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EXPERIMENTAL ARTICLES

The Influence of Salt Concentration on the Copy Number of Plasmid pSH1 Replicating in *Micrococcus* sp. 9

T. I. Lobova*,[1], S. N. Zagrebel'nyi, and L. Yu. Popova***

**Institute of Biophysics, Siberian Division, Russian Academy of Sciences, Krasnoyarsk, 660036 Russia*

***Novosibirsk State University, ul. Pirogova 2, Novosibirsk, 630090 Russia*

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Abstract—The study of heterotrophic bacteria isolated from the brackish waters of Lake Shira has shown that some of them contain plasmid pSH1 of approximately 2.7 kb in size. The number of plasmid copies in plasmid-containing strains cultivated at a minimal concentration of sodium chloride is found to be low, whereas the subculturing of these strains at high salt concentrations increases the plasmid number. The role of natural pSH1 plasmid in the osmotolerance of host bacteria is discussed.

Key words: saline lakes, halotolerance, heterotrophic bacteria, plasmids.

Many genetic determinants that provide for bacterial survival under extreme conditions are located on plasmids. In particular, plasmids are responsible for bacterial resistance to antibiotics, xenobiotics, and heavy metals, as well as for the biodegradation of complex organic compounds [1–6]. There is evidence that plasmids may benefit host bacteria living in habitats with high values of pH, temperature, osmotic pressure, etc. [7]. The halotolerance of the cyanobacterium *Synechococcus* sp. isolated from the coastal waters of the Sea of Japan was reported to be related to plasmid pSY10, which is about 2.7 kb in size [8]. The authors also showed that increasing the sodium chloride concentration from 0 to 3% augmented the copy number of that plasmid. The moderately halotolerant heterotrophic bacteria that we isolated from the brackish waters of Lake Shira in the Khakassia Republic were also found to contain plasmid pSH1 of approximately 2.7 kb in size [9].

The aim of this work was to study the growth of these pSH1-bearing bacteria at different sodium chloride concentrations.

MATERIALS AND METHODS

Objects. Experiments were carried out with heterotrophic bacteria that were isolated from different zones of Lake Shira in the summer (June to August) of 1997. This lake, which has brackish water [10], is located in the south of the Khakassia Republic, Russia, at 54°30′ N, 90°14′ E, and has no outflow. The lake length, width, and depth are 9.35 km, 5.3 km, and 22 m, respectively. The sulfate–chloride–sodium–magnesium lake water is slightly alkaline and contains a salt concentration of up to 30 g/l [11]. The lake water was sampled at four stations, which differed in relation to the content of sodium ions in the water. Station 1 was located in the southeastern part of the lake close to the mouth of the Son River, where the lake depth was 1.2 m and the water contained sodium ions at a concentration of 0.6 g/l. Station 3 was located in the central part of the lake, where the lake depth was 22 m and the sodium content in the water was 3–5 g/l. Station 7 was located in the southwestern part of the lake in the resort area, where the lake depth was 2.3 m and the sodium concentration in the water was 2.7 g/l. Station 8 was located in the northeastern part of the lake near agricultural fields, where the lake depth was 3 m and the sodium concentration was 2.8 g/l.

The isolation of heterotrophic bacteria. Aliquots of water samples were plated onto a slightly modified mineral agar medium M9 containing (g/l) Na_2HPO_4, 6; KH_2PO_4, 3; NaCl, 0.5; NH_4Cl, 1; peptone, 5; and agar, 20. After sterilization by autoclaving, the medium was supplemented with 20% $MgSO_4$ (1 ml), 0.5% $CaCl_2$ (1 ml), and 20% glucose (10 ml) [12]. In order to isolate nonhalophilic and halotolerant bacteria, the medium was supplemented with sodium chloride at concentrations of 0.5, 50, 100, or 200 g/l. The halotolerance of the isolates was tested by the replica method [12], using the M9 medium with different salt concentrations.

