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TEST-SYSTEMS FOR MONITORING OF CORROSION-RELEVANT SULFATE-REDUCING BACTERIA USING REAL-TIME PCR ASSAY

D. R. Abdulina[1]
L. M. Purish[1]
G. A. Iutynska[1]
M. M. Nikitin[2]
A. G. Golikov[2]

[1]Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine, Kyiv

[2]LLC GenBit, Moscow, Russian Federation

E-mail: adara@ukr.net

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The possibility of the designing test-systems for specific detection of corrosive-relevant sulfate-reducing bacteria using real-time PCR assay were investigated. This method of the bacteria identification is based on the detection of the functional genes, encoding key enzymes of dissimilatory sulfate-reduction pathway, i.e. dissimilatory sulfitreductase α subunit *dsrA*. It was established among the six test-systems specificity reveal only three designed on the base of *Desulfotomaculum, Desulfovibrio, Desulfobulbus* genera sequences. The most corrosive-relevant strain *Desulfovibrio* sp. UCM B-11503 *dsrA* gene detected more effectively (threshold cycle was 20.0), than less corrosive-relevant strains *Desulfovibrio* sp. UCM B-11504 (threshold cycle was 28.1) and for *Desulfotomaculum* sp. UCM B-11505 and *Desulfomicrobium* sp. UCM B-11506 were 24.9 and 23.1 cycles, respectively. Test-systems allowed identifying corrosive-relevant sulfate-reducing bacteria faster and more effective. This approach will serve as a base for monitoring of these bacteria for estimating corrosion sites on the high-level dangerous man-caused objects.

Key words: sulfate-reducing bacteria, dissimilatory sulfate-reduction genes, test-systems, real-time PCR.

Sulfate-reducing bacteria (SRB) are widespread in various environments: sea sludge deposits, hydrothermal springs, fresh water systems, soils, anaerobic mud, wastewaters, oil and gas fields and other ecotopes [1–4]. They are known to be the main producers of the biogenic hydrogen sulfide in the biosphere. In the natural conditions biogenic hydrogen sulfide reacts contributing to the formation of sulfur ores, metal sulfide deposits, therapeutic muds, mineral waters and soda lakes. Furthermore, the hydrogen sulfide produced by sulfate-reducers in the industrial systems behaves as a corrosive agent, promoting the biodeterioration of steel, ferroconcrete and metal installations [4]. Therefore, it is actual for monitoring of sulfate-reducing bacteria in the places of the anthropogenic intervention in the underground environment.

As described in our previous works, the zones undergoing man-caused load, namely in the places of underground communication, gas pipeline and hot-water system, get colonized by the sulfate-reducing bacteria *Desulfovibrio Desulfotomaculum* and *Desulfomicrobium* genera [5–7]. The fundamental metabolic feature of isolated SRBs is the hydrogen sulfide production as the result of dissimilatory sulfate reduction.

In all the SRBs described sulfate-reduction pathway is a complex multistage process which catalyzed by more than 17 enzymes, three of them are key enzymes. The initial stage is activation of sulfate by ATP sulfurylase (SAT), (EC 2.7.7.4) the second reaction is reduction of adenosine-5′-phosphosulfate (APS) to adenosine-monophosphate (AMP) and sulfite by enzyme APS reductase (APR) (EC 1.8.99.2), and subsequent further reduction to hydrogen sulfide by dissimilatory sulfite reductases (DSR) (EC 1.8.99.3) [8]. Thus, the functional genes of the sulfate reduction pathway are *aps, apr*-, and *dsr*-genes, encoding the key enzymes [8–10]. Analysis of the *aps*-genes primary are used for differing sulfate- and sulfite-reducing bacteria, cause the last one haven't this gene

[11, 12]. *Dsr*-genes related to the final stage of sulfate reduction are found in all the sulfate- and sulfite-reducing bacteria and may be defined using a certain set of conservative primers.

The use of the molecular biology methods, namely, determination of the functional genes encoding the key enzymes of dissimilatory sulfate-reduction pathway is an efficient way to detect sulfate-reducing bacteria.

