Halophilic Bacteria as a Promising Basis of Biopreparations for Improving the Growth of Autochthonous Strains-Destructors in Salinization Conditions

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Abstract. In some cases, pollution of ecosystems with persistent toxic organic compounds, including polycyclic aromatic hydrocarbons (PAHs), is accompanied by salinization, which significantly inhibits the degradation of these compounds by autochthonous communities of microorganisms. Therefore, new methods of reclamation of such polluted territories are being sought and developed. One of the approaches can be the introduction of bacteria that stimulate the physiological activity of autochthonous destructors. In the course of the conducted study, the moderately halophilic strain *Halomonas* sp. SMB31 was identified as the most competitive for the intermediates of the destruction of naphthalene (model compound PAH), and its effect on the growth of the halotolerant destructor strain *Rhodococcus* sp. SMB38 has been evaluated during the degradation of naphthalene under conditions of high salinity (7% NaCl). It is shown that the joint cultivation *of Rhodococcus* sp. SMB38 and *Halomonas* sp. SMB31 led to a significant reduction in the duration of the period of adaptation to environmental conditions and an increase in the specific growth rate of the destructor strain. Thus, the obtained results showed the prospects of the studied moderately halophilic strain *Halomonas* sp. SMB31 for use as a biological preparation for the purpose of activating the physiological processes of autochthonous microorganisms-destructors under conditions of salinization of the environment.

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) belong to the class of aromatic compounds with condensed nuclei. Widespread use in the chemical and pharmaceutical industries, the presence of coke, gas and oil refining industries in by-products, the formation of various organic materials (coal, oil, gas, wood, garbage, etc.) during combustion has led to the ubiquitousness of PAHs in the environment [1]. Due to their toxic, mutagenic and carcinogenic effects on living organisms, interest in biotechnologies aimed at cleaning the

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environment from compounds of this class has sharply increased [1]. Microbial destruction is one of the main methods of remediation of PAH-contaminated ecosystems. However, it is influenced by changes in physical and chemical environmental factors, including salinization, which reduces the rate of destruction of PAHs by native flora or completely inhibits it [2, 3, 4]. Therefore, the search is underway for technologies that allow the restoration of PAH-contaminated ecosystems burdened by salinization. One of the solutions to this issue may be the use of approaches that increase the physiological activity of autochthonous microorganisms-destructors, including by introducing solutes that perform an osmoprotective role. Previously, it was shown that the introduction of yeast extract into the culture medium accelerated the destruction of xenobiotics in conditions of high salinity [5, 6, 7]. However, the application of this method in practice requires periodic treatment of contaminated areas, which leads to an increase in financial costs for ecosystem remediation. An alternative approach may be the introduction of microorganisms-producers of osmoprotective compounds. Currently, numerous scientific papers have shown that the promising producers of the most effective osmoprotective compound in the world of prokaryotes – ectoine - are moderately halophilic bacteria of the *Halomonadaceae* family [8].

For more than two decades, our research has been devoted to studying the microbial diversity and biotechnological potential of bacteria in the unique ecosystem of the industrial development area of the Verkhnekamsk Salt basin (Russia). A naphthalene-metabolizing consortium of SMB3 bacteria was isolated from saline soil by enrichment cultivation [9], which proved to be an effective agent for bioremediation of saline soils from PAHs [10]. The consortium, isolated and maintained in Raymond liquid mineral medium with 6% NaCl and naphthalene, united 7 strains belonging to the physiological groups of halotolerant and halophilic bacteria [9]. Phylogenetic analysis of almost complete 16S rRNA gene sequences of these strains revealed their belonging to the genera *Glutamicibacter*, *Rhodococcus*, *Microbacterium*, *Salinicola*, *Halomonas* and *Thalassospira* [11]. However, prolonged exposure to higher salinity of the medium (7% NaCl) under the conditions of naphthalene degradation led to a decrease in the species richness of the consortium, retaining halotolerant naphthalene destructor strains SMB37 and SMB38 of the genus *Rhodococcus,* as well as moderately halophilic bacteria of the *Halomonadaceae* family that do not utilize naphthalene [11].

The present study was aimed at selecting the most competitive moderately halophilic strain for the growth substrate and studying its effect on the growth parameters of the naphthalene destructor strain under conditions of high salinity of the medium during co-cultivation.

2 Materials and methods

The selected bacterial consortium and its constituent bacterial strains *Salinicola socius* SMB35 ^{T}, *Halomonas* sp. SMB31, *Rhodococcus* spp. SMB37 and SMB38 were used in the work [11].