[1] Corresponding author; e-mail: yanalobova@mail.ru

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The plasmid profile of bacterial strains isolated from Lake Shira

Isolate	Site from which strain was isolated	Antibiotic resistance		Tolerable NaCl concentration, %	Plasmid size, kb	Plasmid copy number
		Ap	Km			
4	The lake center	+	–	5	11.5	High
9	The lake center	–	+	10	2.7	ND
10	The lake center	+	+	10	2.7	High
					10.6	ND
13	The lake center	+	+	5	11.5	Middle
14	The lake center	+	+	10	2.7	High
18	The lake center	–	+	10	2.7	ND
					10.6	ND
19	The lake center	–	–	0.05	12.5	Low
22	The lake center	+	+	5	12.5	Low
137	The lake center	+	+	5	11.5	ND
164	The lake center	+	–	20	no plasmids	
239	The lake center	+	+	20	no plasmids	
106	The resort	+	+	0.05	4.4; 5.6; 8.8; 11.5	Middle
116	The resort	+	+	0.05	5.6; 8.8, 11.5	ND
118	The resort	+	+	5	3.27; 4.4; 5.6; 8.8; 11.5	ND
125	The resort	+	–	0.05	4.4; 5.6; 8.8; 11.5	Middle
167	The resort	+	+	5	12.5	Low
42	4000 m from the resort	+	+	0.05	12.5	ND
43	4000 m from the resort	–	+	5	4.4; 5.6	Low
49	4000 m from the resort	–	+	5	4.4; 5.6	ND
241	3500 m from the resort	+	+	0.05	12.7	Low
241	3500 m from the resort	+	+	0.05	12.7	Middle
244	3500 m from the resort	+	+	5	12.7	Middle
248	3500 m from the resort	+	–	5	12.7	Middle
250	3500 m from the resort			0.05	12.7	Middle

Note: ND stands for "not determined." The plasmid copy number was considered to be high, medium, and low when it was close to 100, between 10 and 30, and less than 10 per cell, respectively.

Characterization of bacteria with the pSH1 plasmid. The morphology of the bacterial isolates was studied using routine methods [13]. A microscopic examination of isolates 9 and 18 showed that their cells are gram-positive, spherical, small, non-spore-forming, and paired. The cells from isolates 10 and 14 were gram-negative slightly curved rods. Identification of the plasmid-containing isolates according to the taxonomic criteria of *Bergey's Manual* [14] showed that isolates 9 and 18 belong to the genus *Micrococcus*, whereas isolates 10 and 14 belong to the genus *Pseudomonas*.

Isolation of plasmid DNA. The strains were grown in a batch mode to the exponential growth phase. Plasmid DNA was prepared by the method of alkaline lysis [15]. Before electrophoresis, the DNA samples were dissolved in a buffer, whose volume was proportional to the biomass concentration. This procedure was carried out to make the content of plasmid DNA in the samples proportional to its content in the bacterial cells, that is, to the copy number of the plasmids. The control samples contained plasmids with a known number of copies: medium-copy-number plasmids pBMB105 (30 copies) and pBR322 (50 copies) and high-copy-number plasmid pUC18 (200–500 copies). Electrophoresis was carried out in 0.8% agarose. The electropherograms were scanned, and the copy number of the plasmids was estimated with respect to the amount of chromosomal material localized in the band, which corresponded to a molecular mass of 14 kb. The concentration of the 2.7-kb pSH1 plasmid was calculated with the aid of the Scion Image 4.02b program package for Windows.

