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EXPERIMENTAL ARTICLES

Role of the Extracellular Polymer Matrix in Resistance of Bacterial Biofilms to Extreme Environmental Factors

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Abstract—Biofilms of a number of gram-positive and gram-negative bacteria (both environmental strains from the stratal waters of oil fields and collection strains) were found to exhibit higher resistance to extreme physicochemical factors (unfavorable temperature, pH, and salt concentration) than planktonic cultures. The extracellular polymers forming the structure of the biofilm matrix were shown to contribute significantly to this resistance, since suppression of matrix formation by subbacteriostatic concentrations of azithromycin (for *Pseudomonas acephalitica*) or mutation in the *cvil* gene encoding *N*-hexanoyl homoserine lactone synthetase (for *Chromobacterium violaceum* CV026) resulted in the resistance of biofilms being decreased almost to the level of planktonic cultures. The role of the biofilm matrix for bacterial survival under extreme conditions is discussed.

Keywords: biofilms, matrix, extracellular polymers, azithromycin, quorum-sensing system, extreme environmental factors

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The patterns of development of structured microbial communities (biofilms) have been extensively discussed in recent reviews and monographs [1–4]. We were therefore concentrating on one problem, which is closely related to the topic of investigation and has been insufficiently considered in the literature, i.e., on the composition and role of the extracellular polymeric substances (EPS) of the biofilm matrix.

The matrix is responsible for development of the three-dimensional structure of the biofilm, stabilizes the microbial cells, ensures the intercellular contacts, and protects the cells from hydrodynamic shear at the liquid–solid interface. The matrix is thought to play a protective role, providing for increased resistance of the cells to biocides and the agents of the immune system of macroorganisms. Unfortunately, the literature concerning the mechanisms of matrix formation and functioning is scarce. Matrix composition of the biofilms formed by gram-negative bacteria (mostly pseudomonads) and some gram-positive bacteria was investigated [5, 6].

Pseudomonas aeruginosa is the best-studied organism in this respect. It is an agent causing chronic pulmonary infections (cystic fibrosis) in patients with an impaired immune system and may form biofilms on contact lenses or implanted catheters [7].

The matrix of *P. aeruginosa* biofilm is a complex mixture of polysaccharides, proteins, and nucleic acids (mostly DNA) [8]. Polysaccharides are the major structural components. At least three polysaccharides are present in the biofilm matrix of *P. aeruginosa*: Psl (with mannose as the major component), Pel (with glucose as the major component), and alginate (an acidic polysaccharide containing uronic acids). The role of the Psl polysaccharide is fairly well-studied. It is located at the surface of biofilm-forming cells as helical structures and is responsible for cell interactions. In the course of biofilm maturation, Psl is involved in the formation of cavities in three-dimensional bacterial microcolonies. These cavities harbor the motile cells responsible for spreading of the organisms during dissociation of the biofilm. Chemical removal of Psl from the cell surface results in the dissolution of the matrix [5].

Apart from polysaccharides, the matrix contains variable amounts of proteins, which may include the compounds promoting cell adhesion at the interface, as well as the extracellular DNA (eDNA). The functions of the latter are not completely clear, although its involvement in cell adhesion, intercellular interactions, formation of cation gradients, and development of induced resistance to antibiotics are postulated [5].

Among gram-positive bacteria, the species of *Bacillus* (the model microorganism *B. subtilis*), *Staphylococcus*, and *Streptococcus* are the best studied biofilm formers. Importantly, the latter two are involved in formation of multispecies biofilms (dental plaques) and are responsible for nosocomial infections and for biofouling of implanted medical devices [6].

