, and BBL22, which are phylogenetically close to different species of the genus *Halomonas* (*H. alkaliantarctica*, *H. alkaliphila, H. neptunia*, *H. sulfidaeris, H. titanicae*, and *H. utahensis*) based on their 16S rRNA gene sequences. A BLAST search in the genomes of the type strains of these species showed that they lacked *benA* genes. At the same time, TASs closely related to this group of genes were detected in the genomes of *H. sulfidaeris* SST4 (QNTU01000040) and *Halomonas* sp. HL-93 (LJST01000014) (Fig. 2). The *benA* sequences of this cluster had the highest level of identity to the genes of the large subunit of benzoate/2-halobenzoate 1,2-DO and a Rieske cluster-containing protein of *Halomonas* strains isolated from marine ecosystems (Table 2, Fig. 2). The amino acid sequences encoded by the *benA* genes of the strains BBL18 and BBL22 formed a separate branch together with the sequences of 2-halo(chloro)benzoate 1,2-DO of two *Halomonas* strains (Fig. 2).

Based on the 16S rRNA gene sequences, the strains SMB56, NDT27, and D2 are phylogenetically close to *H. taeanensis* DSM 16463^T, and the strain 610-2 is close to *H. ventosae* Al12^T. Comparison of the *benA* sequences of these strains and of *H. taeanensis* DSM 16463^T to homologous sequences from the GenBank database showed that their similarity was the highest to the genes of the α-subunit of benzoate 1,2-DO of the strain *H. aestuarii* Hb3 isolated from a solar saltern in South Korea and the phenol-degrading strain *H. organivorans* CECT 5995^T isolated from saline soil in the south of Spain, as well as to a gene encoding a Rieske cluster-containing protein in a *Halomonas* sp. strain BM-2019 isolated from lake water in Tanzania (Table 2). In the phylogenetic tree, the *benA* TASs of the strains SMB56, NDT27, and D2 (closely related to *H. taeanensis*) and that of the strain 610-2 (closely related to *H. ventosae*) formed two separate clusters (Fig. 2). Interestingly, analysis of the genomes of the *Halomonas* strains present in the GenBank database (https://www.ncbi.nlm.nih.gov/) revealed that the type strain *H. ventosae* CECT 5797^T (SNZJ01000041) and the strain *H. ventosae* USBA 854 (PVTM01000013) possess genes of the α-subunit of benzoate/toluate 1,2-DO. The level of identity between the *benA* sequences of the strain 610-2 and the homologous sequences detected in the genomes of *H. ventosae* strains was 89.66–90.44% (Table 2). In the phylogenetic tree, the corresponding TASs belonged to the same cluster as the *benA* TAS of strain 610-2 (Fig. 2). It was also found that the *benA* TASs of the strains SMB56, NDT27, D2, and the type strain *H. taeanensis* DSM 16463^T (FNCI01000003) formed a separate branch in the tree. The nucleotide sequences of the *benA* genes of these strains had 96.13–98.73% homology. Interestingly, these strains also formed a single cluster in the tree constructed based on the 16S rRNA gene sequences (Fig. 1). Thus, it was established that the *benA* genes of the strains closely related to *H. taeanensis* on the one hand and to *H. ventosae* on the other hand are phylogenetically distinct from each other, as well as from those of other species of the genus *Halomonas*.

Characterization of benzoate-degrading bacteria of the genus *Chromohalobacter*. Among the 52 *Chromohalobacter* strains isolated from various ecotopes of the salt mining area (Korsakova et al., 2013; Olsson et al., 2017; Pyankova et al., 2020), a majority of 35 strains were closely related to *C. canadensis* (99.40–99.93% 16S rRNA gene sequence identity to *C. canadensis* ATCC 43984^T), 16 strains were related to *C. japonicus* (99.54–99.78% identity to *C. japonicus* 43^T), and one strain was close to *C. beijerinckii* (99.85% identity to *C. beijerinckii* ATCC 19372^T). The preliminary screening detected only two strains closely related to *C. canadensis* that were capable of degrading (trans-

Table 2. Comparison of the *benA* gene sequences of strains of the genus *Halomonas* to homologous sequences from the GenBank database

