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EXPERIMENTAL ARTICLES

Effect of Salinity on the Adaptive Capacity of Recombinant Strains of *Escherichia coli* and *Bacillus subtilis*

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Abstract—The effect of different concentrations of salts on natural and recombinant strains of *Bacillus subtilis* and *Escherichia coli* was studied. The recombinant strain of *B. subtilis* was found to be more osmotolerant than the wild-type strain of this bacterium, whereas the opposite situation was observed for the recombinant and wild-type strains of *E. coli.* Some salts exerted a bacteriostatic effect on *E. coli* and *B. subtilis.* The adaptive capacity of recombinant strains depended on the number of plasmid copies in the cells. The introduction of recombinant bacteria into model ecosystems resulted in the generation of their variants with increased osmotolerance.

Key words: genetically modified microorganisms, salinity, salt resistance

Recent advances in genetics and molecular biology gave rise to developments associated with the obtainment of genetically modified microorganisms (GMMs), some of which are intended to be introduced into natural ecosystems [1]. Possible risks related to such an introduction call for the study of the fate of recombinant microorganisms in natural environments.

Relevant investigations may include the evaluation of the effect of various environmental factors on the propagation and survival of GMMs. Such studies should take into account that the number and size of recombinant plasmids, as well as the type of cloned genes, may influence the acclimation of GMMs to the environment. Earlier, we investigated the survival of recombinant microorganisms in fresh water [2–4], which is a habitat of many microorganisms. One of the major environmental factors in aquatic habitats is the concentration of dissolved inorganic ions [5–7], since osmotic shifts can slow down the growth and reproduction of bacteria and even cause their death. Of much interest is the role of the ionic composition of water in bacterial survival.

The survival rate of freshwater microorganisms in saline waters and soils is low. As a result, saline lakes are capable of self-purification from allochthonous microflora [8]. A convenient object for investigating microbial tolerance to salts is Lake Shira in Khakass, whose water, with a salinity of about 20‰, has been extensively studied [9].

The aim of the present work was to study the effect of different concentrations of some salts on the growth and adaptation of recombinant and wild-type strains of *E. coli* and *B. subtilis,* as well as of their variants.

MATERIALS AND METHODS

The strains used in this study were as follows: *Escherichia coli* Z905 (hsdR$^+$, hsdM$^+$, gal$^-$, met$^-$, supE, recA, tet^r), bearing the recombinant plasmid pPHL7 (Ap^{r}Lux$^+$) with the *lux* operon cloned under the *lac* promoter [2]; the wild-type strain *E. coli* K-12; *Bacillus subtilis* 2335/105, harboring the recombinant plasmid pBM105 (Km^{r}Inf$^+$) with the human α2-interferon gene cloned under a constitutive promoter; the wild-type strain *B. subtilis* 2335 [10]; variants of these strains obtained by introducing them into freshwater microecosystems (MESs) or by subculturing on various media [2, 4, 11]; and strains isolated from the water of Lake Shira. Some relevant characteristics of these strains are presented in the table.

Strains were batch-cultivated at 28°C on a shaker in liquid M9 medium [12] supplemented with peptone (5 g/l). The effect of Mg^{2+}, Cl^-, SO_4^{2-}, and Na^+ (these ions were chosen due to their prevalence in the Lake Shira water [9]) was studied by adding sodium chloride, sodium sulfate, magnesium chloride, and magnesium sulfate to the growth medium to give their final concentrations of 0.05, 2, 5, and 10%. Bacterial growth was monitored by measuring the optical density of cul-

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Some characteristics of the strains studied

Strain	Relevant characteristics	Optimum growth concentration of NaCl, %
Escherichia coli K-12	Wild-type plasmid-free strain	0.05
Escherichia coli Z905	Original strain bearing 12.7-kb plasmid pPHL7	0.05
Escherichia coli II-58	Low-plasmid-number variant of *E. coli* Z905 isolated from MES	0.05
Escherichia coli II-67	Low-plasmid-number variant of *E. coli* Z905 isolated from MES	2
Escherichia coli 1-137	Low-plasmid-number variant of *E. coli* Z905 obtained by subculturing in minimal medium without ampicillin	0.05
Bacillus subtilis 2335	Wild-type plasmid-free strain	0.05–2
Bacillus subtilis 2335/105	*B. subtilis* 2335 harboring 5.6-kb plasmid pBMB105	0.05–5
Bacillus subtilis 10-36	Low-plasmid-number variant of *B. subtilis* 2335/105 obtained by subculturing in complete medium without kanamycin	2
Bacillus subtilis 10-116	Low-plasmid-number variant of *B. subtilis* 2335/105 obtained by subculturing in minimal medium with kanamycin	0.05–5
Bacillus subtilis II-9	Plasmid-free variant of *B. subtilis* 2335/105 isolated from MES	0.05–2
49	Strain with 4.4-kb and 5.6-kb plasmids isolated from the central part of Lake Shira	0.05
9	Strain with 2.7-kb and 10.6-kb plasmids isolated from the central part of Lake Shira	5
10	Strain with 10.6-kb plasmid isolated from the central part of Lake Shira	2
173	Plasmid-free strain isolated from the central part of Lake Shira	10

tures at 540 nm in a KFK-2 photoelectrocolorimeter. Experiments were performed in at least five replicates.