The studied *B. subtilis* mutants form two EPS types: an exopolysaccharide and poly-D-glutamate; their

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ratio is variable. Both EPS are required for formation of viable biofilms. Most *S. aureus* strains produce a polymer of *N*-acetylglucosamine, which is involved in biofilm formation, in particular acting as an adhesin. Deficiency in the *ica* operon responsible for the synthesis of this polysaccharide is, however, compensated by production of alternative proteinaceous adhesins (Bap proteins). The matrix of *B. subtilis* biofilms, unlike *S. aureus*, contains a single protein (TasA) acting as an adhesin. Mutants with impaired synthesis of this protein are unable to form biofilms, although they retain the capacity for exopolysaccharide synthesis. TasA was recently shown to exist as extracellular filaments with the properties of an amyloid protein, which play an important structural role in the biofilm matrix [6].

Since there is practically no data in the literature concerning the mechanisms by which the biofilm matrix protects embedded microorganisms from extreme ambient conditions, the goal of the present work was to investigate resistance of the biofilms formed by gram-negative and gram-positive bacteria to heat shock, hyperosmotic, and acidic shock, as well as the effect of the integrity of the matrix on this resistance.

MATERIALS AND METHODS

Subjects of investigation. The cultures of the halotolerant, oil-oxidizing *Dietzia* sp., *Kocuria* sp., the halophilic *Chromohalobacter* sp., and the oil-oxidizing *Pseudomonas acephalitica* were isolated from stratal waters of the Romashkinskoe oil field (Tatarstan, Russia) [9]. Collection strains of *P. chlororaphis* 66, *Chromobacterium violaceum* WT, and of the mutant *C. violaceum* CV026 were kindly provided by I.A. Khmel (Institute of Molecular Genetics, Russian Academy of Sciences).

The microorganisms were stored in LB agar stabs under paraffin oil (10% NaCl was added for the halophilic *Chromohalobacter* sp.) at 4–6°C (*Chromobacterium* cultures were stored at room temperature, with 100 μg/mL kanamycin for the mutant CV026). For inoculation, the bacteria were grown at 29°C in LB medium with shaking (150 rpm) for 20–24 h. The medium for the *C. violaceum* CV026 mutant contained 100 μg/mL kanamycin. These inocula were used for biofilm experiments.

Comparison of the biofilm and planktonic cultures was carried out using the Teflon block method described previously [10]. For biofilm formation, a test tube with 4 mL of LB medium and 2 g of Teflon blocks (2 × 2 × 2 mm) was seeded with 50 μL of the inoculum. After incubation, the planktonic culture was separated and its growth was determined using conditional optical density (adsorption + light scattering) at 540 nm. The blocks were washed with 1% NaCl and the biofilms were fixed with 96% ethanol. After ethanol removal, the biofilms were stained with 0.1% crystal violet (CV). In parallel experiments, staining with 1,9-dimethylmethylene blue (DMMB) was carried out according to the standard procedure [11]. After stain extraction with 96% ethanol, optical densities of the solutions were determined at 590 (for CV) or 540 nm (for DMMB).

Effect of temperature on growth was determined using the biofilms preformed for 6 h at the optimal temperature (the time required for realization of the "biofilm phenotype" [10]). The cultures were then transferred into the incubators at various temperatures.

In experiments with azithromycin, preformation of the biofilms was carried out in the presence of this antibiotic (0.5 μg/mL). Effects of pH and salt concentration on growth were determined using the forming biofilms (without preincubation). The composition and content of osmoprotectors in binary biofilms (from the cultures of the halotolerant *Dietzia* sp. and halophilic *Chromohalobacter* sp.) were determined as described previously [9] for the biofilms grown on cellophane films in petri dishes with LB medium with 10% NaCl. Monocultures of the halophiles grown under the same conditions were used for comparison. The amount of the extract applied to the chromatogram was normalized per biomass of the halophile. For this purpose, the cultures were washed off the filters with 96% ethanol, ectoine was extracted, and the dry biomass was determined. Binary biofilms after ectoine extraction were treated with 10 mL distilled water (pH 8.5) with 1–2 mg DNase and 5 mg $MgSO_4 \cdot 7H_2O$ to cause complete lysis of the halophilic cells. After centrifugation and washing, the residue was dried to determine the dry biomass of the halotolerant oil-oxidizer. The biomass of the halophilic satellite was calculated as a difference between the total biomass and the biomass of the oil-oxidizer. Complete removal of the halophilic cells was confirmed by model experiments.

