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EXPERIMENTAL ARTICLES

Resistance of the Oil-Oxidizing Microorganism *Dietzia* sp. to Hyperosmotic Shock in Reconstituted Biofilms

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Abstract—A number of halotolerant and halophilic bacterial strains were isolated from the Romashkinskoe oil field (Tatarstan) stratal waters having a salinity of up to 100 g/l. The isolation of pure cultures involved biofilm reconstitution on M9 medium with paraffins. The associations obtained were dispersed and reinoculated onto solid media that contained either peptone and yeast extract (PY medium) or paraffins. It was shown that such associations included both oil-oxidizing bacteria and accompanying chemoheterotrophic bacteria incapable of oil oxidation. The pure cultures that were isolated were used for creating binary biofilms. In these biofilms, interactions between halophilic and nonhalophilic bacteria under hypo- and hyperosmotic shocks were investigated. We conducted a detailed study of a biofilm obtained from an oil-oxidizing halotolerant species (with an upper growth limit of 10–12% NaCl) identified as *Dietzia* sp. and an extremely halophilic gram-negative bacterium (growing within the 5–20% NaCl concentration range) of the genus *Chromohalobacter* that did not oxidize paraffins. If these microorganisms were grown in a mixed suspension (planktonic) culture that was not supplemented with an additional amount of NaCl, no viable cells of the halophilic microorganism were detected after reinoculation. In contrast, only halophilic cells were detected at a NaCl concentration of 15%. Thus, no mutual protective influence of the microorganisms manifested itself in suspension culture, either under hypo- or under hyperosmotic shock. Neither could halophile cells be detected after reinoculating a biofilm obtained on a peptone medium without the addition of NaCl. However, biofilms produced at a NaCl concentration of 15% contained approximately equal numbers of cells of the halophilic and halotolerant organisms. Thus, the halophile in biofilms sustaining a hyperosmotic shock exerts a protective influence on the halotolerant microorganism. Preliminary data suggest that this effect is due to release by the halophile of osmoprotective substances (ectoine and glutamate), which are taken up by the halotolerant species. Such substances are diluted by a large medium volume in suspension cultures, whereas, in biofilms, their diffusion into the medium is apparently hampered by their interaction with the intercellular polymer matrix.

Key words: oil field microflora, biofilms, interactions among bacteria in biofilms, protection from osmotic shock, osmoprotectants, ectoine.

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Most studies on the ecology, physiology, and biochemistry of microorganisms are conducted with suspension ("planktonic") cultures. Nevertheless, there is convincing evidence that 90–98% of populations of microorganisms in natural habitats exist in the form of structured associations, such as microbial mats, sediments, etc., which are generally referred to as biofilms. A large number of pathogenic microorganisms form biofilms in the macroorganism infected by them and, in addition, overgrow prostheses (catheters, contact lenses, etc.). Importantly, populations within a biofilm differ from suspension cultures in their essential properties. They are characterized by high density, lowered sensitivity to stress factors and biocides, enhanced physical, chemical, and metabolic stability, and genetic heterogeneity caused by an increased frequency of horizontal gene transfer [1]. These peculiarities are largely due to interactions among the microbial components of structured associations that await further investigation.

Research on the microflora of oil fields usually involves an analysis of the cell suspensions that are washed by water from oil-bearing horizons. According to the data of several researchers [2], it is the interactions among microorganisms in the biofilms formed in an oil reservoir that can significantly influence their biogeochemical activities, particularly if the environmental conditions in an oil field are extreme.

We earlier succeeded in reconstituting biofilms using samples of stratal water from oil fields. Such structured associations included oil-oxidizing microorganisms and accompanying chemoheterotrophic bacteria that were dependent on them and did not oxidize hydrocarbons per se [3]. It was demonstrated that protocooperative relationships exist between the components of these microbial associations: the accompany-

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ing bacteria synthesize activators that enhance hydrocarbon utilization by oil oxidizers [4, 5].

Of special interest are biofilms reconstituted from microbial components of stratal water from oil fields with extreme environmental conditions (e.g., high salinity). In such biofilms, halotolerant oil oxidizers are frequently associated with halophilic accompanying bacteria that presumably contribute to the protection of the halotolerant species from hyperosmotic shock [6]. A similar naphthalene-degrading association has recently been revealed in the technogenic soils of a salt mine [7].

The goal of this work was to investigate the taxonomic composition of microorganisms and the mechanisms of their interactions in biofilms reconstituted from samples of stratal water with high salinity.

