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EXPERIMENTAL ARTICLES

Evaluation of Quantitative and Qualitative Composition of Cultivated Acidophilic Microorganisms by Real-Time PCR and Clone Library Analysis

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Abstract—Quantitative and qualitative composition of the cultivated acidophilic microorganisms obtained from the enrichment cultures on the universal medium inoculated with the samples of Shanuch deposit (Kamchatka peninsula) was investigated. The clone library ($N = 93$) containing eubacterial and archaeal 16S rRNA gene insertions was analyzed. DNA sequences were grouped into 5 ribotypes related to four known genera and one family. Most microorganisms (92%) were shown to belong to the genus *Acidithiobacillus*. One more group of microorganisms was identified as belonging to the family *Acetobacteriaceae* (3%); one microorganism was identified as a member of the genus *Acidiphilium*. Apart from eubacteria, the sequences specific for archaea of the genera *Thermococcus* (3%) and *Ferroplasma* (2%) were found; however, these sequences could not be reliably referred to any known species. Quantitative ratio of the microorganisms from the enrichment cultures was determined using real-time PCR. Species-specific test systems were used to determine that the sequences of *A. ferrooxidans, A. thiooxidans,* and *Ferroplasma acidiphilum* present in the samples in the ratio of 62, 4, and 0.14%, respectively.

Keywords: bioleaching, real-time PCR, clone library analysis, acidophilic microorganisms

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Application of microorganisms in the processes of metal recovery and reduction from ores and ore concentrates (bioleaching and biooxidation) became widespread recently [1]. This is due to the economic attractiveness of the technology and to the necessity for rationalization of mineral mining. For example, application of microorganisms (bacteria of the genera *Thiobacillus*, *Leptospirillum* or thermophilic archaea) makes it possible to increase gold mining efficiency up to 85–95% as compared to 15–30% for conventional technologies [2]. Moreover, bioleaching does not include expensive and complex stages and is environmentally friendly [3].

Currently, all microorganisms used in industrial bioleaching and biooxidation have originated from the region-specific environmental populations. Application of microbial communities rather than of single species is especially promising. This is due to the fact that region-specific communities are well-adapted to the energetic substrates typical for certain biocenosis, and can therefore efficiently utilize the chemical compounds of the ore.

The main processes associated with sulfur oxidation are performed by thiobacteria. Some thiobacteria (*Acidithiobacillus thiooxidans*, *A. ferrooxidans*) have long been used for bioleaching of the metals from sulfide ores and are considered the most efficient producers [4].

In spite of the practical interest in the industrial technologies based on bioleaching and biooxidation, many environmental issues, as well as the composition of complex communities involved, are as yet poorly studied. However, molecular DNA technologies open new possibilities for the analysis of the structure of acidophilic microbial communities and for improvement of the bioleaching efficiency [5]. Most widely used technologies are fluorescent in situ hybridization (FISH), separation of the PCR products in acrylamide gel denaturing gradient gel electrophoresis (DGGE), and clone library analysis [6]. Although FISH may be considered a quantitative method (in the modification involving flow cytometry), the technologies based on fluorescent molecules need optimization of the hybridization reaction, resulting in the high price of the method and making it impractical for routine diagnostics. Electrophoresis-based techniques are qualitative and give no indication of the initial abundance of microorganisms. Clone library analysis gives the information regarding the quantitative ratios of microorganisms in mixed communities; nevertheless,

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Table 1. Species-specific primers used in the study

Specificity	Sense-primer	Antisense-primer
Acidithiobacillus thiooxidans	AtF-TCTTCGGACCTCGCGCTGG	AtR-GTCAACAGCAGGCGATATTAGCAC
Acidithiobacillus ferrooxidans	AfF-AATCTGCTATTGACGTGAATCC	AfR-CATGAACCATACCGTGGTAAC
Ferroplasma acidiphillum	Fer-d-CCTGAAGCTTAACTCCAGAAAGT	Fer-r1-TCTTTGTAATGCGCGTGTAGC
Sulfobacillus thermosulfidooxidans	Sulf-d1-ACCTTCGGGTCAGCGGCGG	Sulf-r-GCCGGTCTTCGTCCCGACA

the method is expensive and has low resolution (often it does not allow analysis at the species level). Therefore, the only accessible technique for the quantitative and qualitative analysis nowadays is real-time polymerase chain reaction (RT-PCR). The method is simple and may be used both in research and in the industry.

