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SHORT COMMUNICATIONS

Calcinating Bacteria in Extreme Ecosystems of the Southern Aral Region

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Abstract—The processes of microbially induced precipitation of calcium carbonates are widespread in natural environments and are an important part of the biogeochemical carbon cycle. These processes comprised the basis of new "biocementation" technologies, which are extensively developing worldwide during the last decade. These technologies are aimed at designing the novel "self-healing" construction materials, as well as at maintaining the strength of various buildings and structures. Since the optimal conditions for calcite formation are high salinity and alkalinity, the search for calcifying microorganisms in a variety of ecosystems, including extreme ones, is of interest. At present, many strains of halophilic and halotolerant bacteria, that induce calcination, have already been isolated and tested in pilot industrial processes. Most of these bacteria possess urease activity, which is the main contributor to the binding of calcium ions to insoluble carbonate. A wide variety of natural ecosystems with optimal conditions for the development of calcifying urobacteria, as well as the economic demand for biocementation technologies, stimulate interest in the search for more and more novel strains of these microorganisms. One of the promising resources to be screened for such organisms is the ecosystem of the drying Aral Sea and the adjacent desert and semi-desert Aral region. Here we present the results of screening various extreme ecosystems of the Aral region for the presence of calcifying microorganisms. We obtained 28 pure cultures of heterotrophic aerobic bacteria from samples of plant residues and soils of the Aral Sea region, 4 of which had urease and calcifying activities. Their activities were compared with those of the strains presently used to produce biocementing mixtures. We have identified the phylotypes of putative calcifying microorganisms in microbial communities of desert soil, thermal waters, and bottom sediments of a salt lake, and described the phylogenetic diversity of these communities. Our results indicated the wide distribution of calcifying microorganisms in the ecosystems of the South Aral region and highlighted the expediency of screening them for the new biotechnologically relevant strains of these organisms.

Keywords: microbially induced calcite precipitation, extreme ecosystems, Aral Sea, biocementation
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The bottom of the drying Aral Sea, one of the extreme biogeocenoses of our planet, is located in Uzbekistan. The drastic decrease in its area resulted in increased water mineralization and elevated concentrations of pesticides and mineral fertilizers, which have been washed from irrigated fields by the rivers Amu Darya and Syr Darya during the 1960s–1990s (Micklin, 2007). In the course of drying, Aral water salinity increased by an order of magnitude, from tens to hundreds g/kg (Zav'yalov et al., 2012). A new sandy-saline Aralkum Desert was formed on most of the Aral territory. The soils of this area are characterized by high salinity and extreme instability of humidity level, up to complete drying (Zav'yalov et al., 2012; Davletmuratova, 2017). The microbiome of this type of soils is usually represented by salt-tolerant, thermotolerant, xerophilic organisms, though in-depth studies of microbial diversity of the Aral region are in their beginning. Since the Aralkum ecosystem is affected by several extreme physicochemical factors simultaneously, its microbial inhabitants may be of possible application in various fields of biotechnology, including biocementation technologies basing on microbially induced calcite precipitation (MICP). The MICP occurs commonly in nature and are an important part of the carbon biogeochemical cycle, promoting immobilization of atmospheric CO_2 in carbonate sedimentary rocks. The global geochemical role of this process is conversion of loose sediments into solid rocks and consolidation of soils and sands (Frankel