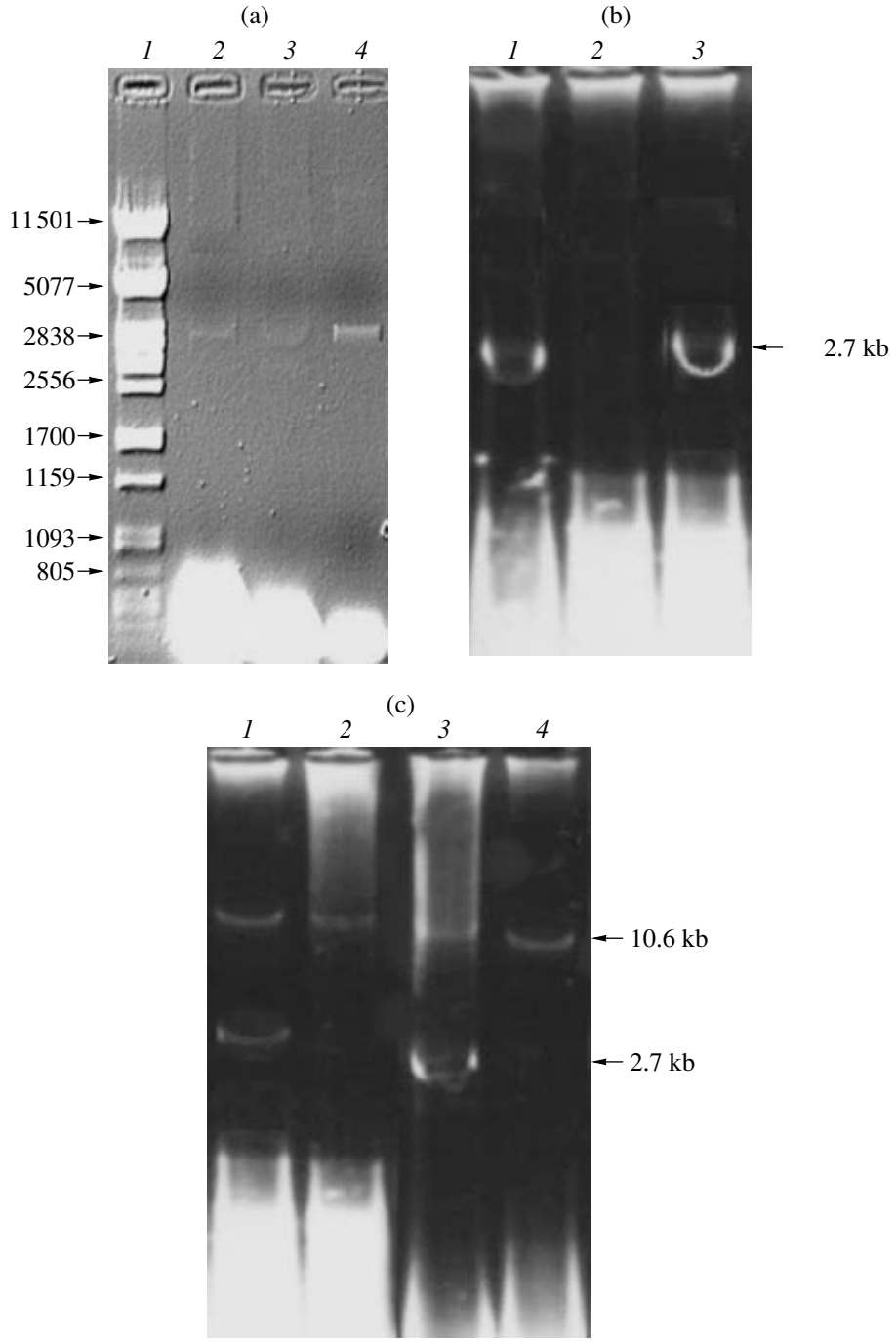

Fig. 1. Electrophoretic analysis of the strains isolated from Lake Shira (the numbers alongside the arrows indicate the plasmid size in kb). Lanes (a): (*1*) control (λ/Pst I); (*2*) *Micrococcus* sp. 9; (*3*) *Micrococcus* sp. 9 adapted to 0.05% NaCl; (*4*) *Micrococcus* sp. 9 adapted to 5% NaCl. Lanes (b): (*1*) *Pseudomonas* sp. 14; (*2*) *Pseudomonas* sp. 14 adapted to 0.05% NaCl; (*3*) *Pseudomonas* sp. 14 adapted to 5% NaCl. Lanes (c): (*1*) *Pseudomonas* sp. 10; (*2*) *Pseudomonas* sp. 10 adapted to 0.05% NaCl; (*3*) *Micrococcus* sp. 18; (*4*) *Micrococcus* sp. 18 adapted to 0.05% NaCl.

Cultivation of heterotrophic bacteria. The bacteria under study were cultivated at 28°C on a shaker (150 rpm) in 50-ml test tubes containing 20 ml of the medium. The culture density was measured at 540 nm in 0.5-cm pathlength cuvettes using a KFK-2 photocolorimeter. The results were expressed in relative units by taking the culture density of the best growing *Micrococcus* sp. strain 9 to be 100%.

The effect of sodium chloride concentration on the copy number of the pSH1 plasmid in the host strains was studied by cultivating them on the peptone-containing M9 medium with NaCl at concentrations of