The goal of this work was to develop the complex of molecular methods for evaluation of the qualitative and quantitative composition of the cultivated communities of acidophilic microorganisms from the Shanuch deposit (western Kamchatka). Enrichment cultures of microorganisms from the reactor samples of the laboratory of geochemistry and geotechnology (Scientific Research Geotechnological Centre, Far Eastern Branch of the Russian Academy of Sciences) were used in the study. The obtained data regarding the composition of microbial communities are important for the understanding and control of bioreactor processes. The methods developed in this study may be used for the construction of highly efficient acidophilic microbial associations and for optimization of the bioleaching technology.

MATERIALS AND METHODS

Culture characteristics and DNA isolation. The quantity and taxonomic composition of microorganisms was determined in the samples of enrichment cultures isolated from sulfide ores of the Shanuch deposit (Kamchatka Peninsula). The enrichment cultures were grown in the universal Silverman-Lundgren 9K culture medium without iron [7]. DNA isolation was performed as described earlier [8].

For the development of species-specific primers for identification of *A. thiooxidans* [9], *A. ferrooxidans* [9], *Sulfobacillus thermosulfidooxidans* [10], and *Ferroplasma acidiphilum* [11], the pure cultures of microorganisms supplied by the laboratory of chemolithotrophic microorganisms, Winogradsky Institute of Microbiology, Russian Academy of Sciences, were used.

Amplification of the 16S rRNA fragments, cloning and sequencing. For amplification of the 16S rRNA gene fragments of bacteria and archaea the primers upr-2d and upr3-r were used [8].

PCR products of the 16S rRNA gene fragments were cloned into the plasmid vector pGEM-T (Promega, United States). The cloning was performed on the base of the Eurogene Co, Moscow, Russia. A total of 93 unique clones containing 16S rRNA gene insertions were selected.

The nucleotide sequence of the insert was determined by sequencing. Preparation of the products was carried out using the standard M13 plasmid primers and labeled dideoxyribonucleotidetriphosphates (Big-Dye Terminator Kit, Applied Biosystems, United States). Reaction was performed in a Tercyc amplifier (DNA-Technology Co.) in accordance with the manufacturer's protocol. Amplification conditions were as follows: 40 cycles at 94°C for 10 s, 56°C for 20 s, 72°C for 20 s. The subsequent preparation of the products included chemical denaturing of the template using denaturing buffer TSR (Template Suppression reagent, Applied Biosystems, United States) according to the manufacturer's recommendations. Sequencing was performed using an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, United States).

Nucleotide sequences were aligned using the ClustalW algorithm of the European Bioinformatic Institute server (www.ebi.ac.uk) and the Seqman (DNAstar) software package. Relation to the certain phylotype was determined in case of 97% sequence similarity using the FastGroup II software package [12]. Relation to the taxa was determined using the classifier of the RDP, version 10.4 database (http://rdp.cme.msu.edu) with the minimal threshold level of 60%, and GenBank database of NCBI, United States (http://www.ncbi.nlm.nih.gov/genbank/).

Real-time PCR. The primers used in this study are shown in Table 1. Nucleotide sequences of the microorganisms were taken from the RDP and GenBank databases. Primer development was carried out using the Oligo 6.0 software package. The primers were synthesized at Syntol, Moscow.