and Bazylinski, 2003; DeJong, 2013; Osinubi et al., 2020; Pacheco et al., 2022). Calcite precipitation is most actively induced by ureolytic bacteria, which hydrolyze urea and uric acid (Joshi et al., 2017; Omoregie et al., 2021). Since urea is the end product of metabolism of nitrogen compounds in many animals, both this compound and the microorganisms utilizing it are widespread in various ecosystems, especially in those enriched with organic matter (OM) of animal origin (Atkinson, 1992). Calcinating ureolytic bacteria are the object of growing interest, and screening for these microorganisms in diverse ecosystems is presently carried out by several research teams worldwide. The most active of the presently retrieved strains belong to various *Bacillus* species (Vahabi et al., 2015; Mutitu et al., 2019; Arias et al., 2019; Ekprasert et al., 2020; Leeprasert et al., 2022). MICP coupled to urea hydrolysis is an easily controlled process, resulting in production of large amounts of calcium carbonate during short periods (Kalenov et al., 2020). Urea degradation is catalyzed by urease; the catalytic activity of this enzyme is considered relatively high, and the mechanism of catalysis has been studied in detail (Karplus et al., 1997). Ammonia produced by urease increases the ambient pH, so free calcium ions may bind with CO_2, resulting from the urease reaction, into insoluble carbonates (Chaparro-Acuña et al., 2020). Due to the high calcinating activity of ureolytic bacteria, the preparations containing these microorganisms may be used for damage recovery (crack bridging) in concrete and building stone, extended operation of ferroconcrete structures, and production of modified construction materials capable of self-healing after mechanical damages. Recent studies showed higher economic efficiency of the technologies of biogenic self-healing of concrete compared to abiotic technologies for crack bridging (Joshi et al., 2017, 2020; Almajed et al., 2021). The importance of this problem for material sciences and construction industry stems from the fact that, alongside with natural environmental factors negatively affecting the preservation of construction materials and structures, anthropogenic factor of environmental pollution with nitrogen and sulfur oxides, resulting in acidic rains and fogs capable of dissolving the calcite matrix of limestone and washing away the soluble components from concrete surface layers, becomes more active (Alonso et al., 2018; Davidyuk et al., 2021; Batyanovskii et al., 2022). Search for novel calcinating microorganisms is therefore an urgent task.

Saline natural and anthropogenic basins are a poorly studied, albeit promising ecological niche for such search. They are inhabited by extremely halophilic bacteria and archaea and their accompanying microbiota, which was shown to be either halotolerant or dependent on the products of metabolism and lysis of true halophiles (e.g., on osmolytes). Preliminary studies revealed non-halophilic members of the order *Bacillales* with high urease activity in microbial communities of saline and soda lakes (Galinski et al., 1994; Ventosa et al., 1998; Panosyan et al., 2018; Kalenov et al., 2020). This research promoted interest in the search for calcinating microorganisms in other extreme ecosystems, since MICP application implies resistance of such microorganisms to high salt concentration and alkaline environment of mortars, as well as their ability to attach to the target surface and survive long periods of desiccation. Such physicochemical conditions are typical of the soils of the Aral region.

The goal of the present work was therefore to assess the potential of microbial communities of several extreme ecosystems of the Southern Aral region as the sources for novel ureolytic calcinating microorganisms usable in the technologies for biocementation and self-healing bioconcrete to be used under the conditions of arid continental climate.

Search for novel ureolytic bacteria was carried out using 18 samples of plant residues and soil of the Southern Aral region (November–October 2021 expedition) and 23 samples of soil, microbial biofilms, water, and sediments of the Aral Sea, Aralkum, and Southern Aral region, as well the samples of thermal water from wells drilled in the former Aral Sea bottom (September 2022 expedition). Material from all samples was fixed on site for subsequent isolation of total DNA and phylogenetic profiling of the microbial communities using 16S rRNA gene sequences.

Chemical analysis of the soil samples collected during the first expedition according to CIS international technical standards GOST 2642(3-8)-85 and GOST 26490-85 was carried out in order to determine the physicochemical characteristics of these extreme ecosystems. Soil samples and the samples of plant residues from these soils were plated on meat infusion agar (MIA), pH 6.8 ± 0.2, 2% agar, 100–150 g/L NaCl to cultivate aerobic salt-tolerant heterotrophic bacteria, a group to which most known ureolytic bacteria with calcinating activity belong (Joshi et al., 2017). The plates were incubated at 35°C. A total of 28 pure cultures of heterotrophic microorganisms were isolated from the colonies using tenfold serial dilutions in liquid medium (meat infusion broth with the same NaCl concentration). Purity of the cultures was determined by microscopy, plating on MIA, and MALDI time-of-flight mass spectrometry (MALDI-TOF MS) on a MALDI Biotyper Microflex complex (Bruker, Germany), which also allowed for primary identification of the cultures. Urease activity of the cultures was tested by streak inoculation on solid Christensen medium, pH 6.8 ± 0.2 with 7.5 g/L $CaCl_2$ and Phenol

red (0.012 g/L) as an indicator. Pink coloration of the medium around the colonies indicated urease activity. Calcite formation was observed by naked eye and by light microscopy (a BA210 Digital Motic microscope, China) as the development of a mineral crust over the colonies or around their edges. Calcination intensity was assessed by the rates of urease activity emergence and calcite crystals formation compared to the reference strains of halotolerant ureolytic bacteria (further on referred to as the SKG1-9 group of strains). These strains have been recently isolated from several different hypersaline ecosystems worldwide and were successfully used in the preparations for improving the functional and protective properties of concrete (Kalenov et al., 2020).