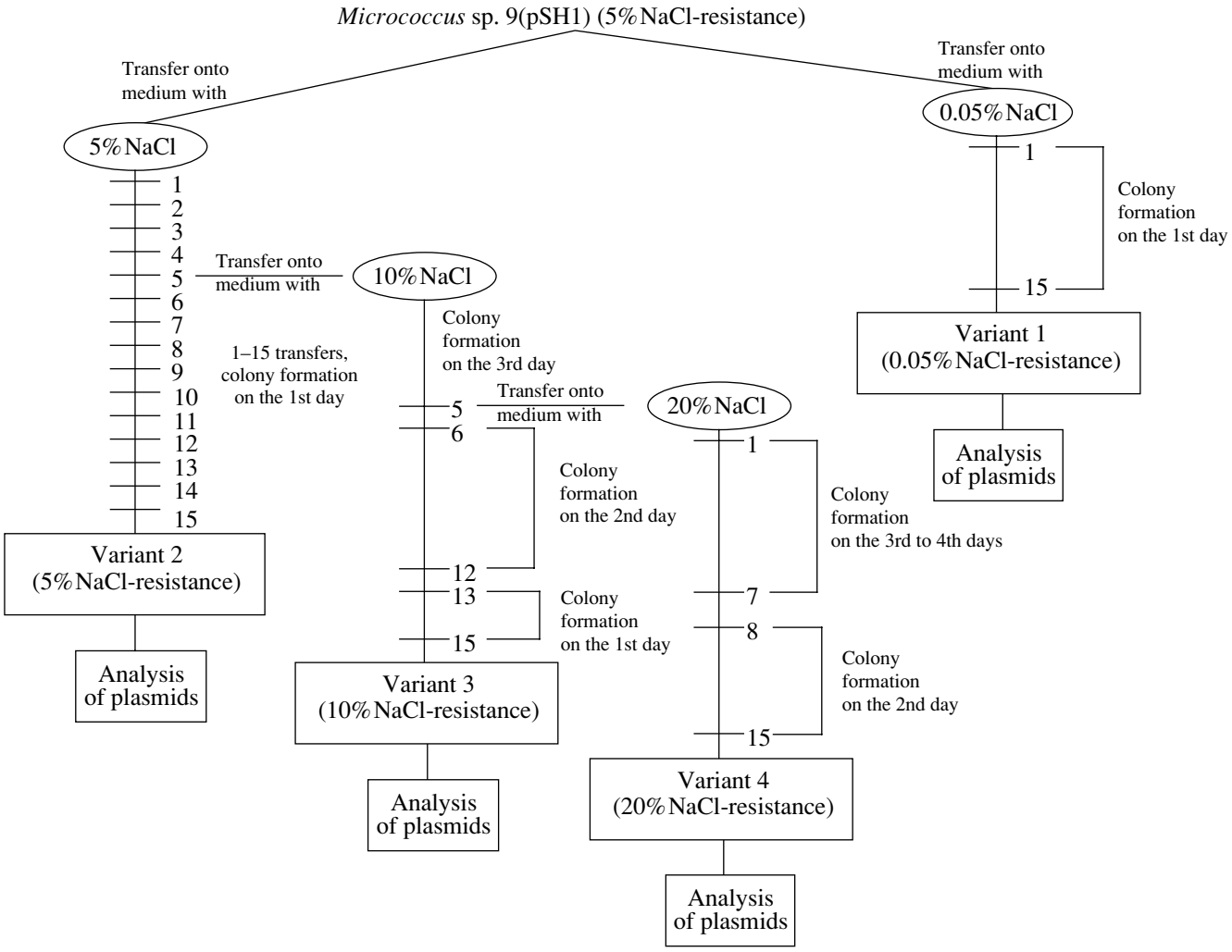

Fig. 2. Scheme showing the subculturing of *Micrococcus* sp. 9(pSH1) on the agar medium at different concentrations of sodium chloride.

0.05, 5, 10, and 20% (this methodological part of the study is described in detail in the Results and Discussion section with reference to *Micrococcus* sp. strain 9).

The antibiotic resistance of the heterotrophic bacteria under study was evaluated using the replica method [17]. The medium was supplemented with antibiotics possessing different mechanisms of action: inhibitors of protein synthesis (kanamycin, erythromycin, tetracycline, and oletetrin) and inhibitors of cell wall synthesis (ampicillin, penicillin, and dicloxacillin). A mass screening of the antibiotic-resistant bacterial isolates from Lake Shira was performed with ampicillin and kanamycin, which are widely used in medical practice in the Lake Shira resorts. The antibiotics were added to the medium, at a concentration of 50 μg/ml, after it had been autoclaved.

RESULTS AND DISCUSSION

General characteristics of the heterotrophic bacteria from Lake Shira. The brackish waters of Lake Shira in the Khakassia Republic are of great curative value, which poses the problem of protecting the lake from the anthropogenic impact of the resort and the settlement situated on the lakeshore. The domestic wastewaters from these places, which are contaminated by human and animal microflora, are discharged into the lake. As a rule, such microflora contains antibiotic resistance genes, which are mostly located on plasmids. During the regular ecological studies of Lake Shira by researchers from the Institute of Biophysics, great attention has been given to the seasonal dynamics of antibiotic-resistant autochthonous and allochthonous bacteria and the possible mechanisms responsible for the retention of allochthonous bacteria in the water of Lake Shira. We investigated the plasmid profile of approximately 100 heterotrophic bacteria isolated from different zones of Lake Shira and resistant either to one antibiotic (ampicillin or kanamycin) or to both. About one-fifth of the bacterial isolates tested were found to carry plasmids of various sizes (see table). After the

