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SHORT COMMUNICATIONS

Diversity of Bacteria of the Family *Halomonadaceae* at the Mining Area of the Verkhnekamsk Salt Deposit

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The Verkhnekamsk deposit is an area of massive development of salt-bearing sediments with strata of rock salt and sylvinite, which is located within the Pre-Urals depression and is bounded from the west and east by the East European craton and the West Ural Fold-and-Thrust zone, respectively [1]. In the course of mining and development of the salts, waste is accumulated at the surface as halite dumps, where NaCl content exceeds 90% [2]. Dissolution of the dump material and salt migration result in formation of salinization zones 1 to 5 m in radius. In the lakes with saline water (brine collectors) formed at the depressions at the base of the dumps, salts are precipitated [3]. Thus, conditions favoring survival of halophilic bacteria develop at the salt mining areas. Bacteria of the family *Halomonadaceae*, which are common in saline biotopes of various ecogeographic zones and show promise for biotechnological application, are especially interesting in this respect [4].

The goal of the present work was to investigate the phylogenetic diversity of the cultured halophilic *Halomonadaceae* bacteria in the biotopes of the zone of industrial mining at the Verkhnekamsk salt deposit.

The samples were collected in June 2011 at the Uralkalii salt-mining plants (Solikamsk, Perm krai). The samples of soils, bottom sediments from the brine collectors, salt crust from the dumps, and material at the bottom of the dumps were collected with disposable sterile spatula into sterile plastic test tubes. Saline water from the surface of the brine collectors was sampled with sterile test tubes. The samples of ores (rock salt and sylvinite), as well as the samples from the slurry tanks of salt-mining plants were provided by the workers of the Mining Institute, Ural Branch, Russian Academy of Sciences. Bacterial strains (SMB31, SMB35, SMB56, and SMB61) used in this work were isolated in 2002 from soil samples close to the salt dumps at Berezniki, Perm krai. Na^+ cations were determined in water extracts prepared according to the State Standard 26423-85 [5] on an AA-6300 atomic absorption spectrophotometer (Shimadzu, Japan) by the workers of the group of physicochemical investigation, Institute of Ecology and Genetics of Microorganisms, Ural Branch, Russian Academy of Sciences. For the isolation of bacteria, both direct plating and enrichment cultures on various media were used. The samples (1 g) from the upper soil layer (0–5 cm) collected 5–10 m from the salt dumps were mixed on a shaker (1 h at 100 rpm) with 100 mL of sterile 3% NaCl. The suspension from the fivefold dilution (0.1 mL) was plated of agarized Rymond rich medium [6] with 5 or 15% NaCl, and the plates were incubated at 28°C to obtain well-isolated colonies. The isolates from the rich Rymond medium with 5 and 15% NaCl were designated M and MH, respectively. This method did not result in bacterial isolation from the samples of ores, wastes (slurry tanks, salt dumps), and brine collectors. The samples (1 g) were therefore incubated for 1 month at 28°C in 10-mL sterile vials with 6 mL of ATCC 213 *Halobacterium* medium with 20% NaCl (www.atcc.org/ATCCAdvancedCatalogSearch). For the isolation of pure cultures, aliquots from these enrichments were plated on agarized ATCC 213 *Halobacterium* medium. Bacteria were also isolated from enrichment cultures obtained by incubation of soil samples in Rymond mineral medium with naphthalene, as described earlier [6]. The isolates obtained from such enrichments were designated SMS (Solikamsk) and SMB (Berezniki). Genomic DNA was isolated by alkaline lysis of whole cells [7]. Amplification of the 16S rRNA gene fragments was carried out as described [8]. Sequencing was carried out on a Genetic Analyzer 3500xl (Applied Biosystems, United States) according to the manufacturer's recommendations. Phylogenetic analysis was carried out according to the description in [8]. DNA typing of the isolates was carried out by BOX-PCR according to the standard procedure [7].

According to the sequencing of the 16S rRNA genes, 55 bacterial isolates were found to belong to the