MATERIALS AND METHODS

Research subjects. This work used bacterial cultures isolated from stratal waters of the Romashkinskoe oil field (Republic of Tatarstan). The salt content of the stratal waters of this oil field reaches 100 g/l. The cultures were isolated via intermediate reconstitution of biofilms (see below). Two strains (2610-1 and 21684-1K), which were identified as *Dietzia* sp. (an oil oxidizer) and *Chromohalobacter* sp. (an accompanying bacterium), were studied in detail.

Medium composition and cultivation conditions. The microorganisms were maintained on PY agar slants containing 1% Casamino acids (Difco), 0.5% yeast extract (Difco), and 1.8% agar–agar (pH 7.5–8.0). 0.5% of hexadecane was added to the medium for oil oxidizers, and 10% of NaCl to that for halophilic microorganisms. Cultivation was carried out in 100- or 250-ml flasks that contained 20 or 50 ml, respectively, of liquid PY medium or synthetic M9 medium with the addition of various NaCl amounts. The microorganisms were cultivated on a shaker (150 rpm) at a temperature of 28–29°C. The growth of the cultures was monitored by determining their optical density and dry biomass. Arbitrary optical density (light absorbance + light scattering) was measured at a wavelength of 540 nm with a KFK-2-UKhL 4.2 nephelometer.

Biofilm reconstitution and pure culture isolation. Stratal water samples stored at 4–5°C were used for biofilm reconstitution. Sterile polycarbonate Nucleopore filters (pore diameter, 0.2 μm, Schleicher & Schuell) were placed on the solid medium surface in petri dishes. The medium composition varied depending on the goal of the experiment. M9 medium with paraffins was used for the primary reconstitution of biofilms. 25–50 μl of stratal water containing planktonic microbial cells washed out of the oil-bearing stratum were placed on filters. The petri dishes were incubated at 28–29°C for 5–10 days until biofilm formation. The biofilms were dispersed in liquid medium or in a salt solution, and dilutions of the suspension were plated onto various solid media. The resulting colonies were redispersed and reinoculated to obtain pure cultures.

Identification of the pure cultures isolated. Culture identification was based on polyphasic taxonomy. Cells washed from a PY slant (with 3 ml of a 1 or 10% NaCl solution for the halotolerant and the halophilic species, respectively) were used as inoculum in physiological and biochemical tests. 0.1 ml of cell suspension was added to 50 ml of the M9 medium with the tested substrate. In studies using solid media, the culture suspension was streaked on the surface. A medium without a carbon source served as the control system. 1% (w/v) of a substrate (with acids, of their sodium salts) was added. All media for halophiles were supplemented with 10% NaCl. The results obtained were assessed 5–7 days after the cessation of the culture optical density increase. The dependence of the growth rate on the pH and temperature values was investigated on the PY and M9 media, using the most utilizable substrates. Other taxonomically relevant traits were described using standard protocols [8]. The 16 S rRNA gene sequence was determined and compared with database sequences according to the method described in [9]. Fragments of 16S rRNA genes were amplified and sequenced using the 11F 5'-GTTTGATCMTGGCTCAG-3' and 1492R 5'-TACGGYTACCTTGTTACGACTT-3' primers. Sequencing was performed by the Sanger method, using a Silver Sequencing kit (Promega, United States) according to the manufacturer's recommendations with minor modifications. Preliminary analysis of the nucleotide sequences of the 16S rRNA genes was carried out using the GenBank database and NCBI software (http://www.ncbi.nlm.nih.gov); this analysis confirmed the affiliation of the oil oxidizer with the genus *Dietzia* of the suborder *Corynebacterineae* and of the halophilic bacterium, with the genus *Chromohalobacter* of the family *Halomonadaceae* (the similarity was 95–96% in both cases).

Microscopic studies were conducted with an Axio Imager D1 microscope (Carl Zeiss) with a EC PLAN–NEOFLUAR 100× objective, in a phase-contrast system and after staining with the fluorescent dyes DAPI and acridine orange.