The goal of the study is to develop a complex of molecular-biology methods and microchip-based test-systems on the biochip for monitoring the corrosion-relevant sulfate-reducing bacteria by real-time multiplex PCR assay.

Materials and Methods

Bacterial cultures and cultivation. Sulfate-reducing bacteria *Desulfovibrio* sp. UCM B-11503, *Desulfovibrio* sp. UCM B-11504, *Desulfotomaculum* sp. UCM B-11505, *Desulfomicrobium* sp. UCM B-11506 were used at this work. Bacterial strains early were isolated from man-caused ecotopes, identified and had established their corrosive activity [6]. Bacteria are stored in the Ukrainian Collection of Microorganisms (UCM)of the Zabolotny Institute of Microbiology and Virology NAS of Ukraine.

Bacteria had cultivated on the Postgate "B" media [14] in anaerobic conditions at 28 °C, during 7 days to the middle of the log phase growth.

Genomic DNA extraction. DNA were extracted from culture biomass using kit "DNA Sorb-B" ("AmpliSens", Russia) according to manufacturer instructions. DNA concentration had measured on the spectrophotometer SmartSpec Plus (Biorad, USA). As a control had used TE-buffer (pH 7.8).

PCR-primers. At this work were used degenerated primers specific to the genes, encoding sulfate-reduction pathway enzymes: dissimilatory sulfite reductase α- and β-subunits *(dsrAB)* and APS reductase α-subunit (*apsA*) [11, 13]. Primers characteristics were shown in Table 1.

Test-systems. Primers and probes had designed on the basis of the available from GenBank database sequences of dissimilatory sulfite reductase α-subunit (*dsrA*) of sulfate-reducing bacteria belonging to different genera: *Desulfotomaculum*, *Desulfovibrio*, *Desulfomicrobium*, *Desulfobulbus*, *Desulfobacter*, *Desulfococcus*. The primers had been selected by the following parameters: specificity to certain genus of sulfate-reducing bacteria, length of the amplified fragment of a gene (not more than 300 bp) and similarity of the annealing temperature. Probes were contained fluorescent label FAM on the 5-end, phosphate group and black hole quencher BHQ-1 on the 3-end. Test-systems were designed using program software Oligo 6 (www.oligo.net/), designed test-systems were checked by the program Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Designed test-systems characteristics were shown in Table 2.

*dsrAB and aps*A *genes amplification* were performed with degenerate primers specified in Table 1 and subsequent reaction products electrophoresis. Reaction mixtures contained, in a volume 20μl: 10 pM of the each primer, Master Mix GenPak PCR Core (Neogene, Ukraine), contain 1.0 U of "hot-start" Taq-polymerase; 0.2 μM mixes of dNTPs; 2.5 μM of $MgCl_2$ and 5 μl template DNA (in concentration 50 μg/ml). Amplification was performed using the thermal cycler 2720 (Applied Biosystems, USA). The thermal profile for amplification was as follows: an initial denaturation step (1 min, 95 °C) was followed by 30 cycles of denaturation (10 s, 95 °C), annealing (20 s, 61 °C), and extension (60 s, 72 °C) and one final extension step (10 min, 72 °C) [2]. Amplification products were analyzed by electrophoresis in 1% agarose gel with TBE-buffer (pH 8.0), at field voltage tension (10 V/sm). Gel was dyed by the ethidium bromide in concentration 1 μg/ml. As a molecular mass marker were used MassRuler DNA LadderMix

Table 1. **Degenerate primers used for dsrAB and apsA gene amplification**

Target gene	Primers pair	Primers sequences (5/-3/)*	PCR product length (bp)	References
dsrAB	DSR1F DSR4R	ACS CAY TGG AAR CAC G GTG TAR CAG TTA CCR CA	1 900	[13]
apsA	APS-FW APS-RV	TGG CAG ATM ATG ATY MAC GG G GGG CCG TAA CCG TCC TTG AA	396	[11]

Note: * – degenerate positions marked bold type: R (G or A); Y (C or T); S (G or C); M (A or C).