Total DNA was isolated from the samples of soil, water, and microbial biofilms collected during the second expedition. Amplicon libraries of the V4 variable region of the 16S rRNA gene were prepared and sequenced, and bioinformatic analysis of the results was performed as described previously (Gavrilov et al., 2022). Raw reads were deposited in the SRA (NCBI) database under the project no. PRJNA925816. The search for the phylotypes which are likely to harbor the microorganisms with calcinating activity was carried out using the data sets obtained. Analysis of the literature data was used to select 10 full 16S rRNA gene sequences of the strins with confirmed urease and calcinating activity. 16S rRNA gene sequences of the halotolerant ureolytic bacteria of the SKG1-9 set were also used for the analysis. These sequences were determined previously (Kalenov et al., 2020), and within the framework of the present study they were deposited in GenBank under accession nos. listed in Table 1. Salt tolerance of these strains was determined in order to assess their applicability as reference organisms for the analysis of extremophilic microbial communities of the Aral region. The final list of sequences used for the search of putative calcifying microorganisms is provided in Table 1. Search for the target phylotypes was carried out using Local blastn; similarity of at least 98.8% (species level) was accepted as the threshold value.

According to the results of chemical analysis, the soils sampled during the first expedition were alkaline and relatively halomorphic, with pH ranging from 8.23 to 9.27. Soil salinity varied within a broad range (from 0.24 to 56.63 g/L), but in most samples it exceeded 10 g/L. NaCl was the main salt, while sulfate content varied widely (on average 73.4 mg Eq/L). These results indicated most of the soil samples to be markedly halomorphic.

Screening of 28 pure cultures of aerobic heterotrophs isolated from plant residues and soils of the Aral region revealed urease and calcinating activity of four strains. Urease activity was observed after 24 h of cultivation (Fig. 1a), while formation of calcite crystals could be observed only on day 5 of cultivation. No formation of calcite crystals occurred in the cultures lackig urease activity. Preliminary identification of the active isolates by MALDI-TOF MS showed that they belonged to the species *Bacillus licheniformis* (cultures AS001 and AS003), *Staphylococcus felis* (AS008), and *Azoarcus indigens* (AS009). Calcinating activity of the novel isolates became visible after 2.5 times longer period than in the case of the SKG1-9 reference strains (species *Lysinibacillus macroides*, *B. licheniformis*, and *B. subtilis*). These strains exhibited calcinating activity on the second day of cultivation (Kalenov et al., 2020). While assessment of salt tolerance of the SKG1-9 strains revealed growth at NaCl concentrations up to 150 g/L, the highest density of the cultures was achieved at up to 50 g/L NaCl and decreased significantly at higher salt concentrations. The yield of *L. macroides* SKG7 remained relatively high at up to 100 g/L NaCl. Thus, the comparison of the SKG1-9 strains and the cultures isolated from the Aral region plant residues with salinity not exceeding 56 g/L was relevant.

Search for the microorganisms closely related to the reference strains of calcinating bacteria (see Table 1) in the samples collected from various Aral region ecosystems during the second expedition revealed 9 phylotypes (ASVs) with high similarity of the V4 regions of the 16S rRNA gene to the strains *Bacillus megaterium* de Bary, *B. licheniformis* SKG1 and SKG4, *B. subtilis* SKG9, *Alkalihalophilus pseudofirmus* DSM 8715, and *L. macroides* SKG7. Most phylotypes of putative calcinating bacteria were related to strain *L. macroides* SKG7 with considerable salt tolerance (Table 2). These phylotypes were found in the communities of the Ustyurt Plateau dry soil (sample K08), Lake Sudochye anoxic sediments (K21), in the thermal freshwater from a well drilled down to 400 m in the former Aral Sea bottom (K01), and in the communities associated with outflow of this water on halomorphic soil (microbial mats and bottom sediments of a thermal spring, K02 and K04)). Interestingly, the sedimental community of the thermal freshwater spring exhibited the best representation of the phylotypes of putative calcinating bacteria related to the haloalkaliphilic strain *A. pseudofirmus* DSM 8715 and to the halotolerant *L. macroides* SKG7. Description of these samples and relative abundance of the phylotypes of calcinating bacteria are presented in Table 2. None of these phylotypes was predominant in these communities, with the share of each single one not exceeding 0.87% of the phylogenetic diversity. This is hardly surprising, considering heterotrophic metabolism of calcinating bacteria and their dependence on urea, an end product of nitrogen compounds degradation, and therefore, on the activity of other numerous