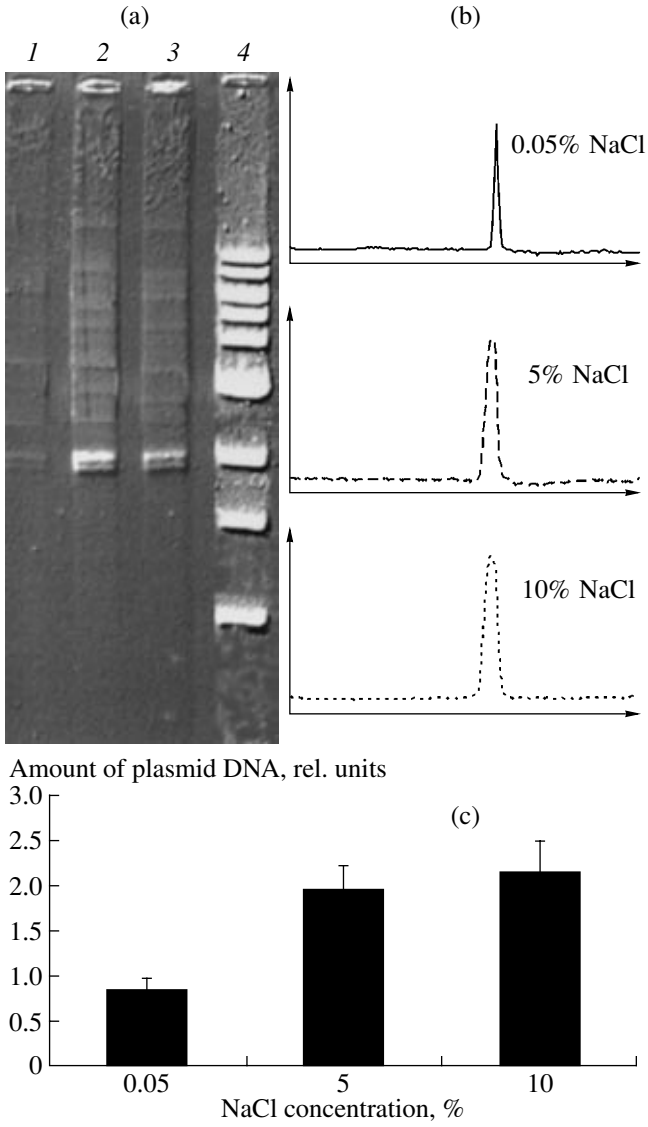

Fig. 3. Evaluation of the amount of pSH1 plasmid in the *Micrococcus* sp. 9(pSH1) variants cultivated on the agar medium with different concentrations of sodium chloride. (a) Electrophoresis of the pSH1 plasmid of *Micrococcus* sp. 9(pSH1) adapted to 0.05% NaCl (variant 1, lane *1*), 5% NaCl (variant 2, lane *2*), and 10% NaCl (variant 3, lane *3*). Lane *4*: the control (λ/Pst I). (b) Evaluation of the concentration of the pSH1 plasmid with the aid of the Scion Image program. (c) Diagram showing the relative amount of pSH1 plasmid in the *Micrococcus* sp. 9(pSH1) variants cultivated in the presence of different NaCl concentrations.

2-month storage of the plasmid-containing isolates on the M9 medium with peptone, the isolates still contained plasmid DNA, although the concentration of the 2.7-kb plasmid in strains *Micrococcus* sp. 9, *Micrococcus* sp. 18, *Pseudomonas* sp. 10, and *Pseudomonas* sp. 14 had considerably decreased (Fig. 1a, lanes *2, 3*; Fig. 1b, lanes *1, 2*; Fig. 1c, lanes *2, 4*). Further studies were performed with two isolates, *Pseudomonas* sp. 14 and *Micrococcus* sp. 9, that contained only the pSH1 plasmid.

Study of the functional role of the pSH1 plasmid. In order to reveal the factor influencing the copy number of the pSH1 plasmid, we subcultured the bacterial isolates on the medium with the antibiotics. These experiments showed that neither pSH1 nor the other plasmids changed their copy number in response to the presence of antibiotics in the cultivation medium. Presumably, the antibiotic resistance of the isolates is associated with chromosomal rather than plasmid DNA. Taking into account the fact that the water of Lake Shira contains substantial amounts of sodium and chloride ions, we suggest that sodium chloride may favor the maintenance of the pSH1 plasmid in bacteria isolated from this water. The batch cultivation of the plasmid-containing bacterial isolates for 18 h in the liquid M9 medium [12] with peptone and 5% NaCl showed that the concentration of plasmid DNA in *Micrococcus* sp. 9 and *Pseudomonas* sp. 14 increased (Fig. 1a, lane *4* and Fig. 1b, lane *3*, respectively). In other words, NaCl does influence the copy number of the 2.7-kb pSH1 plasmid, as in the case of the 2.7-kb pSY10 plasmid found in cyanobacterial cells [8].