Table 2. **Specific test-systems used for real-time PCR**

Specificity to sulfate-reducing genera	Test-system name	Primers and probes sequences (5′-3′)	PCR product length (bp)
Desulfotomaculum	SRBF1 SRBR1 SRBProbe1	ACC CAC TGG AAA CAC GG CGC AGG AAG TCG CTC TT FAM-CTTACTGGCTGGCTGGTTGAC-BHQ1	161
Desulfovibrio	SRBF2 SRBR2 SRBProbe2	ACC CAC TGG AAG CAC G ACG GTG TGG AAG TGC G FAM-CGGGCTGGTCACAGTAACGG-BHQ1	113
Desulfomicrobium	SRBF3 SRBR3 SRBProbe3	GAC CAG CCC CAG ATG TT ATG AAA ATG AGC AAC GCC G FAM-CGGCGTTGCTCATTTTCAT-BHQ1	95
Desulfobulbus	SRBF4 SRBR4 SRBProbe4	GTC TGC CGA CCT TCC TC CCC AGC CAC CAG GTA CT FAM-TGCCGACCTTCCTCAGCG-BHQ1	194
Desulfobacter	SRBF1 SRBR5 SRBProbe5	ACC CAC TGG AAA CAC GG CTT ACC GCA GGG CTG AT FAM-CGGGGAACAGTTTGGGCT-BHQ1	138
Desulfococcus	SRBF6 SRBR6 SRBProbe6	GCG TTA TCG GTC GTT ACT TGT GGG TCA GTT CAA AGA A FAM-CCCAGGAAGATGATGTCGCC-BHQ1	240

SM0403(Fermentas, Lithuania). Gels were documented with the gel documentation system INGENIUS LHR (Syngene, USA).

dsrA-gene real-time PCR-amplification were performed with designed test-systems on the *dsrA*-gene α-subunit (Table 2). Reaction mixtures contained, in a volume 10μl: 0.4 μM of each primer; 0.2 μM of probe; 10× buffer solution for DNA polymerase; 0.25 μM of mixes dNTPs; 0.1 U./μl DNA Taq-polymerase ("Eurogen", Russia) and 2 μl template DNA (in concentration 50 μg/ml). Reaction mixture loaded (in volume 1.2 μl) in aluminum microchip wells (LLC "GenBit", Russia). The thermal profile for amplification was as follows: an initial denaturation step (3 min, 94 °C), was followed by 45 cycles of denaturation (5 s, 94 °C) and annealing (30 s, 60 °C). Real-time PCR amplification was performed using microchip thermal cycler AriaDNA (LLC "Lumex-Marketing", Russia). Results were analyzed using program software "AriaDNA". It was shown results of the typical experiments.

Results and Discussion

Comparative microbiological studies of the soils near gas mains had shown that on the emergency areas the quantity of the sulfate-reducing bacteria was increased in 3–4 orders [7]. Recently sulfate-reducing bacteria from man-caused ecotopes were isolated and identified. Revealed that these bacteria had corrosive activity and synthesized large amount of the hydrogen sulfide (Table 3).

It was performed comparative study of the corrosive-relevant sulfate-reducing bacteria using PCR with amplification products electrophoresis. In all the investigated bacteria were appeared genes encoding sulfate-reduction pathway enzymes: dissimilatory sulfite reductase (*dsrAB*) and APS-reductase (*apsA*) (Fig. 1, tracks 1–6). Using PCR amplification of the *dsrAB* and *apsA* genes from sulfate-reducing bacteria were obtained positive result indicating that amplicons were expected sizes 1900 bp and 396 bp, respectively.