Table 1. Complete reference sequences of the 16S rRNA gene used for screening the phylotypes of potentially calcinating microorganisms in microbial communities of the Southern Aral region ecosystems

Strain	Mention in the publication on calcinating activity	Database identifier of the sequences	Reference to the publication on calcinating activity
Myxococcus xanthus ATCC 25232	*Myxococcus xanthus* CECT 422	NR_043945.1	https://doi.org/10.1128/AEM.69.4.2182-2193.2003
Sporosarcina pasteurii ATCC 6453	*Bacillus pasteurii* ATCC 6453	16S rRNA from the genome of ATCC 6453	https://doi.org/10.1016/S0038-0717(99)00082-6
Bacillus megaterium de Bary ATCC 14581, VKM B-512	*Bacillus megaterium* ATCC 14581	16S rRNA from the genome of ATCC 14581	https://doi.org/10.1016/j.ecoleng.2010.11.009
Sporosarcina ureilytica	*Bacillus sphaericus* LMG 22257	CP017560.1	https://doi.org/10.1016/j.conbuildmat.2006.12.011
Bacillus megaterium SS3	*Bacillus megaterium* SS3	KC121060.1	https://doi.org/10.1007/s11274-013-1408-z
Bacillus sp. CT5	*Bacillus* sp. CT5	FJ973470.1	https://ascelibrary.org/doi/full/10.1061/(ASCE)MT.1943-5533.0000159
Alkalihalobacterium alkalinitrilicum FK3	*Bacillus alkalinitrilicus*	ON834547.1	https://doi.org/10.1016/j.cemconcomp.2011.03.012
Sutcliffiella cohnii RSH	*Bacillus cohnii* CCM 4369	X76437.1	https://doi.org/10.1016/j.cemconres.2014.06.003
Alkalihalophilus pseudofirmus DSM 8715	*Bacillus pseudofirmus*	NR_026139.1	https://research.tudelft.nl/en/publications/crack-repair-by-concrete-immobilized-bacteria
Pseudomonas aeruginosa type strain	*Pseudomonas aeruginosa*	X06684.1	https://doi.org/10.14359/10154
Bacillus licheniformis SKG1	*Bacillus licheniformis*	OQ299525[1]	https://doi.org/10.21519/0234-2758-2020-36-4-21-28
Bacillus subtilis SKG2	*Bacillus subtilis*	OQ299526[1]	https://doi.org/10.21519/0234-2758-2020-36-4-21-28
Bacillus subtilis SKG3	*Bacillus subtilis*	OQ299527[1]	https://doi.org/10.21519/0234-2758-2020-36-4-21-28
Bacillus licheniformis SKG4	*Bacillus licheniformis*	OQ299528[1]	dhttps://doi.org/10.21519/0234-2758-2020-36-4-21-28
Bacillus subtilis SKG5	*Bacillus subtilis*	OQ299529[1]	https://doi.org/10.21519/0234-2758-2020-36-4-21-28
Bacillus subtilis SKG6	*Bacillus subtilis*	OQ299530[1]	https://doi.org/10.21519/0234-2758-2020-36-4-21-28
Lysinibacillus macroides SKG7	*Lysinibacillus macroides*	OQ299531[1]	https://doi.org/10.21519/0234-2758-2020-36-4-21-28
Bacillus badius SKG8	Was not mentioned earlier	OQ299532[1]	https://doi.org/10.21519/0234-2758-2020-36-4-21-28
Bacillus subtilis SKG9	*Bacillus subtilis*	OQ299533[1]	https://doi.org/10.21519/0234-2758-2020-36-4-21-28

[1] The sequences were determined previously by Kalenov et al (2020), but were not deposited to the database prior to the present work.