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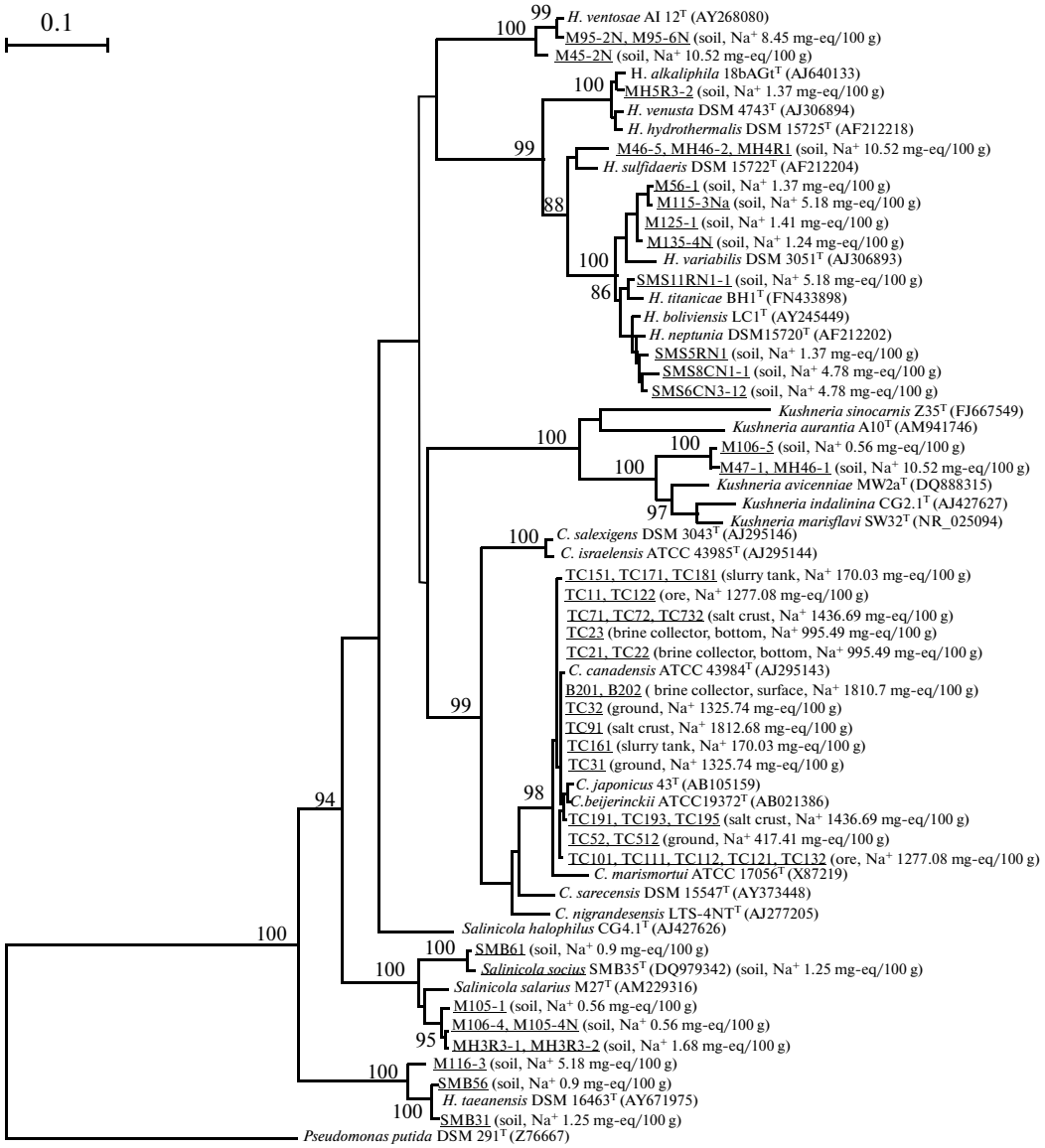

Fig. 1. Phylogenetic tree showing the position of the new isolates according to the sequences of their 16S rRNA gene fragments (1248 bp). The tree was constructed using the neighbor-joining algorithm. The scale bar corresponds to 10 nucleotide replacements per 100 nucleotides. The numerals indicate statistical reliability of the branching order determined by bootstrap analysis of 1000 alternative trees (the values above 50% are shown). The names of the isolates with identical 16S rRNA gene sequences obtained from the same sample are separated by commas. The sites of isolation and Na^+ content in the samples are listed in parentheses.

genera *Halomonas, Chromohalobacter, Kushneria*, and *Salinicola* of the family *Halomonadaceae* (Fig. 1). The 16S rRNA genes of the isolates exhibited high similarity to those of the type strains of valid species (98.6 to 99.9%). Although some *Chromohalobacter* and *Halomonas* species are known to have the 16S rRNA gene homology of 99.9%, and sometimes even 100%, DNA–DNA hybridization of closely related strains yielded values below 70%, the recommended threshold for bacterial species [9]. Thus, the isolates probably represent new taxa within the family *Halomonadaceae*. We have previously isolated and described a new member of this family, *Salinicola socius*, from the same ecological niche [8].

Microbial communities from soils with Na^+ content of 0.56–10.52 mg-eq/100 g, which corresponds to 0.03–0.6% NaCl, exhibited the highest phylogenetic diversity. Mostly *Halomonas* strains were isolated from these samples (64.3% of all *Halomonadaceae*), which were closely related to *H. ventosae, H. alkaliphila, H. sulfidaeris, H. titanicae, H. neptunia, H. variabilis, H. boliviensis,* and *H. taeanensis* (Fig. 1). Six isolates belonged to the genus *Salinicola*, which grouped with *S. socius* and *S. salarius* on the phylogenetic tree.