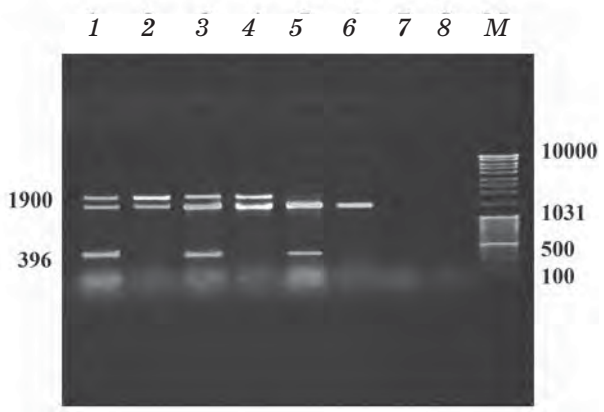

Fig. 1. **Electrophoregram of PCR amplification of the sulfate-reduction pathway genes *dsrAB* and *apsA*:**
tracks *1, 2* — *Desulfovibrio* sp. B-11503;
3, 4 — *Desulfotomaculum* sp. B-11505;
5, 6 — *Desulfomicrobium* sp. B-11506;
7, 8 — negative control; *M* — molecular mass ruller DNA Ladder Mix SM0403

Table 3. **Corrosive activity of the sulfate-reducing bacteria**

Bacterial culture	Hydrogen sulfide production, μg/l	Steel corrosion rate, $g/m^2 \times hour$
Desulfovibrio sp. UKM B-11503	418.6 ± 20.9	0.090 ± 0.004
Desulfovibrio sp. UKM B-11504	426.8 ± 19.2	0.048 ± 0.002
Desulfotomaculum sp. UKM B-11505	405.3 ± 16.2	0.049 ± 0.0021
Desulfomicrobium sp. UKM B-11506	451.5 ± 15.7	0.055 ± 0.0024

On the tracks *1*, *3*, *5* it's shown the presence amplification products belonging both studied genes, cause there were loaded in wells mixes of the two primers pairs (DSR1F/DSR4R and APS-FW/APS-RV). On the tracks *2*, *4*, *6* detected amplification products belong to dissimilatory sulfite reductase (*dsrAB*). In this case there were loaded only one primers pairs (DSR1F/DSR4R).

It should be noted that PCR assay with subsequent amplification products electrophoresis had disadvantage because degenerate primers often insufficiently, so this is decrease the sulfate-reducers identification accuracy. Moreover, this approach should establish only presence of the sulfate-reduction genes, but not activity and corrosiveness of the sulfate-reducing bacteria.

The usage of the real-time PCR for sulfate-reduction bacteria detection reveals more effective. Selected specific primers and probes for this method allow detecting quantity and activity of the sulfate-reducing bacteria at different samples [15]. It had been found that this bacterial cell contain only one *dsr*-gene copy [3], so identifying this genes it is possible to identify relative quantity of sulfate-reducing bacteria in studied samples.

During the study we had developed test-systems (primers and probes) for specific real-time detection of the sulfate-reducing bacteria (table 3). Primer pairs (SRBF/SRBR) and probes (SRB ProbeN) were selected by the following parameters as specificity to certain genus of the sulfate-reducing bacteria: test-system SRB1 (*Desulfotomaculum*), SRB2 — *Desulfovibrio*, SRB3 — *Desulfomicrobium*, SRB4 — *Desulfobulbus*, SRB5 — *Desulfobacter*, SRB6 — *Desulfococcus*. Also take in to account length of the amplified gene fragment (not more than 300 bp), similarity of the annealing temperature (60 °C), as the several test-systems loaded to the one microchip well. Each primer pairs tested with all the studied sulfate-reducing bacteria. Fluorescent probe signal intensity during the real-time PCR was increased in direct ratio to the amplicons accumulation, displaying dynamics of the PCR product accumulation.

To all the investigated corrosive-relevant sulfate-reducing bacteria among the six test-systems specificity revealed three of them designed on the base of such genera *Desulfotomaculum* (SRB1), *Desulfovibrio* (SRB2) and *Desulfobulbus* (SRB4). Due to this test-systems on the microchip different sulfate-reducing bacteria were detected simultaneously, i.e. this method were multiplex (Fig. 2).