Statistical reliability of the results was achieved by selection of the typical experiment by the nonparametric method of pairwise comparison using the sign criterion [12].

RESULTS AND DISCUSSION

Effect of temperature. As was already mentioned, the biofilms may exhibit increased resistance not only to antibacterial agents, but also to unfavorable physicochemical factors of the environments (heat, osmotic, or acidic shock) [13, 14], although the mechanisms of such resistance remain unknown.

It was therefore desirable to investigate the effect of physicochemical factors on biofilm formation. In the case of temperature dependence, we compared planktonic cultures and preformed biofilms. The results for *Dietzia* sp., *Kocuria* sp., and *P. chlororaphis* are shown of Fig. 1.

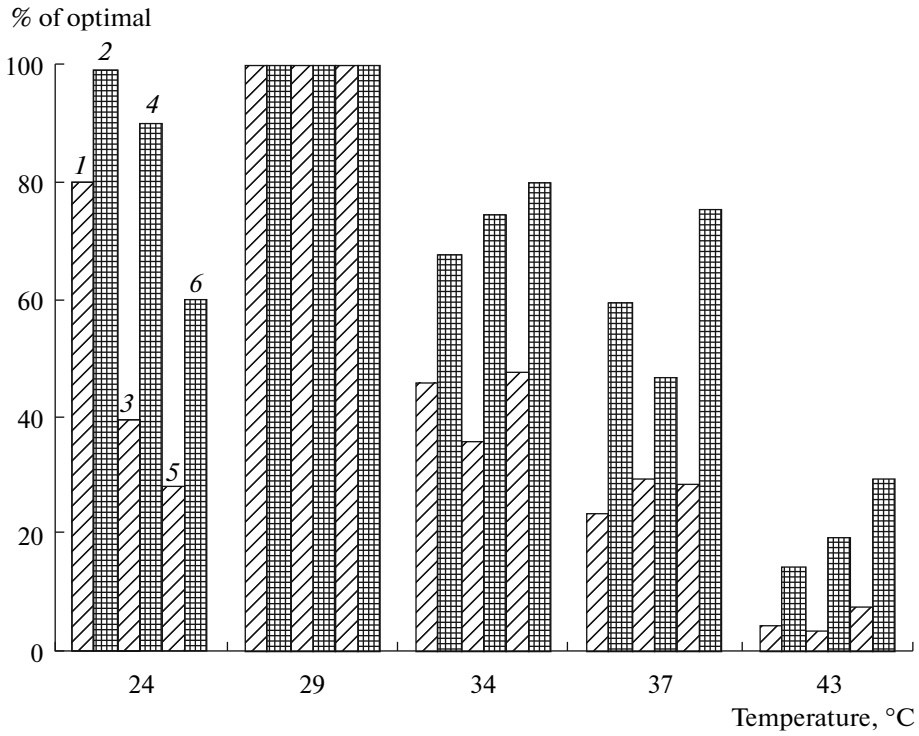

Fig. 1. Growth of planktonic cultures (*1, 3, 5*) and preformed biofilms (*2, 4, 6*) (by CV staining) depending on temperature: *Dietzia* sp. (*1, 2*); *Kocuria* sp. (*3, 4*), and *P. chlororaphis* (*5, 6*). The Y axis shows the percentage of the growth at optimal temperature (29°C) for each culture.

It can be seen that biofilms exhibited better growth (1.5–2.0 times) than planktonic cultures at sub- and especially supraoptimal temperatures, while the temperature optimum remained the same.

Since resistance to heat stress was more pronounced in preformed biofilms, the mechanism of biofilm protection at sub- and supraoptimal temperatures is probably associated with the extracellular polymer matrix. The following working hypothesis may be proposed: the biofilm matrix is known to limit the rates of diffusion of both high- and low-molecular compounds [15]. At suboptimal temperatures the membrane becomes more rigid, resulting in a leak of certain important metabolites into the environment. The growth rate therefore decreases. In the case of biofilms, the matrix prevents, or at least decreases this leakage (as will be shown below) and facilitates reverse adsorption of these metabolites. This role of the matrix, which provides for increased growth rates at nonoptimal temperatures, may certainly be termed protective.