The intracellular pool of low-molecular-weight substances was investigated by the method described in [10], with certain modifications. Strain 21684-1K was grown until the early stationary phase on M9 medium with fumarate and 10% NaCl. The cells were separated by centrifugation (5000 *g*), and the supernatant was completely removed. The sediment was not washed, because washing may result in the loss of intracellular low-molecular-weight substances, even if an isotonic solution is used. The growth medium employed contained no organic substances except fumarate. The cell sediment was twice extracted by a 20-fold volume of 80% ethanol, and the extract was evaporated to dryness at room temperature. The solid material obtained was dissolved in a minimal volume (0.1 ml) of 96% ethanol, and 10 μl of the solution was

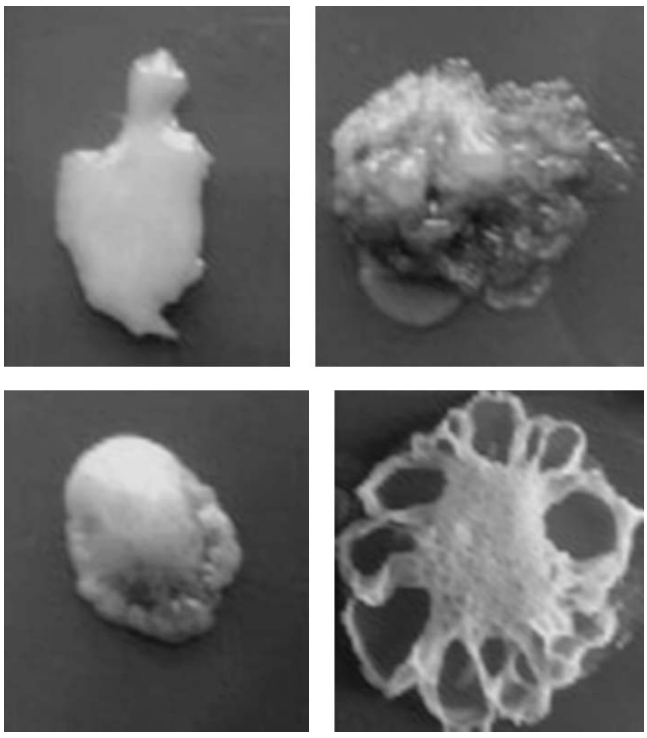

Fig. 1. Examples of biofilms reconstituted from stratal waters of oil fields. Magnification, 4×.

applied on a thin-layer chromatography plate (Merck) covered with silica gel. The moving phase consisted of *n*-butanol, acetic acid, and water in a volume ratio of 12 : 3 : 5. Commercial preparations of ectoine (Bio-Chemika, 99.0%, HPLC) and amino acids were used as standards.

The statistical significance of the results obtained is based on the fact that we used the data of a typical experiment selected from 3–5 trials.

RESULTS AND DISCUSSION

Biofilm reconstitution and characterization. To obtain biofilms, we used polycarbonate filters, which are resistant to physical, chemical, and microbial factors. Such filters were earlier used by other researchers to obtain stationary biofilms to be investigated by means of atomic force microscopy [11]. In contrast to these studies, our experiments were conducted with crude stratal water samples, which contained native mixtures of microorganisms and not a suspension of a pure bacterial culture. In natural ecotopes, an equilibrium exists between structured associations and the planktonic populations that result from their degradation [2]. Upon placing stratal water samples on a suitable surface, biofilms are regenerated from planktonic populations. The biofilms obtained by this method from various stratal water samples are shown in Fig. 1.

In contrast to bacterial macrocolonies that display a homogeneous structure, examining the biofilms