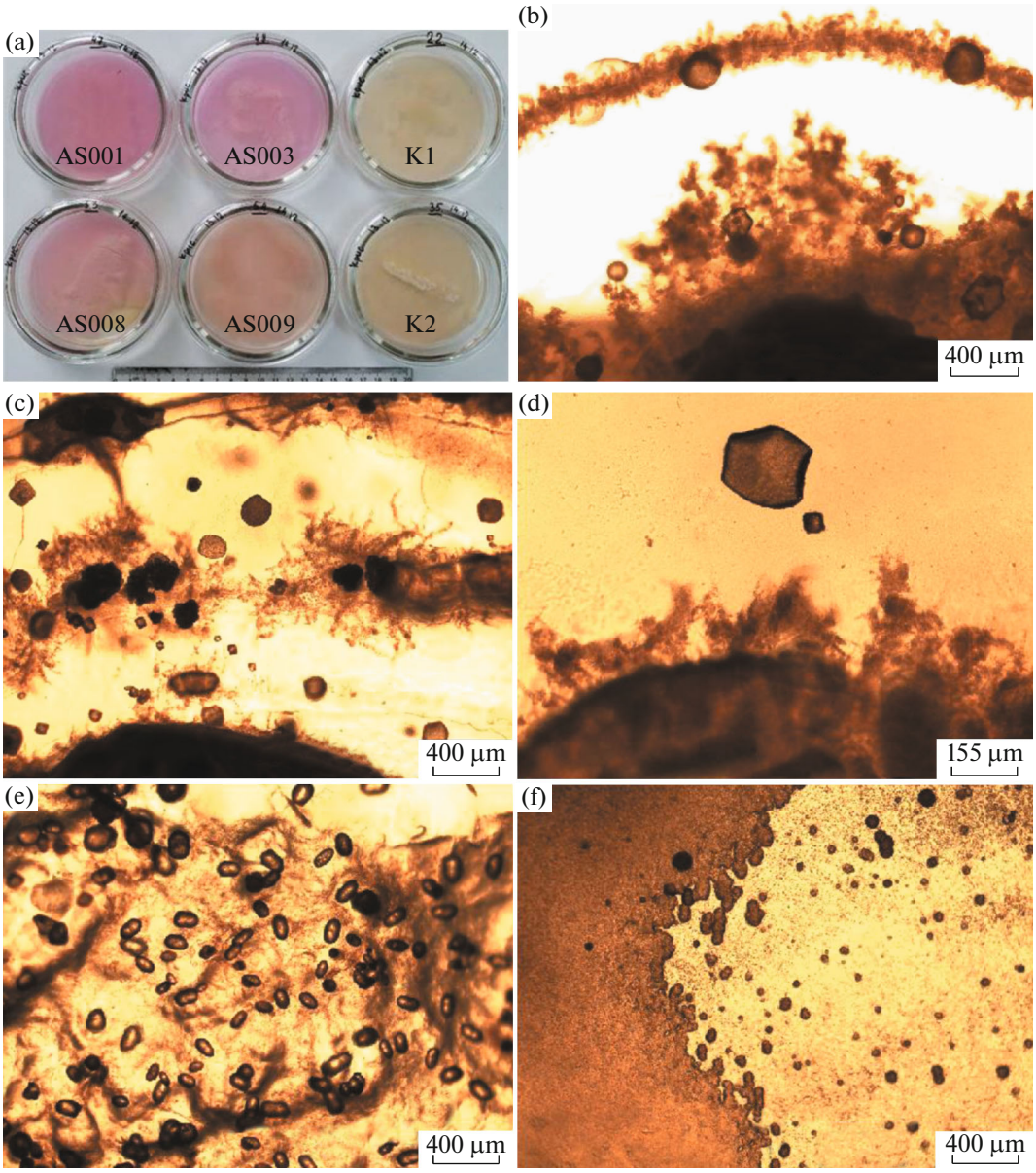

Fig. 1. Urease and calcination activities of pure cultures of aerobic heterotrophs isolated from Aral region soils: a Christensen agar plate 24 h after streak inoculation with active (AS001, AS003, AS008, AS009) and inactive cultures (K1 and K2) (a); calcite crystals produced by urease-positive cultures (b–e): small crystals around an AS001 colony (b); small and large crystals on the surface of an AS003 colony (c); calcinated AS009 colony (d); large crystals on the surface of an AS008 colony (e); and colony edge of the inactive culture K2 (f).