At supraoptimal temperatures (43–45°C for mesophilic bacteria), denaturation of a number of key enzymes occurs in planktonic cultures, so incubation for over 24 h results usually in growth suppression and cell lysis. The matrix promotes accumulation of the thermoprotectors formed by microorganisms within the biofilm, while in planktonic cultures such protective compounds are not accumulated in the cells, but rather diffuse freely into the medium. Growth rates of the biofilms under such denaturing conditions exceeding those for planktonic cultures by 1.5–2 times may be of ecophysiological importance for the microorganisms, as they may preserve their viability and metabolic activity under extreme conditions.

The above suggestion is supported by the following experimental findings.

In the first series of experiments, we used the macrolide antibiotic azithromycin (sumamed), low (subbacteriostatic) concentrations of which were reported to suppress biofilm formation and biosynthesis of the biofilm matrix in some strains of *Haemophilus influenzae* [16] and *P. aeruginosa* [17].

Screening of the collection *Pseudomonas* strains and those isolated from oil fields revealed a *P. acephalitica* strain in which biofilm formation was more sensitive to azithromycin than the growth of planktonic culture (Fig. 2).

It can be seen from Fig. 2 that while 0.5 μg/mL azithromycin had practically no effect on the growth of planktonic cultures, formation of the biofilm matrix (determined by DMMB staining) did not exceed 40% of the control (without the antibiotic). Comparative investigation of the growth of *P. acephalitica* as planktonic cultures and biofilms at different temperatures was carried out with and without azithromycin (0.5 μg/mL). The results are presented on Fig. 3.

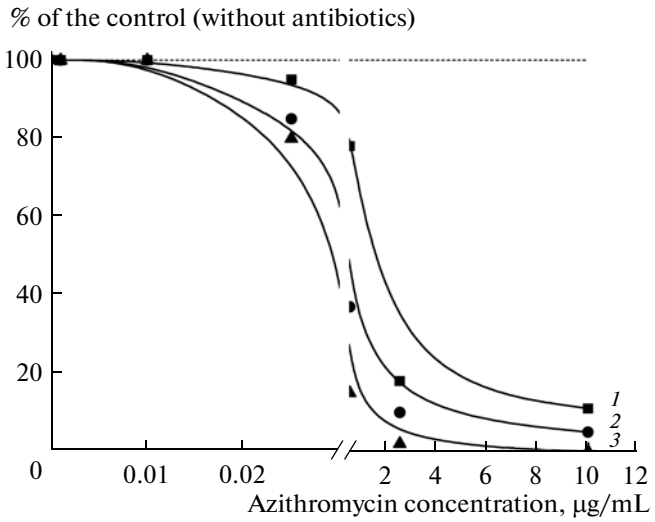

Fig. 2. Effect of azithromycin on *P. acephalitica* growth in planktonic culture (*1*) and in biofilms stained with CV (*2*) and DMMB (*3*). Broken line shows the control, accepted as 100%.