RT-PCR was carried out with Sybr Green I dye using the reagent kits of DNA-Technology, Russia as described earlier [8]. Amplification was performed using a DT-96 termocycler (DNA-Technology, Russia) as follows: 95°C for 10 s, 62°C for 10 s, 72°C for

10 s, 40 cycles. Optical measurements were carried out at 62°C. RT-PCR results were used for determination of the relative representation of *A. thiooxidans, A. ferrooxidans, S. thermosulfidooxidans*, and *F. acidiphilum* in the studied culture samples.

PCR efficiency was determined by serial dilutions and building the normalization curve (interval of dilutions 0.5 order of magnitude) and using the DT-96 software package DT-96 (DNA-Technology, Russia) [8]. Electrophoresis was carried out in 1% agarose gel as described [13]. Relative abundance of microorganisms was determined as described previously [14]. The number of copies of the 16S rRNA gene fragments was determined using the Rrndb Internet database (http://rrndb.mmg.msu.edu) [15].

RESULTS AND DISCUSSION

Structure and composition of enrichment cultures obtained from the bioreactors of the Scientific Research Geotechnological Centre (Far Eastern Branch, Russian Academy of Sciences) were described in this study. For the analysis, a bacterial clone library was constructed carrying specific inserts of the 16S rRNA gene fragments of eubacteria and archaea. Using nucleotide sequence analysis, the qualitative composition of mixed microbial communities may be determined at the level of genera, although determination of the species is usually not possible due to the limitations of the method. If the universal primers are well-chosen, the quantitative composition of the community may be evaluated from the number of various ribotype sequences.

More precise analysis was carried out using RT-PCR. Moreover, application of species-specific primers made it possible to perform taxonomical analysis of the previously described microorganisms as the main representatives of the community.

Clone library analysis. A total of 93 clones were obtained and analyzed. All 16S rRNA sequences were grouped into 5 ribotypes belonging to 4 known genera and 1 group of similar sequences identified at the level of family. The grouping was carried out using the FastGroupII online algorithm. The sequences were grouped according to the percentage of similar sequences for two analyzed clones (PSI algorithm) [12]. The minimal level of sequence similarity was accepted as 97%. Most of the sequences (92%) were found to belong to microorganisms of the genus *Acidithiobacillus*. The level of similarity within this ribotype sequence was relatively high (over 98%), prohibiting more detailed analysis within the group. Apart from bacteria of the genus *Acidithiobacillus*, 3 sequences of the *Alphaproteobacteria* from the family *Acetobacteriaceae* were found. Two of them couldn't be identified at the genus level (uncultured acetobacteria). The only sequence which was identified at the genus level belonged to *Acidiphilium* sp. (class *Alphaproteobacteria*, phylum *Rhodospirillales*, family *Acetobacteriaceae*). Three other sequences detected in the community were identified as archaeal (type *Euryarchaeota*). Two were classified as *Thermococcus* sp. (class *Thermococci*, phylum *Thermococcales*, family *Thermococcaceae*), the third one, as *Ferroplasma* sp. (class *Thermoplasmata*, phylum *Thermoplasmatales*, family *Ferroplasmaceae*).

Development of species-specific test-systems. A combination of the morphological and cultural methods revealed that *A. thiooxidans, A. ferrooxidans, S. thermosulfidooxidans*, and *F. acidiphilum* were the predominant native microorganisms in the Shanuch deposit [16]. However, these data were not related to the quantitative composition of the communities. Moreover, they were insufficient for discrimination between closely related and morphologically identical species. To provide qualitative and quantitative analysis of the communities, the complex of PCR test-systems detecting the results of reaction in real-time mode was developed.

The modification of the method used in this study can not be characterized as truly quantitative. It does not determine the absolute copy number of the fragment under consideration. For this purpose, the standard samples with known nucleic acids concentration should be introduced into the experiment. However, if an efficiency of PCR for each pair of primers is known as well as the total bacterial mass (TBM) in a sample, a reasonably precise evaluation of abundance of specific fragments relating to the TBM can be made. Since this approach was successfully used in assessment of the relative microbial abundance in the complex communities of bacterial biocenoses in human urogenital tract, it was adapted for the purpose of the current study [17].