To verify this supposition, *Micrococcus* sp. strain 9(pSH1) was serially subcultured on the peptone-containing agar M9 medium supplemented with NaCl at different concentrations (Fig. 2). The control medium contained NaCl at a concentration (0.05%) that is not beneficial for the maintenance of plasmids (Fig. 1a, lane *2*). During the subculturing of this strain on the medium with 5% NaCl (five sequential subcultures), colonies appeared on the plates on the 1st day of incubation. After one passage on the medium with 10% NaCl, the formation of colonies on the medium with 5% NaCl was delayed by 3 days. This delay, however, tended to shorten to 1 day after serial passages on the medium with 5% NaCl. In the next set of experiments, *Micrococcus* sp. strain 9(pSH1) was subcultured five times on the medium with 10% NaCl and then once on the medium with 20% NaCl. As the number of subcultures on the medium with 10% NaCl was increased, the colonies were formed within a shorter time period (for instance, on the first day after 13 passages). During subculturing on the medium with 20% NaCl, the growth rate of the colonies also increased; as a result, after eight subcultures, the colonies appeared on the second day. Further increasing the number of subcultures did not reduce the time of colony formation.

The electrophoretic analysis of *Micrococcus* sp. 9(pSH1) cells subcultured 15 times on the medium with different sodium chloride concentrations showed that the presence of NaCl in the medium favored the maintenance of the 2.7-kb pSH1 plasmid. The maximum content of this plasmid in the cells was observed when the medium contained 5% NaCl (Fig. 3). It should be noted that, after 15 passages on the medium with 20% NaCl, the cells became resistant to lysis, so that it was difficult to perform their electrophoretic analysis (data not shown).

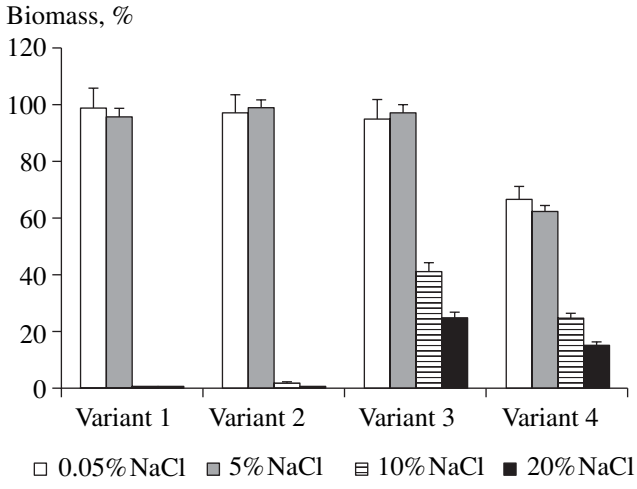

Fig. 4. Growth of the *Micrococcus* sp. 9(pSH1) variants cultivated in a batch mode in the presence of different NaCl concentrations. Variants 1, 2, 3, and 4 were adapted to, respectively, 0.05, 5, 10, and 20% NaCl by means of 15 passages on a particular salt concentration.