OM degraders in the community. Diversity of the analyzed communities containing the phylotypes of putative calcinating bacteria was relatively high (Fig. 2). The thermal water community was represented mainly by *Gammaproteobacteria* of the genus *Thiobacillus* and uncultured genus-level groups of the families *Hydrogenophilaceae* and *Rhodocyclaceae*, which together comprised 71% of the 16S rRNA gene reads. Various *Bacillota* (former *Firmicutes*) also constituted a significant part of the community (7%, Fig. 2). Communities of the green mat collected at the well outlet and of the sediments of the thermal spring originating there were much more diverse. The mat community was represented by 88 phylotypes of *Alpha-* and *Gammaproteobacteria* (together responsible for 40% of the community), 21 cyanobacterial phylotypes (16% of the community), and 40 phylotypes of planctomycetes (7% of the community), while 20% of the diversity was represented by a single phylotype of nitrifying bacteria of the genus *Nitrospira*. In the community of the thermal spring sediments, 65 phylotypes of *Alpha-* and *Gammaproteobacteria* constituted 35% of the total

Table 2. Environmental samples, in which the phylotypes related to known calcinating microorganisms were detected using molecular techniques

Sample	Description	Reference strain	Similarity of the 16S rRNA gene sequences, %	Numbers of the phylotypes (ASVs) related to the reference strains	ASV's taxonomic position (*Bacilli*; *Bacillales*...)	ASV's relative abundance in the community, %
K01	Thermal freshwater from a well in the Aral Sea bottom (41°C, pH 7.0)	*L. macroides* SKG7	98.8 98.8	ASV1050 ASV1314	*Planococcaceae*; *Planomicrobium*/*Planococcus*	0.19 0.21
K02	Green mat from a tube at the well outlet	*B. megaterium* de Bary ATCC 14581	100.0	ASV919	*Bacillaceae*; *Bacillus*	0.23
K04	Black sediment from a thermal water spring, 50 m of the well	*L. macroides* SKG7 *A. pseudofirmus* DSM 8715	99.8 99.2 98.8	ASV1667 ASV408 ASV988	*Planococcaceae*; *Psychrobacillus*/*Planococcus* *Bacillaceae* uncultured	0.16 0.87 0.64
K08	Dry soil from the Ustyurt Plateau slope	*B. licheniformis* SKG1, SKG4 *A. pseudofirmus* DSM 8715	100.0 98.8	ASV1142 ASV2576	*Bacillaceae*; *Bacillus* *Bacillaceae* uncultured	0.42 0.10
K21	Lake Sudochye black loose sediment (15 cm below the sediment surface)	*B. subtilis* SKG2, SKG3, SKG5, SKG6, SKG9	99.6	ASV1742	*Bacillaceae*; *Bacillus*	0.15

diversity. The groups contributing significantly to the microbial diversity included also microalgae detected by chloroplasts (16% of the 16S rRNA gene reads) and various bacteria of the phyla *Desulfobacterota* (9%), *Chloroflexota* (15%, 37 phylotypes), and *Bacillota* (former *Firmicutes*, 8%). The latter phylum was represented by 16 phylotypes, two of which were closely related to the calcinating strains *L. macroides* SKG7 and *A. pseudofirmus* DSM 8715. The community of the Ustyurt Plateau soil at the sampling site was represented mainly by actinobacteria (phylum *Actinomycetota*, 51% of the 16S rRNA gene reads, 55 phylotypes), *Alpha*- and *Gammaproteobacteria* (totally, 22%), *Chloroflexota* (7%, 28 phylotypes), and *Bacteroidota* (6%, 27 phylotypes). On the contrary, in the Lake Sudochye sediments, *Alpha*- and *Gammaproteobacteria* (26% of the community) and *Desulfobacterota* (19%) were predominant, while members of the phyla *Bacteroidota* (8%) and *Cyanobacteria* (7%) were less abundant, although they were represented by 44 and 17 phylotypes, respectively (Fig. 2). Members of various taxa within the phylum *Bacillota* constituted ~4% in the community of Lake Sudochye sediments, and one of their phylotypes exhibited high 16S rRNA gene similarity with the calcinating strains *B. subtilis* SKG2,3,5,6,9 (Table 2).

Our results revealed broad distribution of the microorganisms possessing urease activity and able to induce calcite formation in diverse ecosystems of the Southern Aral region, including saline soils, sediments of a saline lake, and fresh thermal water. Detection of calcinating microorganisms in phylogenetically diverse microbial communities supports the previously known tendency of association of these prokaryotes with organic-rich ecosystems, in which combined activity of many OM degraders, including extremophiles, provides the calcinating strains with carbon sources, energy substrates, and protection from deleterious environmental factors, in particular by osmolyte secretion (Galinski et al., 1994; Jebbar, 1997; Panosyan et al., 2018; Kalenov et al., 2020).