Strains	Homologous genes in GenBank	GenBank acc. no.	Identity, %	Isolation site	Reference
	Gene of the α-subunit of 2-halobenzoate 1,2-DO of *Halomonas* sp. KO116	CP011052	92.15 94.67 94.46 93.32 94.03 93.39 95.50 93.66	Surface water of the Mediterranean Sea, delta of the Nile, Egypt	O'Dell et al., 2015
	Gene of the α-subunit of 2-halobenzoate 1,2-DO of *H. titanicae* GPM3	CP054580	91.88 94.46 94.46 93.50 94.03 93.39 95.09 94.08	Red alga *Pyropiatenera,* South Korea	ND
NDT28 M56-2 M125-1 M135-4Nt	Gene of a Rieske [2Fe-2S] cluster-containing protein of *Halomonas* sp. PA16-9	CP040451	91.88 94.68 94.66 93.91 94.24 92.98 93.87 91.97	Bottom sediment, Pacific Ocean	ND
PD13-5 8CN1-1 BBL18 BBL22	*benA* of *H. sulfidaeris* SST4	QNTU01000040	92.15 94.01 94.46 93.70 94.03 93.80 95.30 92.60	Marine sediments, Antarctica	Abdel-Mageed et al., 2020
	benA of *Halomonas* sp. HL-93	LJST01000014	ND 87.58 87.01 86.50 86.60 85.42 87.63 ND	Microbial mat of a hypersaline lake, United States	Nelson et al., 2015
	Gene of the α-subunit of benzoate/toluate 1,2-DO of *H. ventosae* CECT 5797^T	SNZJ01000041	86.70 88.99 89.63 89.08 89.60 88.02 88.64 ND	Saline soil, Spain	ND

Table 2. (Contd.)

Strains	Homologous genes in GenBank	GenBank acc. no.	Identity, %	Isolation site	Reference
	Gene of the α-subunit of benzoate/toluate 1,2-DO of *H. ventosae* USBA 854	PVTM01000013	91.02 90.00 90.59 90.42 90.00 92.94 91.02 ND	Saline spring, the Andes, Columbia	ND
DCM16463^T NDT27 D2 DR1 SMB56 610-2	*benA of H. aestuarii Hb3*	CP018139	89.48 89.00 89.38 89.32 88.91 88.71	Solar saltern, South Korea	ND
	benA of *H. organivorans* CECT 5995^T	FN997646	85.92 87.68 86.74 86.86 87.37 84.70	Saline soil, southern Spain	Moreno et al., 2011
	Gene of a Rieske [2Fe-2S] cluster-containing protein of *Halomonas* sp. BM-2019	CP071922	86.56 88.38 87.90 87.82 87.03 86.34	Lake water, Tanzania	ND
	benA of *H. sulfidaeris* SST4	QNTU01000040	82.85 84.06 83.44 83.54 83.92 ND	Marine sediments, Antarctica	Abdel-Mageed et al., 2020
	Gene of the α-subunit of benzoate/toluate 1,2-DO of *H. ventosae* CECT 5797^T	SNZJ01000041	ND 89.00 88.96 88.89 88.49 90.44	Saline soil, Spain	ND
	Gene of the α-subunit of benzoate/toluate 1,2-DO of *H. ventosae* USBA 854	PVTM01000013	ND 90.80 89.66 89.66 90.59 89.66	Saline spring, the Andes, Columbia	ND
	Gene of the α-subunit of benzoate/toluate 1,2-DO of *H. taeanensis* BH539^T	FNCI01000003	100 97.28 94.86 94.83 97.28 83.98	Solar saltern, Korea	ND

ND, no data.

Table 3. Comparison of the nucleotide sequences of the *benA* genes of strains of the genus *Halomonas* (family *Halomonadaceae,* class *Gammaproteobacteria*) to the closest homologous sequences of bacteria of other taxa (BLAST analysis)*