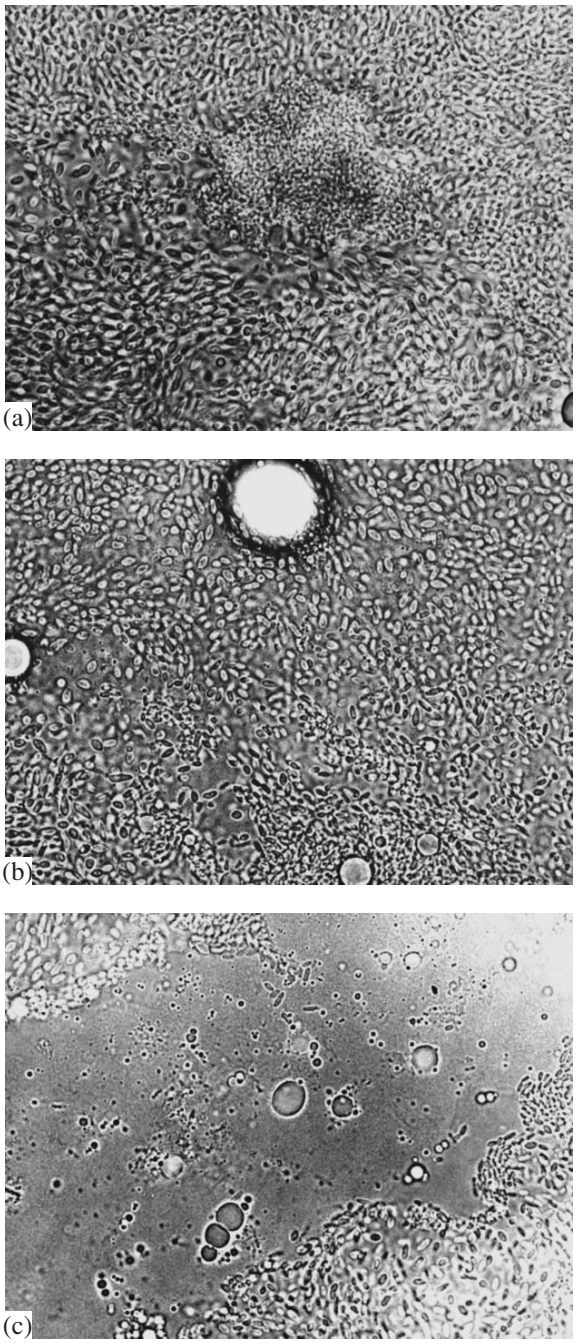

Fig. 2. Light microscopy of reconstituted biofilms (phase contrast images): (a) microcolonies of various microorganisms; (b) sticking of microbial cells to the paraffin drop surface; (c) internal channels in a biofilm.

obtained in a light microscope revealed their pronounced heterogeneity (Fig. 2) due to the formation of microcolonies of different microorganisms (Fig. 2a), synthesis of an extracellular matrix enclosing the substrates (hexadecane droplets in our case, Fig. 2b), and presence of internal channels (Fig. 2c).

The most important difference between macrocolonies and biofilms is that in biofilms microorganisms

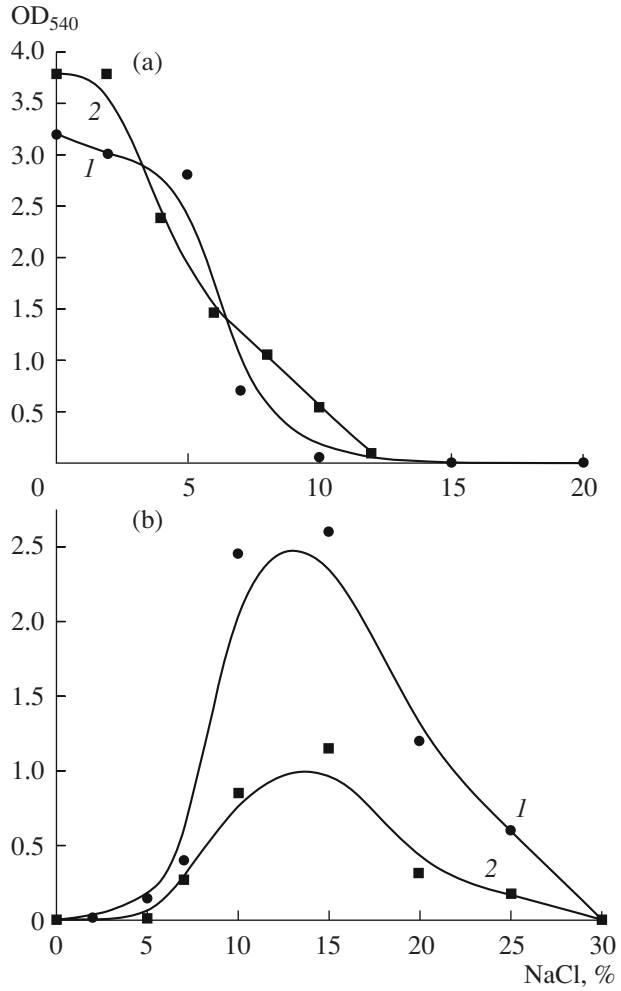

Fig. 3. Dependence of the growth of (a) strain 2610-1 and (b) strain 21684-1K on the NaCl concentration in the medium: (*1*) PY medium; (*2*) M9 medium with hexadecane (a) or glycerol (b).

exhibit "biofilm phenotype" due to the selective expression of a large number of genes, resulting in significant differences between the physiological and biochemical properties of biofilm cells and cells from macrocolonies or suspension cultures [1]. We demonstrated earlier that these differences also manifest themselves in reconstituted biofilms [4, 5].