Pure cultures for experiments were grown on an agarized Raymond nutrient medium (NRM) [12] containing 3% NaCl. The concentration of sodium chloride was chosen taking into account the salinity range of the medium in which bacterial strains can grow [9]. The consortium was cultured in Raymond liquid mineral medium (RMM) [12] containing 7% NaCl and naphthalene at a final concentration of 1 g/l. The cultivation conditions corresponded to those of the consortium selection [11].

Periodic cultivation was carried out in 250 ml flasks in 100 ml of RMM on an orbital shaker UVMT-12-250 with a flask rotation speed of 100 rpm at 28 °C.

Screening of strains for the ability to use acetate as the only source of carbon and energy, the final concentration of which was 0.5 g/l, was carried out by cultivating strains in a liquid RMM containing 3% NaCl. Growth was assessed by changes in the optical density of the culture fluid using UV-Visible BioSpec-mini (Shimadzu, Japan) at a wavelength of 540 nm. The length of the optical path of the cuvette was 0.5-cm.

The bacterial consortium was grown on acetate in a liquid RMM containing 7% NaCl until the stationary growth phase. The final concentration of the substrate was 0.5 g/l. The inoculum was a consortium grown in liquid RMM containing 7% sodium chloride and naphthalene. The consortium structure was studied by denaturating gradient gel electrophoresis (DGGE).

To conduct an experiment on the co-cultivation of pure cultures, the strain-destructor *Rhodococcus* sp. SMB38 was grown in naphthalene vapors on agarized RMM with 3% NaCl. Naphthalene crystals were added to the lid of a Petri dish. A moderately halophilic strain of *Halomonas* sp. SMB31, which does not utilize naphthalene, was cultured on an agarized NRM with 3% NaCl. The cells were washed off with a sterile solution of 3% NaCl, washed and adjusted to the same optical density of the solution (OD_{540}) 0.8 units. Then the same volumes of strain inoculates were introduced into liquid RMM with 7% NaCl and naphthalene. A similar volume of culture fluid of the destructor strain was introduced into a liquid RMM with 7% NaCl and naphthalene. The account of viable cells of the halotolerant naphthalene destructor strain was carried out by the method of serial dilutions followed by seeding on dense NRM without additional supplementation of sodium chloride. The results were expressed in the form of colony-forming units (CFU). The initial concentration of cells was $1.2*10^{5}$. The specific growth rate (μ) and the duration of the *lag* phase (T_{l}) were calculated according to standard formulas [13]. To confirm the preservation of the halophilic strain as part of an artificially created association, the DGGE method was used.

The total genomic DNA was isolated from the bacterial biomass of the consortium grown in RMM with acetate in the presence of 7% NaCl (see above), as well as from an artificially created association of strains of *Rhodococcus* sp. SMB38 and *Halomonas* sp. SMB31 (see above). The cells were precipitated by centrifugation of 5 ml of culture medium at 8000×g for 10 minutes at room temperature and resuspended in a TE buffer. Pure cultures (SMB31, SMB35, SMB37 and SMB38) were grown on an agarized NRM with 3% NaCl, then one colony was placed in 1.5 ml of eppendorf containing 100 µl of TE buffer. Genomic DNA was isolated from bacterial biomass according to the described SDS-CTAB method [14]. Amplification was performed with primers flanking a fragment of the 16S rDNA gene with variable V1-V3 regions. The direct primer included a GC-rich sequence 40 bp long (GC-tail) at the 5' end [15]. The PCR products had a total length of 566 bp according to the reference strain of *Escherichia coli* (J01695). Amplicons were separated in a 6% (mass/volume) polyacrylamide gel with a denaturing gradient from 30 to 60% (where 100% of the denaturant contains 7M urea and 40% formamide) for 4 hours at 130 V and 60°C on a universal DcodeTM mutation system (Bio-Rad, USA). The gradient is made using the Gradient Delivery System model 475. After electrophoresis, the gel was stained in a solution of ethidium bromide (0.5 mcg/ml) and documented using Gel Doc XR (Bio-Rad, USA). Identification of bands in the consortium's DGGE profiles was carried out by comparison with the position of 16S rDNA fragments of pure cultures.