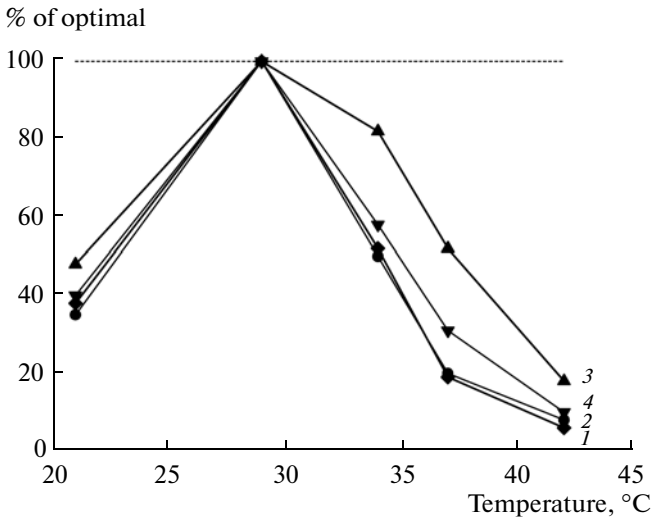

Fig. 3. Growth of planktonic cultures and biofilms of *P. acephalitica* depending on temperature: planktonic culture (*1*), planktonic culture with 0.5 μg/mL azithromycin (*2*), biofilm (DMMB staining) (*3*), and biofilm + 0.5 μg/mL azithromycin (DMMB staining) (*4*). Broken line shows the growth at optimal temperature (similar to Fig. 1).

Addition of azithromycin resulted in a drastic (by 1.3–2.0 times) decrease in the heat resistance of the biofilm, so that its sensitivity became close to that of the planktonic culture. Decreased DMMB staining confirmed suppression of the matrix formation in the presence of the antibiotic.

In another series of experiments, the strain *C. violaceum* WT and its mutant CV026 were used. The latter has an impaired quorum-sensing (QS) system. In this mutant, insertion of the mini-Tn5 transposon resulted in inactivation of the *cvil* gene encoding *N*-hexanoyl homoserine lactone synthetase [18].

Various opinions exist concerning the biochemical mechanisms of the QS involvement in biofilm formation by different microorganisms. One of the first works on the topic reported, however, that the *P. aeruginosa* mutant with deficient Las QS system formed flat, undifferentiated biofilms, which were similar to planktonic cultures in their sensitivity to the biocide (sodium dodecyl sulfate) [19]. These properties of the mutant biofilms clearly indicate impaired formation of the extracellular polymeric matrix.

Direct evidence of involvement of the QS system in the regulation of the biosynthesis of the matrix components was published recently [20]. The authors showed that transcription of the *pel* operon, which is involved in the biosynthesis of the glucose-containing matrix polysaccharides, decreased drastically in the *lasI* and *rhlI* mutants, while addition of 3-oxo-dodecanoyl homoserine lactone to the culture of the *lasI* mutant restored both transcription of the *pel* operon (to the wild type level) and the capacity for forming full-grown biofilms.

Temperature dependence of growth of *C. violaceum* cultures is shown of Fig. 4. The biofilms were stained with DMMB to reveal the differences in the matrix formation.

These results show no significant difference in the effect of temperature on growth of the planktonic cultures of both strains. Biofilm formation by the mutant was, however, much more sensitive to supraoptimal temperatures than the same process in the wild type *C. violaceum* strain.

Effect of pH. Effect of pH on the growth of planktonic cultures and biofilms is shown on Fig. 5.

The pH range for growth of planktonic cultures and biofilms was essentially the same, although the biofilms of *Dietzia* sp. and wild-type *C. violaceum* WT accumulated more biomass at unfavorable pH values than planktonic cultures.

This phenomenon may be explained by the fact that acidic components of the exopolymer matrix may act as a barrier limiting the proton permeability and protecting the biofilm from unfavorable pH. This suggestion is supported by decreased pH resistance of the biofilms of the *C. violaceum* CV026 mutant, in which the process of matrix formation is impaired.

Effect of NaCl concentration. We have previously shown that in reconstructed binary biofilms the halophilic satellite *Chromohalobacter* sp. protected the petroleum oxidized *Dietzia* sp. from hyperosmotic shock. Increased resistance of a nonhalophilic organism to hyperosmotic conditions in a binary biofilm was hypothesized to originate from the halophilic organism producing an osmoprotector (ectoine), which did