Physiological and biochemical properties and taxonomic status of pure cultures 2610-1 and 21684-1K. Isolates 2610-1 and 21684-1K were used in further studies. Strain 2610-1 is an aerobic gram-positive microorganism that forms short nonmotile rod-shaped and coccoid cells about 1×2 μm in size. No spores are formed. The colonies on solid media are round, shiny, and orange to red in color (depending on their age). The strain utilizes paraffins and shows good growth on media with D-glucose, L-arabinose, maltose, trehalose, acetate, butyrate, and L-serine. Weak growth occurs on media with L-rhamnose, glycerol, propionate, citrate, and L-glutamate. The strain does not utilize D-cellobiose, D-raffinose, mannitol, formate, succinate, or fumarate. It hydrolyzes starch but not gelatine. The strain lacks oxidase and nitrate reductase activities and is catalase-positive.

The 21684-1K strain is also aerobic but gram-negative. Its cells are motile rods about 0.8×4–6 μm in size. No spores are formed. The colonies on solid media are round, mucous, and beige in color. Paraffins are not utilized. The strain grows well on media with D-glucose, L-arabinose, maltose, trehalose, mannitol, glycerol, acetate, succinate, fumarate, citrate, L-serine, and L-glutamate, but not with D-cellobiose, D-raffinose, L-rhamnose, formate, propionate, or butyrate. It does not hydrolyze starch or gelatine and exhibits oxidase, nitrate reductase, and catalase activities.

To determine the dependence of the growth of the tested strains on the NaCl content in the medium, we used PY medium (for both strains) and M9 medium with hexadecane (for strain 2610-1) or glycerol (for strain 21684-1K). The data, shown in Fig. 3, demonstrate that strain 21684-1K is a typical halophile whose optimum salt concentration is 12%. The minimum concentration that enables it to grow is 5% (Fig. 3b). Strain 2610-1 is a halotolerant organism whose optimum salt concentration is within the 0–2% range; i.e., it is close to salt concentrations in the media not supplemented with additional salt). The maximum concentration at which weak growth still occurs is 10–12 % (Fig. 3a). Thus, the salt concentration ranges that sustain the growth of the two strains overlap, which indirectly supports the idea that the two strains coexist in a natural ecotope.

The pH dependence of the growth of the strains studied was investigated on PY medium. Its initial pH value was adjusted to 5.0–10.0 by adding 1 M HCl or NaOH solution. 10% NaCl was added to the halophile's medium. The pH value did not change over the first two days of cultivation and increased over the subsequent days. Therefore, the effect of pH was judged from the biomass accumulated after two days of cultivation. No buffer solutions were used, because scanning a wide pH range would have necessitated the employment of a number of different buffers that may influence the growth of microorganisms. The results are presented in Fig. 4. It is evident that weakly alkaline medium (pH 8.0–8.2) was optimum for both strains. Strain 2610-1 was somewhat more alkaliphilic.

The dependence of the growth of the strains studied on temperature was examined on PY medium at a pH value of 8.0; 10% NaCl was added to the halophile's medium. The results are shown in Fig. 5. The optimum temperature range was 28–30°C for both strains.

Based on the tested biochemical properties, we preliminarily identified the halotolerant oil oxidizing strain 2610-1 as a representative of the genus *Rhodococcus* or *Dietzia* within the suborder *Corynebacterineae*; the halophilic accompanying bacterium (21684-1K) was

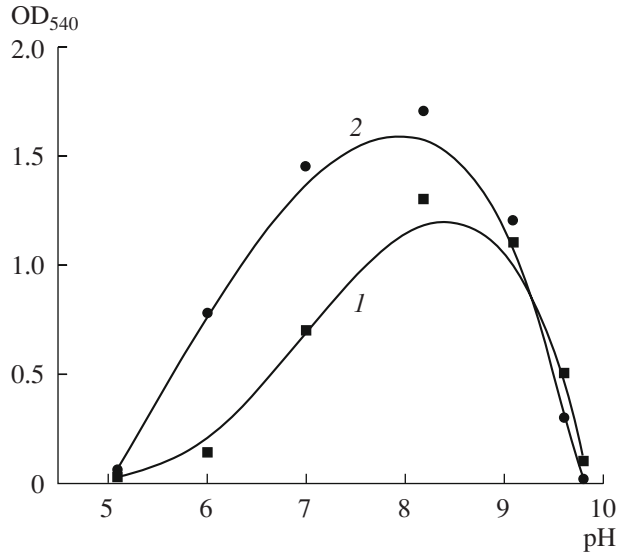

Fig. 4. Dependence of the growth of (*1*) strain 2610-1 and (*2*) strain 21684-1K on pH of the medium.