Real-time PCR quantitative criteria had been estimated on the base of the values of the "threshold cycle". Threshold cycle (*Ct*) is the cycle *n*, when reached reported fluorescence level P*Ct* = const. The results were reliable when threshold cycle was less than 35. Line Ch1 is the threshold line for first fluorescence probe channel (FAM), which underline above the backgroung automatically by the program. Intersection the fluorescence signal curve with threshold line (*Ch1*) gave the threshold cycle C*t*.

Relevant quantity of the sulfite-reductase (*dsrA*) gene copies number was expressed as a account of the genome equivalent which proportional to the bacteria account. Number of target gene copies were increased in order for 3.4 cycle of the PCR program. Values of the threshold cycle C*t* mathematically with formula counted into lg10 genome copies [16]:

$$10^{(45\text{-}Ct)/3.4},$$

where 45 — threshold cycle, after that target gene fragment registration impossible.

Values of the threshold cycles and relative *dsrA* gene fragment copy numbers shown in the Table 4.

The most specific were test-system SRB2, designed on the base of *Desulfovibrio* genus. It had been established that best *dsrA* gene detection were in the most corrosiveness strain *Desulfovibrio* sp. UCM B-11503, threshold cycle was 20.0 cycles. For less corrosiveness strain *Desulfovibrio* sp. UCM B-11504 threshold cycle was 28.1; and