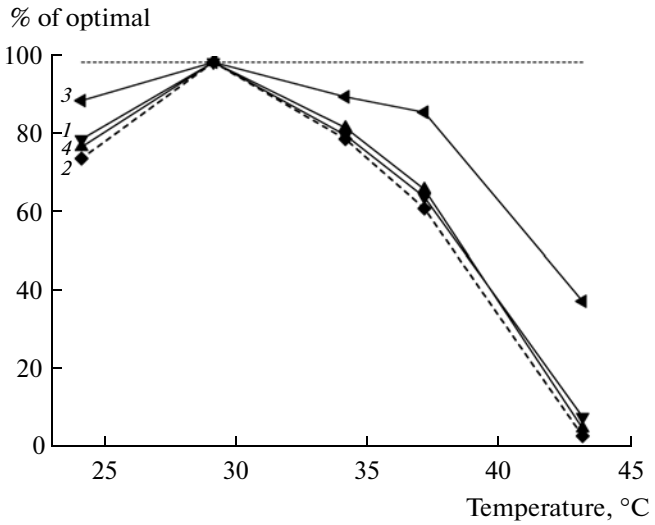

Fig. 4. Growth of planktonic cultures and biofilms of *C. violaceum* WT and the CV026 depending on temperature: WT, planktonic culture (*1*); CV026 planktonic culture (*2*), WT biofilm (DMMB staining) (*3*), and CV026 biofilm (DMMB staining) (*4*).

not dissipate in the environments due to the barrier action of the matrix [9].

To verify this hypothesis, binary biofilms reconstructed from the pure cultures of *Dietzia* sp. and its satellite *Chromohalobacter* sp. were used. Relations of protocooperation have been demonstrated for these organisms.

Comparative chromatographic analysis of the extracts from the biofilms formed by the halophilic *Chromohalobacter* sp. alone and by its mixture with the halotolerant *Dietzia* sp. in LB medium with 10% NaCl revealed a 1.3 to 1.6 higher ectoine content in the binary biofilm (Fig. 6).

Adsorption of a fraction of ectoine formed by *Chromohalobacter* sp. by the halotolerant *Dietzia* sp, resulting in a decrease of the extracellular ectoine in the biofilm and therefore in the derepression of the system of ectoine biosynthesis, may be an explanation for this effect.

The degree to which ectoine content in the biofilm increased depends on its amount accumulated in the cells of the halotolerant microorganism required for successful osmoprotection. After "saturation" of the *Dietzia* cells with ectoine, its extracellular content reaches the threshold level when ectoine overproduction is blocked by the regulatory mechanism. We have shown previously that exogenous ectoine could indeed protect the halotolerant *Dietzia* sp. from hyperosmotic shock [9]

Our results show the significant role of EPS (which are responsible for formation of the biofilm matrix) in development of resistance of these structured communities to extreme environmental factors, which may constantly or periodically affect the microorganisms