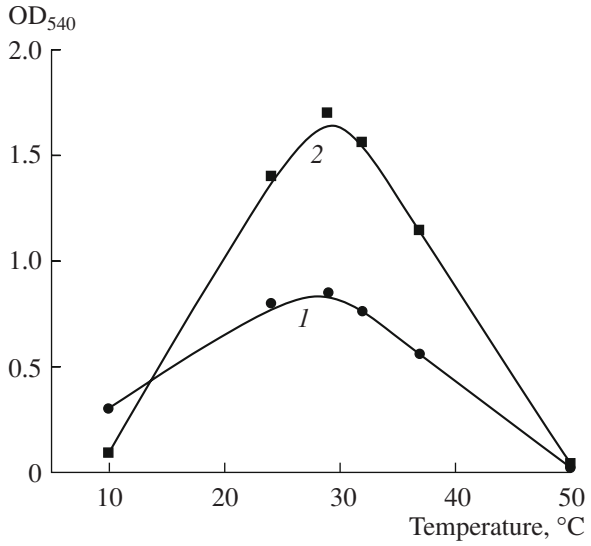

Fig. 5. Dependence of the growth of (*1*) strain 2610-1 and (*2*) strain 21684-1K on the temperature.

identified as a representative of the family *Halomonadaceae* within the class *Gammaproteobacteria*. More precise results were obtained by determining the 16S rRNA gene sequences. The bacteria were identified as *Dietzia* sp. and *Chromohalobacter* sp., respectively, with a probability of 95–96%. The species affiliation was not determined.

Interactions between the isolates in suspension cultures and biofilms. The interactions between the halotolerant oil oxidizer 2610-1 and the halophilic accompanying bacterium 21684-1K were studied by the binary culture method. The experimental protocol is shown in Fig. 6. Two variants of liquid PY medium were used in studies with suspension cultures: (i) without the addition of NaCl (causing a hypoosmotic shock in the halophile) and (ii) with the addition of 15% NaCl (causing a hyperosmotic shock in the halotolerant species) (Fig. 6a). Both medium variants were inoculated with suspensions of the tested strains obtained by washing their cells off of the solid PY medium. The suspensions of both strains contained the same CFU number (table). After incubating the cultures on a shaker for 7 days, the CFU number changes were determined by plating onto the same two solid PY medium variants, selective for the halophilic or the halotolerant species. In this system, viable halotolerant cells were only detected in the liquid medium without the addition of salt, whereas viable halophilic cells only occurred in the medium that contained 15% NaCl (Fig. 6a, table). Therefore, in suspension cultures, the mutual protective influence of the microorganisms manifested itself under neither hypo- nor hyperosmotic shock.

A different pattern was revealed in binary biofilms (Fig. 6b). Cultivating the tested strains in binary biofilms enabled not only viability of the halotolerant strain in the presence of 15% NaCl but also an increase in the number of its viable cells by an order of magnitude after 7 days (table). Hence, within a biofilm, the halophilic microorganism produces a manifest protective effect on the halotolerant strain under conditions of hyperosmotic shock. This important fact provides insight into the physiological and biochemical basis of

Results of cocultivation of the halotolerant and halophilic strains in liquid suspension culture and within biofilms

Medium composition	Cultivation conditions	CFU/ml in the binary culture			
		Initially		After 7 days	
		2610-1	21684-1K	2610-1	21684-1K
PY medium without NaCl addition	Suspension culture	1.5×10^7	1.5×10^7	2.5×10^8	n.d.
	Biofilm	1.5×10^7	1.5×10^7	8.3×10^8	n.d.
PY medium + 15% NaCl	Suspension culture	1.5×10^7	1.5×10^7	n.d.	8.8×10^8
	Biofilm	1.5×10^7	1.5×10^7	3.7×10^8	7.6×10^8

Notes: n.d., not detected. The liquid culture volume was 20 ml, and the final biofilm volume was 0.1 ml.