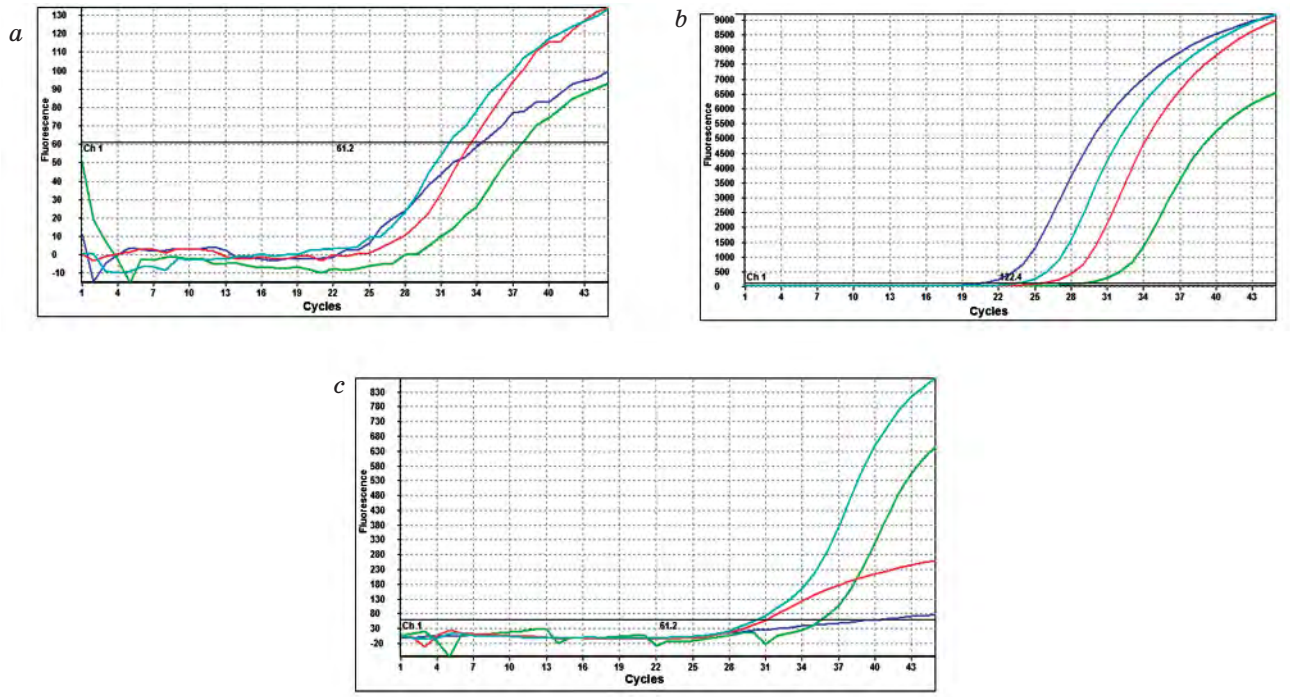

Fig. 2. **Real-time PCR kinetic curves:**
a — test-system with specificity to *Desulfotomaculum* (SRB1); *b* — test-system with specificity to *Desulfovibrio* (SRB2); *c* — test-system with specificity to *Desulfobulbus* (SRB4).
Samples: *1* — *Desulfovibrio* sp. UCM B-11503 (blue); *2* — *Desulfovibrio* sp. UCM B-11504 (green); *3* — *Desulfotomaculum* sp. UCM B-11505 (red); *4* — *Desulfomicrobium* sp. UCM B-11506 (cyan)

Table 4. **Criteria of real-time PCR quantitative assessment**

Bacterial culture	Threshold cycle *Ct*			Gene copy number *dsrA*, lg10		
	SRB1	SRB2	SRB4	SRB1	SRB2	SRB4
Desulfovibrio sp. UCM B-11503	34.5	20.0	–*	3.1	7.3	–
Desulfovibrio sp. UCM B-11504	–	28.1	–	–	5.0	–
Desulfotomaculum sp. UCM B-11505	33.6	24.9	31.3	3.3	5.9	4.0
Desulfomicrobium sp. UCM B-11506	31.8	23.1	30.5	3.8	6.4	4.3

Note: * — threshold cycle > 35.

for *Desulfotomaculum* sp. UCM B-11505, *Desulfomicrobium* sp. UCM B-11506 24.9 and 23.1 cycles, respectively. It was shown that relative account of the *dsrA* gene were in a range from lg 5.0 for *Desulfovibrio* sp. B-11504 to lg 7.3 — for *Desulfovibrio* sp. B-11503. Comparing studies of the obtained results with corrosiveness activity data (Table 1) showed that relative quantity of the sulfitereductase gene (*dsrA*) corresponded to the corrosiveness activity of the studied sulfate-reducing bacteria.

To analyze the results should be noted that detection of the sulfate-reduction bacteria by the microbiological method, included pure bacterial culture isolation and subsequent determination of their cultural and biochemical properties, is a labour-intensive and time-consuming approach and allows to identify only cultivated microorganisms.

Recently, begin applying polyphasic analysis with usage of the several methods, including molecular genetics. To detect the sulfate-reducing bacteria use the PCR assay with marker genes of the dissimilatory sulfate-reduction [3, 4, 8, 10, 12].

As a phylogenetic markers widespread genes encoding ribosomal subunit 16 S rRNA. It had been designed large number of the primers for sulfate-reducing bacteria genes amplification [17]. Such approach used in metagenomic analysis for investigation the

phylotypes diversity in microbial communities in particularly oil fields, sulfate-reducing bacteria are caused corrosion where damages of oil equipment [1, 4, 18]. Sulfate-reducing bacteria 16 S rRNA gene analysis is complicated due to its multicopy [3, 19, 20] and high heterogeneity of the gene terminal sites [21].

Usage of the functional gene encoding key enzymes of the sulfate-reduction pathway revealed more effective method for sulfate-reducing bacteria detection [8, 10, 13]. Conserved gene used in this study allowed us designing specific primers and probes; this made investigated bacterial group detection more direct and reliable.

Thus, designed test-systems for real-time PCR enable to detect corrosive-relevant bacteria more effective than current existing methods. These test-systems could found the application for further monitoring of the sulfate-reducing bacteria in oil, oil products as well as for mapping corrosive aggressiveness of soils, this maps could use for prediction potential dangerous sites during the planning and building technical objects.