Test systems for specific detection of microorganisms (*A. thiooxidans, A. ferrooxidans, S. thermosulfidooxidans*, and *F. acidiphilum* and for relative quantitative evaluation of the number of copies of the target 16S rRNA fragments were developed. The action of these test systems is based on the usage of Sybr Green I intercalating dye. An important property of intercalating dyes is their ability to embed into the DNA double-helix. A fluorescent signal obtained during the reaction is an accurate representation of dynamics of accumulation of the reaction product and fits well the quantitative analysis of nucleic acids. The only disadvantage of the methods using intercalating dyes is the possible registration of accumulation of the nonspecific amplification products. To verify the results of RT-PCR, the possibility of cross-reactions between the developed species-specific oligonucleotides was evaluated. For this purpose each pair of primers was tested against all available samples of pure cultures in a series of 4 experiments (Fig. 1). Nonspecific amplification was not observed in any of the parallel reactions. Moreover, the nucleotide sequences of PCR products from 5 reactions of each experiment were determined. In all cases the obtained sequence was

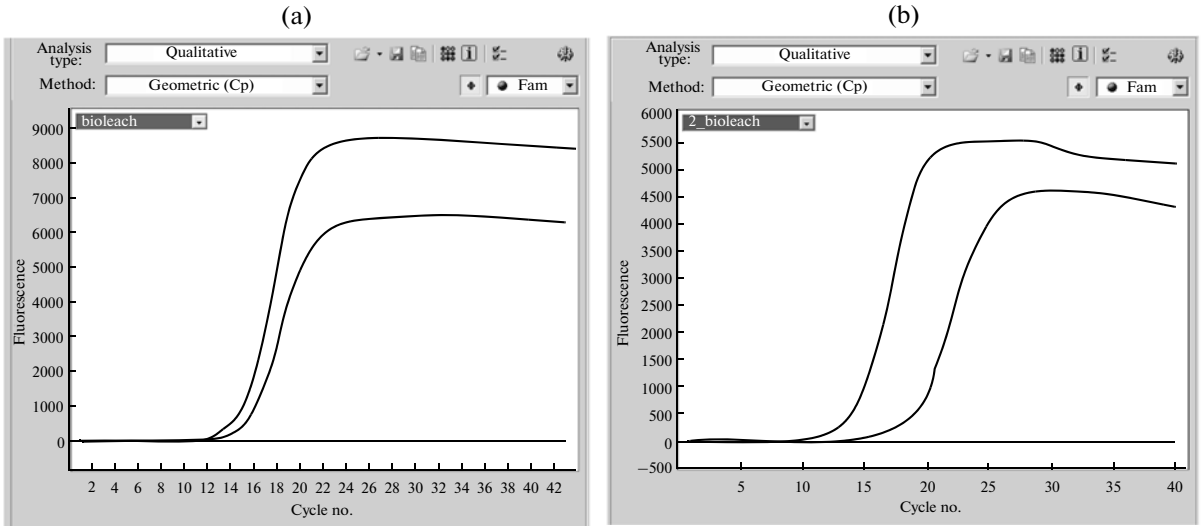

Fig. 1. RT-PCR results in the experiments indicating the possibility of the cross-reaction of developed species- and genus-specific primers. Using pure cultures of *A. thiooxidans, A. ferrooxidans, S. thermosulfidooxidans*, and *Ferroplasma* sp., the pairs of primers AtF, AtR and AfF, AfR (a) and Fer-d, Fer-r1 and Sulf-d1, Sulf-r (b) were tested.

identical to those of the target 16S rRNA gene fragments. Thus, the developed test systems may be used for evaluation of the composition of the mixed communities of bioleaching microorganisms and for the qualitative and quantitative diagnostics of microorganisms: *A. thiooxidans, A. ferrooxidans, S. thermosulfidooxidans*, and *F. acidiphilum*.