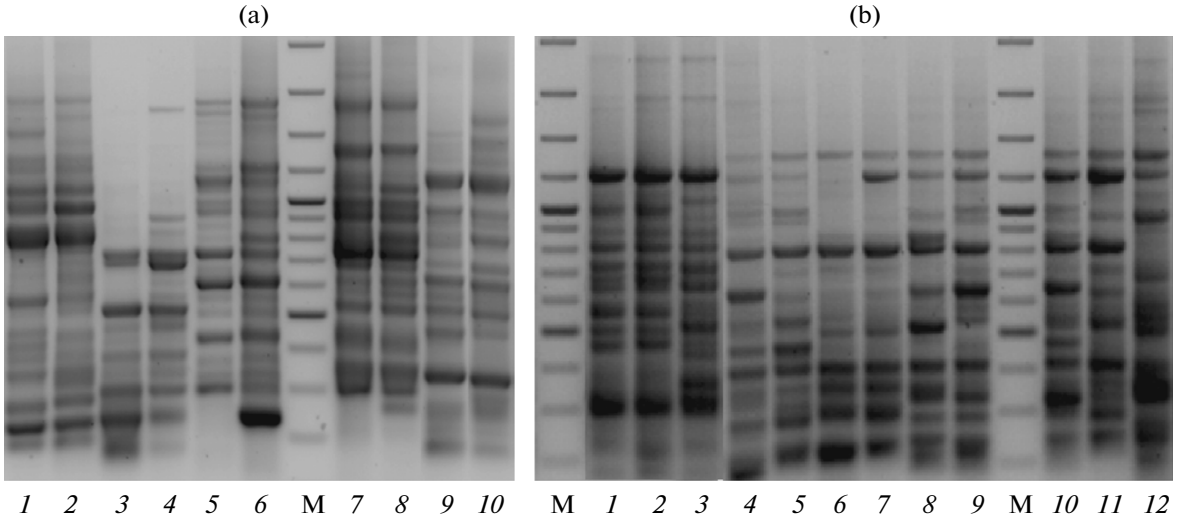

Fig. 2. BOX profiles of bacteria of the genera *Halomonas, Kushneria*, and *Salinicola* (a): M46-5, MH46-2 (*1*), MH4R1 (*2*), M95-2N (*3*), M95-6N (*4*), M47-1 (*5*), MH46-1 (*6*), SMB31 (*7*), SMB56 (*8*), M106-4, M105-4N (*9*), and MH3R3-1, MH3R3-2 (*10*); and of the genus *Chromohalobacter* (b): TC101, TC111, TC112, TC121, TC132 (*1*); TC52, TC512 (*2*); TC91, TC193, TC195 (*3*); TC21, TC23 (*4*); TC22 (*5*); TC31, TC161 (*6*); TC32, TC91 (*7*); TC71 (*8*); TC72 (*9*); TC732 (*10*); TC11, TC122, TC151, TC171, TC181 (*11*); and B201, B202 (*12*). M is the O'GeneRuler™ 100 bp Plus DNA Ladder (Fermentas, Lithuania).

Three strains (M106-5, M47-1, and MH46-1) fell into a subcluster within the genus *Kushneria* (Fig. 1). Analysis of the literature data on the physiology of closely related species revealed that most of them were able to grow without NaCl or at a concentration not exceeding 0.5%, with the exception of *H. ventosae*, which required at least 3% NaCl [10]. The strains exhibiting high similarity to *H. ventosae* were isolated from soil samples with the highest Na^+ content (8.45–10.52 mg-eq/100 g). Apart from the phylogenetic diversity of halomonads in the soil samples, BOX-PCR revealed genetic polymorphism of the strains with identical 16S rRNA genes which manifested itself in the differences in the size and number of the PCR fragments (Fig. 2a).

The samples of ores and mineral waste with high Na^+ content (170.03–1812.68 mg-eq/100 g) contained *Chromohalobacter* bacteria closely related to *C. canadensis* and *C. japonicus*, which require salt for growth (3 to 25% NaCl) [4]. The strains related to *C. japonicus* were isolated from the same biotope (TC191, TC193, and TC195 from the salt crust of the dump; TC52 and TC512 from the soil near the dump; and TC101, TC111, TC112, TC121, and TC132 from the ore) and were characterized by the same set of BOX-PCR products, while the strains of this group isolated from various biotopes differed from each other (Fig. 2b). The isolates related to *C. canadensis* exhibited much higher genetic diversity (Fig. 2b). Genetic polymorphism of the strains of this phylogenetic cluster isolated from one biotope (bottom of the brine collector drainage, soil near the salt dump, and salt crust of the dump) was revealed, while the isolates from different biotopes were present in one genomic group (Figs 1 and 2b). For example, strains TC11 and TC122 (from ore) and strains TC151, TC171, and TC181 (from slurry tanks) had identical BOX profiles (Fig. 2b). These data indicate the presence of *Chromohalobacter* bacteria in the natural and anthropogenic highly mineralized ecosystems of the sites of salt recovery in the Perm krai.

Thus, the phylogenetic diversity and wide occurrence of halophilic bacteria of the family *Halomonadaceae* was shown for the biotopes of the sites of industrial development of the Verkhnekamsk salt deposit.

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