3 Results and discussion

Since the selected consortium included representatives of moderately halophilic bacteria of different genera (strains of *Halomonas* sp. SMB31 and *Salinicola socius* SMB35 ^{T}) of the *Halomonadaceae* family, it was decided to investigate their competitiveness for an easily

digestible source of carbon and energy in conditions of high mineralization of the medium (7% NaCl). Acetate was used as a growth substrate, since it is the end product of the metabolism of various organic compounds, including those related to PAHs [16, 17, 18, 19]. Despite the fact that both members of the *Halomonadaceae* family and the genus *Rhodococcus* were capable of growth on acetate (the optical densities of the culture medium reached 0.2-0.3 units), the cultivation of a consortium of bacteria on acetate led to the absolute dominance of the moderately halophilic strain *Halomonas* sp. SMB31 (Fig. 1). The data obtained indicate its advantages in competition for a growth substrate, and, consequently, the ability to occupy an ecological niche of consumption of naphthalene metabolism products in new communities.

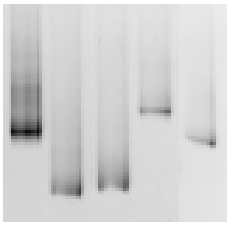

Fig. 1. DGGE of 16S rRNA gene fragments amplified with genomic DNA: 1 – consortium grown at 7% NaCl in liquid RMM with acetate, 2 – strain SMB38, 3 – strain SMB37, 4 – strain SMB35, 5 – strain SMB31.

Earlier, by the method of proton magnetic resonance, it was found that the main osmoprotective substance of the cells of the strain *Halomonas* sp. SMB31 was an ectoine, the share of signal intensities of its protons among such water-soluble compounds ranged from 67.9 to 82.5% [20]. Taking into account the above, a moderately halophilic strain of *Halomonas* sp. SMB31 was selected to study its effect on halotolerant naphthalene-degrading bacteria during co-cultivation under conditions of high salinity of the medium. Of the two destructor strains of the consortium, the most effective one was included in the experimental scheme - the strain *Rhodococcus* sp. SMB38, which performs 100% destruction of naphthalene (0.1 g/l) in 48 hours in RMM without salt [21]. However, an increase in the osmotic pressure of the culture medium to 6% NaCl reduced the destruction of naphthalene by this culture to 31.5% in 72 hours [11].

It should be noted that an increase in the salt resistance of the halotolerant strain was previously detected during co-cultivation in biofilms with halophilic bacteria [22]. The authors of the work used an agarized culture medium, including casamic acids and yeast extract, on which representatives of both physiological groups grew. In this regard, it is extremely difficult to assume whether trophic relationships have developed in the studied artificial association. In addition, the work did not concern the assessment of kinetic parameters of strain growth.

This study takes a different approach. Firstly, the selected strains were co-cultured in liquid RMM, the only organic compound in which was naphthalene. Thus, a moderately halophilic strain of *Halomonas* sp. SMB31, for which there was no decrease in naphthalene in experiments on the quantitative assessment of its destruction [11], in an artificially created association could occupy only an ecological niche of consumption of intermediates of naphthalene degradation. Secondly, a comparative analysis of the growth kinetics of the *Rhodococcus* sp. SMB38 naphthalene destructor strain was carried out when grown both in pure culture and as part of an artificially created association (*Rhodococcus* sp. SMB38/*Halomonas* sp. SMB31) (table). It is shown that the joint cultivation of a halotolerant destructor with a halophilic strain led to a reduction in the period of adaptation

to environmental conditions of the first by 25.5%. In addition, the specific growth rate of the destructor *Rhodococcus* sp. SMB38 increased by 33.3% (table). In both cases, the CFU of the destructor strain grew to 3 orders of magnitude.

Table 1. Growth parameters of the SMB38 destructor strain in monoculture and in association

Cultivation method	*lag*-Phase (h)	μ (h^{-1})	CFU
monoculture	165.80	0.06	$3.26x10^8$
association	123.60	0.08	$7.26\ x10^7$

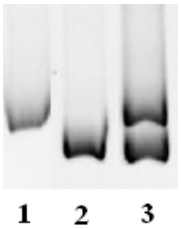

Fig. 2. DGGE amplified with genomic DNA fragments of the 16S rRNA gene: 1 – strain of *Halomonas* sp. SMB31, 2 – strain of *Rhodococcus* sp. SMB38, 3 – association

Despite the duration of the experiments (about 350 hours), the moderately halophilic strain *Halomonas* sp. SMB31 was preserved as part of an artificially created association (Fig. 2). The results obtained indicate the formation of a trophic chain, the links of which were the naphthalene destructor strain *Rhodococcus* sp. SMB38 and the strain *Halomonas* sp. SMB31 consuming the end products of its metabolism.

4 Conclusions

Thus, the obtained results demonstrated the prospects of the studied moderately halophilic strain *Halomonas* sp. SMB31 for use as a biological preparation for the purpose of activating the physiological processes of autochthonous destructor strains in conditions of high salinity of the medium.

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