Isolation of pure cultures of four novel strains of calcinating ureolytic bacteria from 18 soil samples and detection of these phylotypes in 5 out of 28 analyzed microbial communities indicates high application perspectiveness of the Aral region extreme ecosystems as a source of new biotechnologically valuable prokaryotes usable for development of biocementing technologies. The relatively low calcination rate in the cultures of our isolates indicates that both selection of the optimal conditions for this process and the search for more active calcinating microorganisms in the previ-

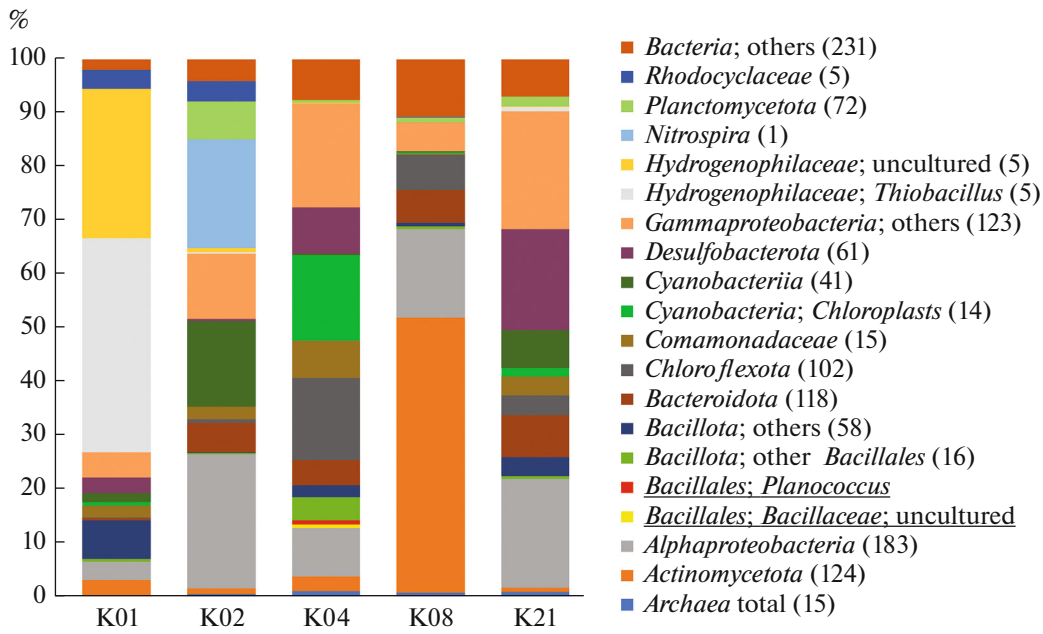

Fig. 2. Phylogenetic composition of the communities in which the phylotypes related to the known calcinating microorganisms were detected, according to the 16S rRNA gene sequencing. The *X* axis shows the samples numbered according to Table 2: K01—thermal freshwater from a well; K02—green mat from a tube at the well outlet; K04—black sediment from a thermal water spring coming out of the well; K08—dry soil from the Ustyurt Plateau slope; and K21—Lake Sudochye anoxic sediment. The phylotypes related to the known calcinating *Bacillus* strains with relative abundance of ~1%, which may be visualized on the chart, are underlined. Close to the taxa names, total abundance of their phylotypes (ASVs) detected in all the analyzed samples is shown in parentheses.

ously unexplored extremophilic microbial communities of the Aralkum and Aral region should be continued. Further work should be aimed at the isolation of such organisms in pure cultures and assessment of the biotechnological potential of these isolates. It should be noted that application of biocementing microbial preparations is especially promising in highly seismic areas, where the frequency of earthquakes, especially minor ones, is high and results in increased and accelerated wear of ferroconcrete structures. Calcinating microorganisms of the Aral region with potentially high tolerance not only to ambient salinity, but also to dehydration, high temperatures and temperature contrasts, and to relatively high insolation, could be the major candidates for development of biocementing preparations for Uzbekistan and other regions of the world with pronouncedly continental, arid climate, considering the similarity of the physicochemical conditions in saline, arid Aralkum soils and on the surface of concrete constructions in these regions.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest.

This article does not contain any studies involving animals or human participants performed by any of the authors.

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