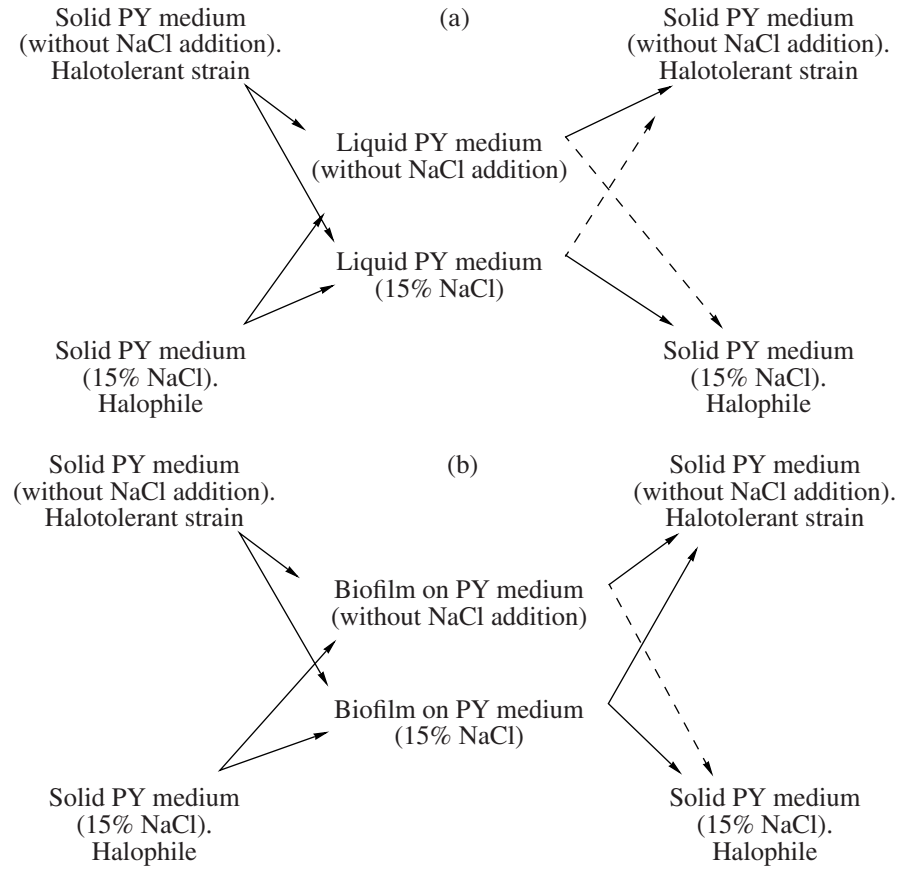

Fig. 6. Experimental protocol for investigation of the interactions between the isolated strains in their binary cultures: (a) binary suspension cultures and (b) binary biofilms on solid medium. Dotted line means lack of growth. See text for explanations.

the stable association between the halotolerant oil oxidizer and the halophilic accompanying bacterium, which requires hydrocarbon degradation products. As mentioned above, a similar association was revealed in soils contaminated with aromatic hydrocarbons [7].

What is the possible mechanism of the protective effect of the halophile on the halotolerant strain? Presumably, the halophile releases excess osmoprotective substances, and they are taken up by the halotolerant strain. In suspension culture, the protective effect does not occur due to the dilution of the osmoprotectants by the large volume of liquid medium. The biofilms promote contacts among cells in the populations. In addition, extracellular matrix hampers the diffusion of the released substances outside the biofilm.

Identification of osmoprotective substances formed by the halophilic strain 21684-1K. To test the above hypothesis, we identified the low-molecular-weight substances of the intracellular pool of strain 21684-1K. The results are given in Fig. 7. Several components were detected in the cell extract. The substance whose R_f is close to 0.7 is ectoine. This conclusion is corroborated by the enhancement of this spot upon the addition of ectoine to the extract. Additional chromatographic research using other standards and visualizing agents (ninhydrin, isatin, etc.) revealed that the substance with an R_f of 0.25 is glutamate (or a glutamate–glutamine mixture). The substances that move near the front include aromatic amino acids (phenylalanine and tyrosine). In general, these results are consistent with the data available in the literature concerning the halophilic bacteria of the family *Halomonadaceae*.

Modelling the protective effect of the halophile. If the protective effect of the halophile on the halotolerant strain is due to the release of osmoprotectants into the medium, then the supplementation of the medium with ectoine should produce the same effect as the cocultivation of these strains. However, for the effect to manifest itself, the ectoine content in a suspension culture should equal that in binary biofilms. Direct determination of the ectoine content in biofilms presents difficulties because a large quantity of the tested material is to be available. Therefore, we investigated the effect of a wide range of ectoine concentrations on the growth of the halotolerant strain at 15% NaCl, i.e., at a salt concentration that, according to our data, virtually prevented growth (Fig. 3a). From the data of Fig. 8, it is evident that the effect was produced at an ectoine concentration as low as 50 μg/ml, while the maximum effect was attained within the 500–1500 μg/ml concen-