Strain	Homologous genes in GenBank	Class	GenBank accession no.**	Identity, %
M135-4Nt	Gene of benzoate/toluate 1,2-DO of *Chromohalobacter salexigens* 40a TX	*Gammaproteobacteria*	QGTY01000001/ PWW42699	82.18
	Gene of benzoate/toluate 1,2-DO of *Marinobacter hydrocarbonoclasticus* ATCC 49840^T	*Gammaproteobacteria*	FO203363/ CCG93781	80.54
	Gene of a Rieske [2Fe-2S] cluster-containing protein of *Pseudomonas* sp. DTU12.1	*Gammaproteobacteria*	CP045254/ QHG23773	80.25
	Gene of a Rieske [2Fe-2S] cluster-containing protein of *Oceanimonas* sp. GK1	*Gammaproteobacteria*	CP003171/ AEY00109	79.21
	benA of *Pseudogulbenkiania* sp. NH8B	*Betaproteobacteria*	AP012224/ BAK76567	77.50
	benA of *Polaromonas naphthalenivorans* CJ2^T	*Betaproteobacteria*	CP000529/ ABM37421	77.41
BBL18	Gene of benzoate/toluate 1,2-DO of *Chromohalobacter salexigens* 40a_TX	*Gammaproteobacteria*	QGTY01000001/ PWW42699	83.82
	benA of *Chromohalobacter salexigens* 40a TX	*Gammaproteobacteria*	QGTY01000032/ PWW32861	80.87
	Gene of a Rieske [2Fe-2S] cluster-containing protein of *Pseudomonas* sp. DTU12.1	*Gammaproteobacteria*	CP045254/ QHG23773	79.47
	Gene of benzoate/toluate 1,2-DO of *Marinobacter hydrocarbonoclasticus* ATCC 49840^T	*Gammaproteobacteria*	FO203363/ CCG93781	78.31
	Gene of benzene 1,2-DO of *Zobellella denitrificans* F13-1	*Gammaproteobacteria*	CP012621/ ATG72450	78.12
	Gene of benzene 1,2-DO of *Kocuria palustris* MU14/1	*Actinobacteria*	CP012507/ ALB03862	77.92
	benA of *Rhodococcus opacus* KT112-7	*Actinobacteria*	CP072193/ ND	77.51
	benA of *Pseudarthrobacter* sp. NIBRBAC000502771	*Actinobacteria*	CP041187/ QDG62019	77.44
	Gene of a Rieske [2Fe-2S] cluster-containing protein of *Oceanimonas* sp. GK1	*Gammaproteobacteria*	CP003171/ AEY00109	77.18
	Gene of 2-halobenzoate 1,2-DO of *Serratia rubidaea* NCTC10848	*Gammaproteobacteria*	LS483492/ SQJ16490	76.76
	Gene of a Rieske [2Fe-2S] cluster-containing protein of *Comamonas piscis* BIGb0172	*Betaproteobacteria*	CP058554/ QMV73311	76.53
	benA of *Rhizobium leguminosarum* bv. *trifolii* 22B	*Alphaproteobacteria*	CP050092/ QIO68893	76.12
	benA of *Citricoccus* sp. SGAir0253	*Actinobacteria*	CP039424/ QCU78183	75.91
610-2	Gene of benzoate/toluate 1,2-DO of *Massilia* sp. WG5	*Betaproteobacteria*	CP012640/ ALK97586	78.92
	benA of *Pseudomonas sihuiensis* KCTC 32246^T	*Gammaproteobacteria*	LT629797/ SDU90471	75.95
	benA of *Rhodococcus* sp. DMU1	*Actinobacteria*	CP050952/ QIX52496	75.80
	Gene of 2-halobenzoate 1,2-DO of *Sinorhizobium americanum* CCGM7	*Alphaproteobacteria*	CP013054/ APG87352	75.00

Table 3. (Contd.)

Strain	Homologous genes in GenBank	Class	GenBank accession no.**	Identity, %
DSM 16463T	*benA* of *Chromohalobacter salexigens* 40a TX	*Gammaproteobacteria*	QGTY01000032/ PWW32861	82.25
	Gene of benzoate/toluate 1,2-DO of *Chromohalobacter salexigens* 40a TX	*Gammaproteobacteria*	QGTY01000001/ PWW42699	82.04
	Gene of a Rieske [2Fe-2S] cluster-containing protein of *Pseudoxanthomonas spadix* BD-a59	*Gammaproteobacteria*	CP003093/ AER57629	77.13
	benA of Pseudomonas fluorescens PF08	*Gammaproteobacteria*	CP032618/ AYG08044	76.99
	Gene of benzoate/toluate 1,2-DO of *Marinobacter hydrocarbonoclasticus* ATCC 49840^T	*Gammaproteobacteria*	FO203363/ CCG93781	76.70
	Gene of benzene 1,2-DO of *Zobellella denitrificans* F13-1	*Gammaproteobacteria*	CP012621/ ATG72450	76.23
	Gene of a Rieske [2Fe-2S] cluster-containing protein of *Aeromonas simiae* A6	*Gammaproteobacteria*	CP040449/ QFI54779	76.16
	Gene of a Rieske [2Fe-2S] cluster-containing protein of *Oceanimonas* sp. GK1	*Gammaproteobacteria*	CP003171/ AEY00109	76.01
	benA of *Rhizobium leguminosarum* Vaf10	*Alphaproteobacteria*	CP016287/ ANP89222	75.88
	Gene of benzoate/toluate 1,2-DO of *Burkholderia gladioli* pv. *gladioli* KACC 11889	*Betaproteobacteria*	CP022006/ ASD81778	75.72
	benA of *Sphingobium wenxiniae* JZ-1^T	*Alphaproteobacteria*	KJ620836/ AHZ46835	75.62
	benA of *Achromobacter* sp. AONIH1	*Betaproteobacteria*	CP026124/ AUT46373	75.35
	Gene of 2-halobenzoate 1,2-DO of *Raoultella terrigena* NCTC 13098	*Gammaproteobacteria*	LR131271/ VDR27448	75.15