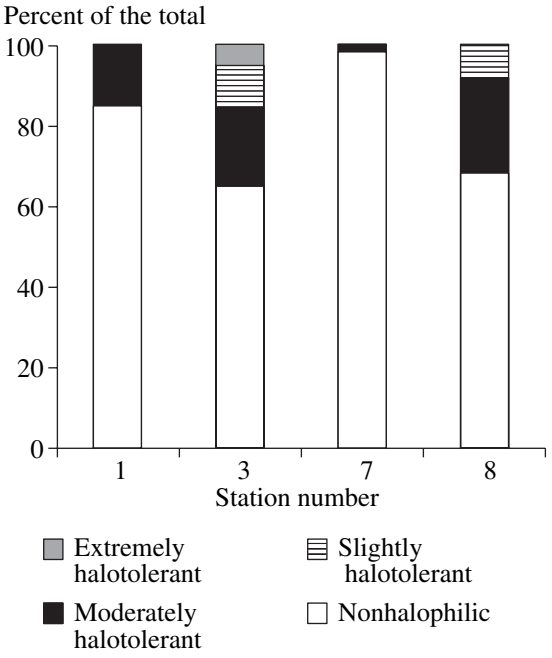

Fig. 5. Distribution of heterotrophic bacteria isolated in 1997 from different stations on Lake Shira with respect to the degree of their halotolerance.

Batch cultivation of strain *Micrococcus* sp. 9(pSH1) preliminarily subcultured on the medium with different NaCl concentrations. As is evident from the results presented in Fig. 4, the subculturing of this strain on the medium when it contained high NaCl concentrations made the strain resistant to salt. This resistance is especially evident in the case of experimental variant 3 (serial subculturing in the presence of 10% NaCl). Unlike the cells adapted to 5% NaCl by serial subculturing in the presence of this salt concentration (experimental variant 2) and the cells adapted to 0.05% NaCl (variant 1), the cells adapted to 10% NaCl (variant 3) grew well and were tolerant to 20% NaCl. Thus, the elevated concentration of NaCl in the medium increases the copy number of the pSH1 plasmid in the host cells, making them capable of growing in the presence of NaCl at concentrations of up to 20%. It should be noted that the growth rate of the cells adapted to 20% NaCl was relatively low in the media with both high and low salt concentrations (Fig. 4, experimental variant 4). This result is probably due to a modification of the metabolism of such cells toward defense against high osmotic pressure. As is evident from Fig. 2, *Micrococcus* sp. 9(pSH1) can produce colonies in the presence of 10% NaCl and higher salt concentrations.

Distribution of heterotrophic bacteria with different halotolerance in Lake Shira. The heterotrophic strains, both plasmid-containing and plasmidless, isolated from Lake Shira were tested for the degree of their halotolerance. Our earlier studies [17] showed that the heterotrophic bacteria of Lake Shira are dominated by nonhalophilic bacteria tolerant to no more than 3% NaCl and slightly halotolerant bacteria withstanding no more than 5% NaCl. Some bacterial isolates were found to be moderately halotolerant (withstanding up to 15% NaCl) and even extremely halotolerant (withstanding up to 20% NaCl). The number of the latter bacteria was very low (Fig. 5). The difference in the proportion of bacteria with different halotolerance is probably due to the different salinity of the waters from which these bacteria were isolated. It should be noted that most of the halotolerant plasmid-containing strains were found to contain the 2.7-kb plasmid. At the same time, most of the extremely halotolerant bacteria isolated from Lake Shira were plasmidless (table, isolates 164 and 239).

Thus, increased concentrations of NaCl in the medium favor the halotolerance of bacteria containing the 2.7-kb pSH1 plasmid. The role of the other plasmids (often of the same size) found in the Lake Shira isolates remains unknown. In order to gain further insight into the functional role of the plasmids in the isolates, it is necessary to reveal the factors that can influence the concentration of these extrachromosomal elements. Of great importance is the biochemical nature of bacterial tolerance to high salt concentrations. As follows from an analysis of the available databases, the functional role of most proteins encoded by plasmid open reading frames in halophilic bacteria is far from being well understood. Among the proteins whose function is known, there are oxidoreductases, hydrolases, transferases, and proteins involved in the formation of vacuoles, i.e., proteins which are unlikely to be directly responsible for halotolerance. Some proteins are homologous to rep proteins, which are involved in the regulation of the replication of plasmids [18, 19].

Our future studies will focus on the structure of pSH1 and investigation of the proteins encoded by this plasmid.

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