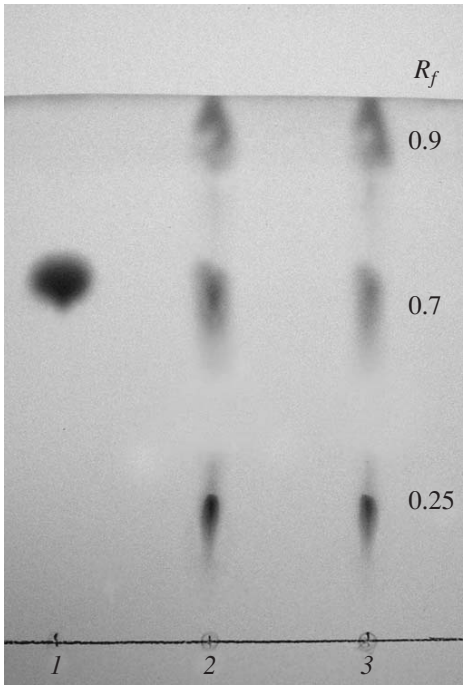

Fig. 7. Results of thin-layer chromatography of strain 21684-1K cell extract: (*1*) ectoine (25 μg); (*2*) cell extract + ectoine (5 μg); (*3*) cell extract. Visualization was done with iodine vapor.

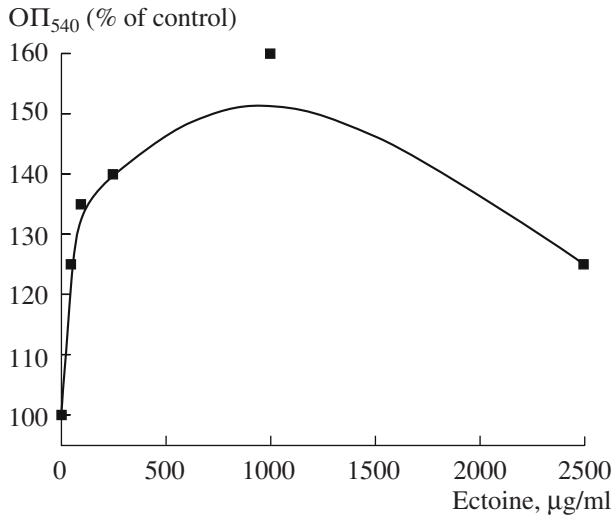

Fig. 8. Dependence of the growth (% of control) of strain 2610-1 strain on PY medium with 15% NaCl on the added amount of ectoine.

tration range. Further concentration increase resulted in a decrease in the effect. The addition of 1000 μg/ml ectoine to the control culture (without salt) did not influence its growth (data not shown).

Comparison of the CFU numbers in the control system without ectoine and in the variant with an ectoine concentration of 1000 μg/ml showed that, in the absence of ectoine, the viable cell number remained at the inoculation level (about 10^7 cells/ml) over the first two days of cultivation and drastically decreased subsequently. In the presence of ectoine, the CFU number increased 4–5-fold on the second day.

Thus, ectoine indeed exerts a protective effect on the halotolerant oil oxidizer 2610-1 under hyperosmotic shock.

The question to be raised is to what extent the ectoine concentrations used in our experiments with suspension cultures approximate those in binary biofilms. To cope with this issue, we consulted the literature concerning the intracellular ectoine content of *Brevibacterium linens* [10], a bacterium close to strain 21684-1K. According to these data, the ectoine content of *B. lineus* cells is about 1 μmol/mg of dry biomass (or 140 μg/mg). This is consistent with the results obtained by us when determining ectoine chromatographically. According to our data, the dry biomass of the tested binary biofilms is approximately 5 mg per biofilm, and the average biofilm volume is 0.1 ml. A simple calculation demonstrates that the ectoine concentration in the biofilm can be as high as 3000 μg/ml, provided that thermodynamic equilibrium is established between the intra- and extracellular concentrations. This corresponds to the concentration that produced the effect in suspension cultures. However, if the biosynthesis and excretion of ectoine proceeded in suspension cultures at the same rate, then, given that the halophile's dry biomass was about 500 mg/l, the ectoine concentration could not exceed 30 μg/ml; i.e., it was too low to produce the effect. Even though these calculations only yield approximate data, they demonstrate that the osmoprotectant concentration inside a biofilm can exceed that in a suspension culture by two orders of magnitude.

In conclusion, it should be emphasized that this paper is the first to present data on the physiological role of the halophilic accompanying bacterium associated with a halotolerant oil-oxidizing microorganism. We demonstrate the ecological value of associations of halophiles, which synthesize and excrete osmoprotectants, and halotolerant microorganisms, which absorb these substances from the medium.

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