ND, no data. * Table 3 includes gene sequences with at least 75% identity to the nucleotide sequences studied. ** Nucleotide/amino acid sequences.

forming) benzoate (Table 1). The signs of benzoate degradation were substrate loss and appearance of dark coloration of the medium that increased in the course of strain cultivation in RMM with benzoate in the presence of 30 and 50 g/L NaCl (Table 2). For instance, for strains 55 and B201, substrate loss after 5 days of incubation in the presence of 30 g/L NaCl constituted 10 and 11% of the initial amount (1 g/L), respectively. Based on the available data concerning the metabolic pathways of benzoate degradation in bacteria (https://www.genome.jp/pathway/ map00362+ C00180), it can be supposed that benzoate was oxidized to hydroxylated aromatic metabolites (Li et al., 2013), which were not further degraded by the enzyme systems of the strains studied and accumulated in the medium. Additional experiments are required to identify the products of benzoate degradation. Our study showed that the type strain *C. canadensis* DSM 6769^T exhibited similar activity when growing on benzoate in the presence of 30 g/L NaCl (Table 1); the substrate loss constituted 33% after 5 days. The type strains *C. beijerinckii* DSM 7218^T, *C. japonicus* CECT 7219^T, and *C. salarius* CECT 5903^T did not degrade or transform benzoate.

PCR with the *benA*-specific primers (Baggi et al., 2008) and DNA templates of the *Chromohalobacter* strains studied did not detect the target nucleotide sequences (*benA* genes). The results of our experiments suggest that benzoate transformation in the *Chromohalobacter* strains studied involves enzyme systems different from the classical pathway of benzoate degradation in bacteria (Li et al., 2013). Analysis of the genome of *C. canadensis* DSM 6769^T showed that it lacked *benA* genes, which confirms this notion. A search in the GenBank database identified *benA* genes in only two strains of the genus *Chromohalobacter*: a gene encoding the α-subunit of benzoate 1,2-DO (EU155151) in *Chromohalobacter* sp. HS-2 and genes of the α-subunit of benzoate 1,2-DO and benzoate-toluate 1,2-DO (QGTY01000032 and QGTY01000001) in *C. salexigens* 40a TX (Fig. 2, Table 3).