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ТЕСТ-СИСТЕМИ ДЛЯ МОНІТОРИНГУ КОРОЗІЙНО-АГРЕСИВНИХ СУЛЬФАТРЕДУКУВАЛЬНИХ БАКТЕРІЙ НА ОСНОВІ МЕТОДУ ПЛР В РЕЖИМІ РЕАЛЬНОГО ЧАСУ

Д. Р. Абдуліна[1]
Л. М. Пуріш[1]
Г. О. Іутинська[1]
М. М. Нікітін[2]
О. Г. Голіков[2]

[1]Інститут мікробіології і вірусології ім. Д. К. Заболотного НАН України, Київ
[2]ТОВ «ГенБіт», Російська Федерація, Москва

E-mail: adara@ukr.net

Методом ПЛР в режимі реального часу з'ясовували можливість створення тест-систем для специфічної детекції корозійно-агресивних сульфатвідновлювальних бактерій. Метод виявлення цих бактерій базується на визначенні функціональних генів, що кодують ключові ензими шляхів дисиміляційної сульфатредукції, зокрема α-субодиницю дисиміляційної сульфітредуктази *dsrA*. Встановлено, що із шести розроблених тест-систем специфічність виявили три на основі родів *Desulfotomaculum, Desulfovibrio, Desulfobulbus*. У найбільш корозійно-агресивного штаму *Desulfovibrio* sp. УКМ В-11503 ген *dsrA* виявлявся ефективніше, пороговий рівень визначення становив 20,0 циклів, для менш агресивного *Desulfovibrio* sp. УКМ В-11504 — 28,1, а для *Desulfotomaculum* sp. УКМ В-11505 та *Desulfomicrobium* sp. УКМ В-11506 — 24,9 і 23,1 відповідно. Ці тест-системи дають змогу швидше й ефективніше виявляти корозійно-агресивні сульфат відновлювальні бактерії, що може слугувати основою для їх моніторингу з метою виявлення осередків корозії на об'єктах із підвищеною техногенною небезпекою.

Ключові слова: сульфатвідновлювальні бактерії, гени дисиміляційної сульфатредукції, тест-системи, ПЛР в режимі реального часу.

ТЕСТ-СИСТЕМЫ ДЛЯ МОНИТОРИНГА КОРРОЗИОННО-АГРЕССИВНЫХ СУЛЬФАТРЕДУЦИРУЮЩИХ БАКТЕРИЙ НА ОСНОВЕ МЕТОДА ПЦР В РЕЖИМЕ РЕАЛЬНОГО ВРЕМЕНИ

Д. Р. Абдулина[1]
Л. М. Пуриш[1]
Г. А. Иутинская[1]
М. М. Никитин[2]
А. Г. Голиков[2]

[1]Институт микробиологии и вирусологии им. Д. К. Заболотного НАН Украины, Киев
[2]ООО «ГенБит», РФ, Москва

E-mail: adara@ukr.net

Методом ПЦР в режиме реального времени исследовали возможность создания тест-систем для специфической детекции коррозионно-агрессивных сульфатредуцирующих бактерий. Метод выявления этих бактерий базируется на определении функциональных генов, кодирующих ключевые энзимы путей диссимиляционной сульфатредукции, в частности α-субъединицы диссимиляционной сульфитредуктазы *dsrA*. Установлено, что из шести тест-систем специфичность проявили три на основе родов *Desulfotomaculum, Desulfovibrio, Desulfobulbus*. У наиболее коррозионно-агрессивного штамма *Desulfovibrio* sp. УКМ В-11503 ген *dsrA* выявлялся эффективнее, пороговый уровень определения составил 20,0 циклов, для менее агрессивного *Desulfovibrio* sp. УКМ В-11504 — 28,1, а для *Desulfotomaculum* sp. УКМ В-11505 и *Desulfomicrobium* sp. УКМ В-11506 — 24,9 и 23,1, соответственно. Эти тест-системы позволяют быстрее и эффективнее определять коррозионно-агрессивные сульфатредуцирующие бактерии, что послужит основой их мониторинга с целью выявления очагов коррозии на объектах с повышенной техногенной опасностью.

Ключевые слова: сульфатредуцирующие бактерии, гены диссимиляционной сульфатредукции, тест-системы, ПЦР в режиме реального времени.