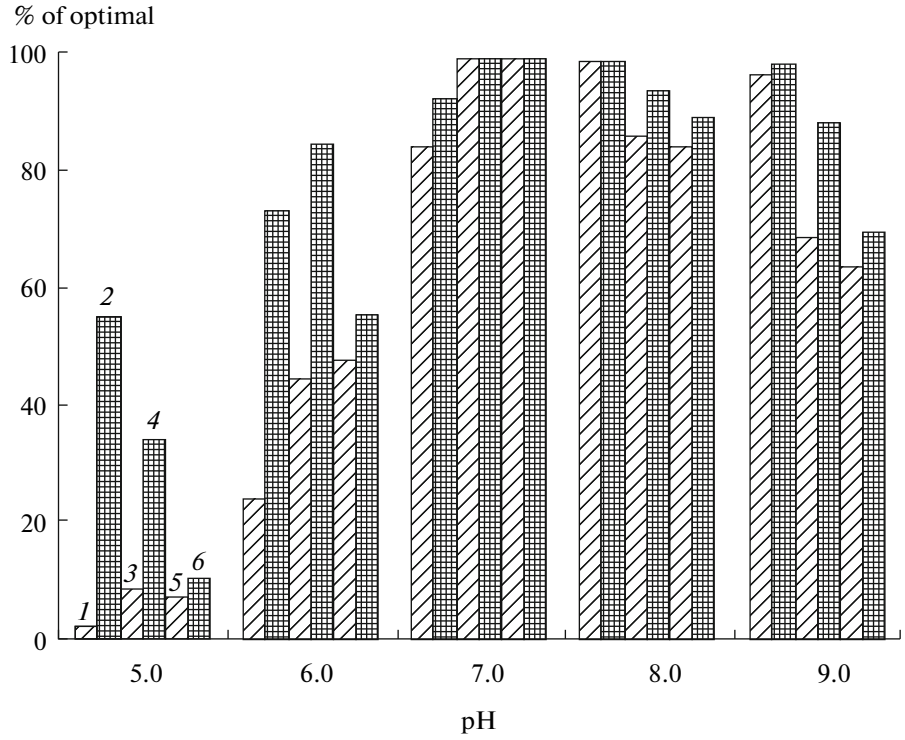

Fig. 5. Growth of planktonic cultures (*1, 3, 5*) and biofilms (*2, 4, 6; 2*, CV staining; *4*, *6*, DMMB staining) depending on pH: *Dietzia* sp. (*1, 2*); *C. violaceum* WT (*3, 4*), and *C. violaceum* CV026 (*5, 6*).

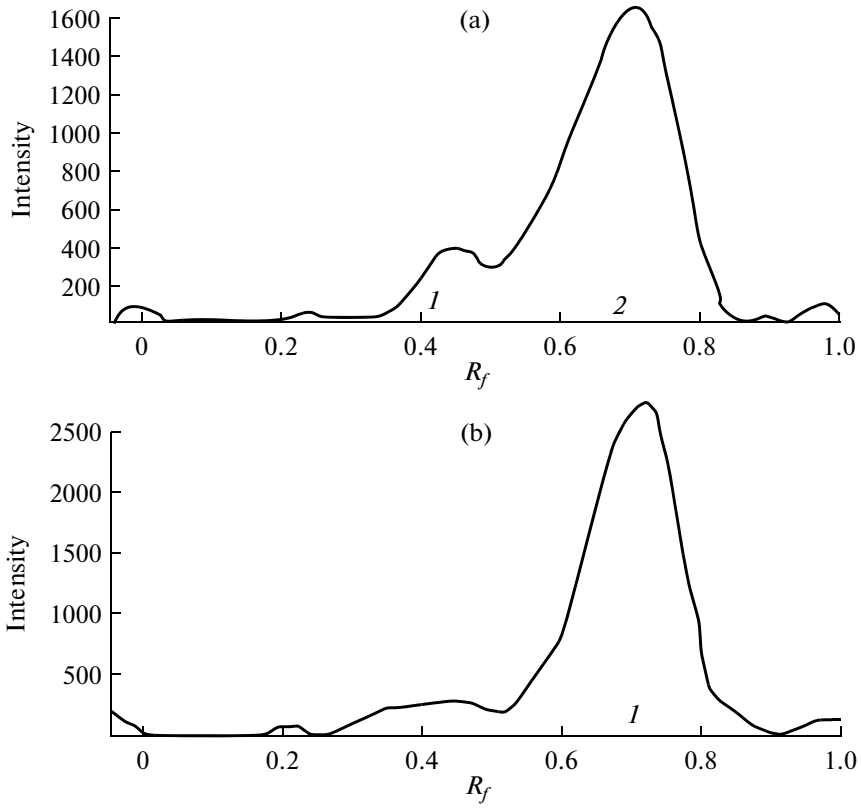

Fig. 6. Ectoine accumulation in the biofilm formed by *Chromohalobacter* sp. (a) and in the binary biofilm reconstructed from the halotolerant *Dietzia* sp. and its halophilic satellite *Chromohalobacter* sp. (b). Ectoine peak areas are 96307 and 132856 pixels, respectively.

in their ecotopes, including soil, water, and oilfields. Moreover, the biofilm matrix is a promising target for countering biofilm formation in undesired places (in medical and technological applications).

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