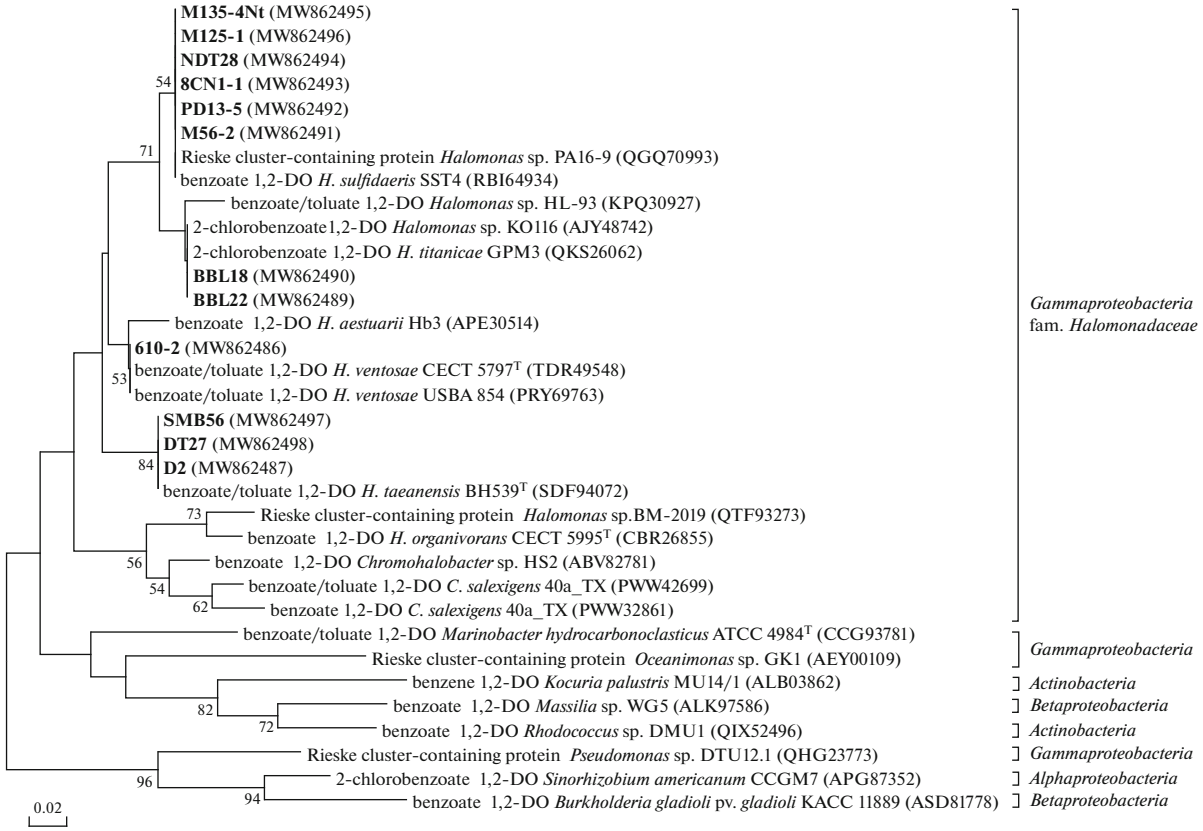

Fig. 2. Positions of the *benA* genes of the *Halomonas* strains studied in the phylogenetic tree constructed using the neighbor-joining approach based on comparison of the amino acid sequences translated from these *benA* genes. The evolutionary distances were calculated using the p-distance method. Numbers indicate the statistical significance of the branching order determined by bootstrap analysis of 1000 alternative trees (only the values higher than 50% are shown). The scale bar corresponds to 2 amino acid substitutions per 100 amino acids. The GenBank accession numbers are given in parentheses.

To sum up, our study of diversity of benzoate-degrading bacteria of the family *Halomonadaceae* in microbial communities of the salt mining area of the Upper Kama deposit (Perm krai) showed that, among the vast variety of isolated and characterized members of the family (genera *Halomonas*, *Chromohalobacter*, *Salinicola*, and *Kushneria*), active benzoate degraders represented the genus *Halomonas*. The degrader strains were phylogenetically close to *H. alkaliantarctica*, *H. neptunia*, *H. olivaria*, *H. taeanensis*, *H. titanicae*, *H. ventosae*, *H. radices*, and *H. utahensis*, which have not been previously described to be capable of degrading benzoate. It was shown that the identified strains of the genus *Halomonas* can utilize benzoate as the only source of carbon and energy when growing in the mineral medium in the presence of 30–70 g/L NaCl. It is known that the industrial site of the salt mining area is characterized by the presence of large amounts of organic pollutants, including various mono- and polyaromatic compounds, and high levels of salinity (Bachurin and Odintsova, 2009; Korsakova et al., 2013). Our study showed that halophilic bacteria of the genus *Halomonas* can degrade benzoate and thus contribute to destruction of aromatic pollutants as members of the unique microbial communities developing in this industrial area. For the first time, the diversity of *benA* genes involved in the initial stage of benzoate oxidation has been studied in members of the genus *Halomonas* (isolated in the Upper Kama deposit region). Analysis of the *benA* nucleotide sequences and the corresponding amino acid sequences showed that the identity level was the highest for homologous sequences of the members of the genera *Halomonas* and *Chromohalobacter* (Tables 2, 3; Fig. 2). In the phylogenetic tree of amino acid sequences translated from *benA* genes, bacteria of the family *Halomonadaceae* formed a separate cluster; within this group, the level of homology among the *benA* nucleotide sequences was 95.50–80.87%. Amino acid sequences translated from *benA* genes representing other taxa of proteobacteria and actinobacteria were separated in the tree from the group "*Halomonadaceae*" (Fig. 2). Recognition of the *benA* genes of the family *Halomonadaceae* as a separate group makes them a promising phylogenetic marker that can be employed to investigate the diversity and activity of benzoate degraders, in particular, members of the genera *Halomonas* and *Chromohalobacter*. Furthermore,

halophilic benzoate-degrading bacteria characterized in the present work are of practical significance for development of new techniques of bioremediation and monitoring of saline soils polluted with toxic organic compounds.

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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 participants performed by any of the authors.

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