Community structure analysis according to RT-PCR. Using species-specific primers, the relative abundance of microorganisms comprising the community was determined. A relative number of a specific group of microorganisms may be shown as the percentage of the total bacterial mass. Quantitative indexes were evaluated basing on the number of threshold cycle (*Ct*) when the positive reaction is first registered. The amount of DNA of certain microorganisms was shown as a number of gene-equivalents (GE) proportional to the number of microorganisms considering the number of the 16S rRNA gene copies in bacterial genome.

In all cases the efficiency of PCR was more than 99% (data not shown). Such efficiency provides a tenfold increase in the copy number of DNA fragments under purpose after 3.4 cycles of the PCR program. The test system possesses sufficient sensitivity to detect single copies of the target fragment [18]. For convenient comparison of the target fragment copy number, *Ct* values were transformed into Log10 of the number of the genome copies according to the formula: $(50 - Ct)/3.4$, where 50 is *Ct* after which the registration of a fragment becomes impossible. As a result, the number of necessary DNA fragments was expressed as 10^n [14]. The formula was adapted considering the differences in the 16S rRNA gene copy number in the *rrn* operon of various species belonging to different bacterial and archaea taxa. Moreover, in a series of experiments in order to determine the PCR efficiency, the maximal *Ct* value (after which the positive signal could not be detected) for the developed test-systems was shown to be 40 cycles. GE numbers

Table 2. Criteria of the quantitative evaluation of samples

	Ct	16S rRNA gene copy number	Target fragment copy number	% TBM
A. ferrooxidans	15.8	2	$10^{7.1}$	62
A. thiooxidans	19.8	2	$10^{5.9}$	4
S. thermosulfidooxidans	29.8	8*	–	–
F. acidiphilum	25.8	1**	$10^{4.2}$	0.14
TBM	15.1	2***	$10^{7.3}$	100

Notes: * Average value for the order *Clostridiales*.

** Average value for the order *Thermoplasmatales*.

*** Average value for microorganisms determined using clone library analysis (genera *Acidithiobacillus* (2), *Thermococcus* (1), family *Acetobacteriaceae* (4), and order *Thermoplasmatales* (1)), considering their abundance.

necessary for evaluation of the relative abundance of microorganisms was calculated:

$$10^{(40-Ct)/3.4}/x,$$

where x is the 16S rRNA gene copy number in the *rrn* operon of bacteria and archaea studied.

Table 2 presents the values of the assessed characteristics.

Relative quantitative analysis demonstrated that *A. thiooxidans*, *A. ferrooxidans*, and *F. acidiphilum* were present in the community under study in a ratio of 62 : 4 : 0.14%, respectively. Thus, these microorganisms comprised ~65% of the community. *Sulfobacillus thermosulfidooxidans* was not detected in the samples. Comparison of these results with clone library analysis suggests species diversity within the determined taxa to be significantly higher. For example, considering the copy number of the 16S rRNA gene, the genus *Acidithiobacillus* was shown to comprise over 90% of the community. Probably, one third of the diversity of acidithiobacilli is due to the presence of other species of this genus. The same is correct for the genus *Ferroplasma*.

The data obtained using molecular methods confirmed the results of microbiological studies. Cultivated microorganisms from the Shanuch deposit mainly belonged to the genus *Acidithiobacillus*. Approximately 62% belonged to one of the most efficient species of iron-oxidizing bacteria, *A. ferrooxidans*. The mixed cultures contained also thermophilic archaea providing higher oxidation rates of sulfides at elevated temperatures.

The techniques used in this study demonstrated high accuracy and sensitivity and may be recommended for use in further investigations in the field of ecology of acidophilic microbial communities. The developed test systems may be adapted for screening research in order to reveal the communities with enhanced producing ability.

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