Plasmid DNA was analyzed by conventional methods [13] at the Research, Design, and Technology Institute of Biologically Active Substances SRC VB Vector, Berdsk. To estimate the number of plasmid copies relative to their number in the original recombinant strains, cells were grown, in a batch mode, to the exponential phase and then were subjected to alkaline lysis. The relative number of plasmids was estimated by electrophoresis. Before applying the plasmid DNA isolated from cell lysates onto agarose gels, each DNA sample was dissolved in a buffer volume that was proportional to the biomass from which this DNA sample had been isolated.

RESULTS AND DISCUSSION

Effect of different concentrations of salts on *E. coli* and *B. subtilis* strains. As can be seen from the data presented in Figs. 1a and 1b, NaCl, Na_2SO_4, $MgCl_2$, and $MgSO_4$ at concentrations of 2, 5, and 10% inhibited, to different degrees, the growth of both K-12 and Z905 strains of *E. coli* as compared with the control (growth of these strains in the presence of 0.05% salts).

At a concentration of 10%, NaCl, Na_2SO_4, $MgCl_2$, and $MgSO_4$ completely inhibited the growth of *E. coli* strains, whereas 10% $MgSO_4$ inhibited growth only partially. The tolerance of *E. coli* K-12 to 5% salts was higher than that of *E. coli* Z905. At a concentration of 2%, NaCl and Na_2SO_4 but little affected the growth of *E. coli* Z905; however, the effect of these salts at a concentration of 5% was more pronounced. The maximum inhibitory effect was observed for $MgCl_2$: this salt considerably inhibited bacterial growth even at a concentration of 2%. In our opinion, the more profound effect of $MgCl_2$ in comparison with $MgSO_4$ can be explained by a combined detrimental effect of Mg^{2+} and Cl^- on microorganisms. It is likely that the bacteriostatic effect of Mg^{2+} on microorganisms is due to its implication in their metabolism.

The study of the effect of salts on *B. subtilis* strains (Figs. 1c and 1d) showed that, generally, these strains were more salt resistant than *E. coli* strains, which agrees with the data of other authors who reported a high survival rate of bacilli in saline habitats [14]. As for the relative salt tolerance of *B. subtilis* strains, the plasmid-free strain of this bacterium was more sensitive to 2% Na_2SO_4 than the recombinant strain.

Effect of the osmotic factor on microorganisms. These studies were carried out using NaCl, an osmoticum that is traditional for such investigations [15, 16].

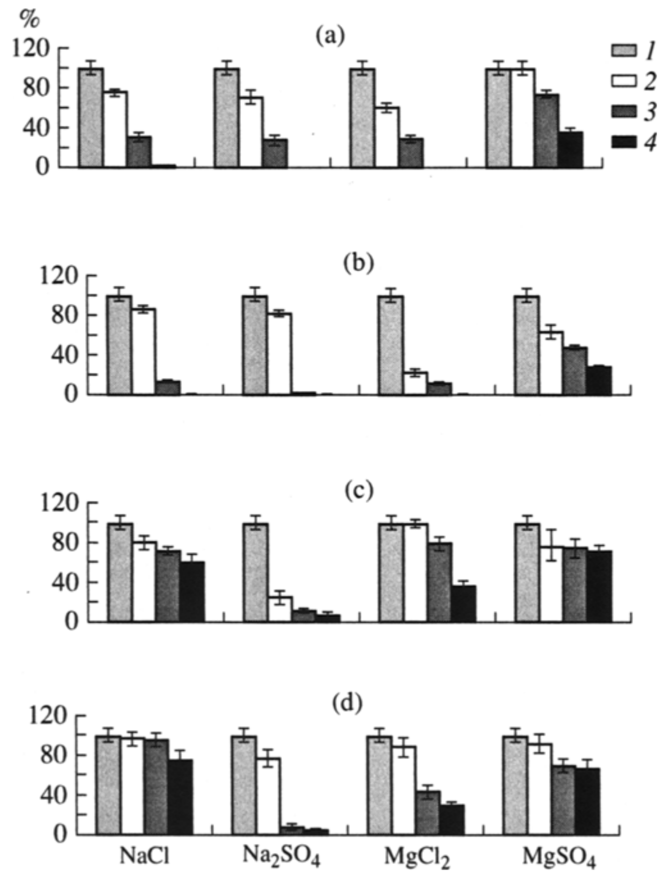

Fig. 1. Relative increase in the biomass of (a) *E. coli* K-12, (b) *E. coli* Z905, (c) *B. subtilis* 2335, and (d) *B. subtilis* 2335/105 strains during 15-h batch cultivation in the presence of different salts added at concentrations (%): (*1*) 0.05; (*2*) 2; (*3*) 5; and (*4*) 10. For all salts, the increase in biomass at a 0.05% salt concentration is taken as 100%.

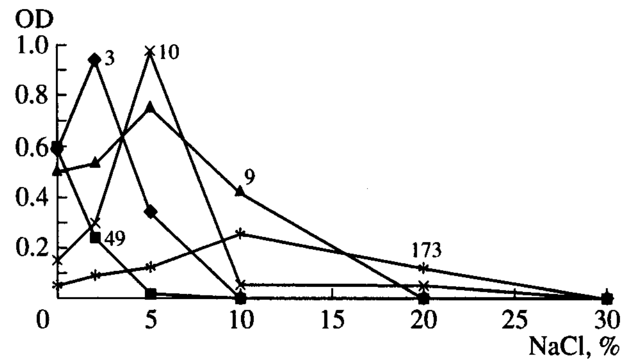

Fig. 2. Increase in the biomass of some isolates from Lake Shira during 10-h batch cultivation in the presence of different concentrations of NaCl. Biomass is expressed in units of optical density measured at 540 nm (OD_{540}).

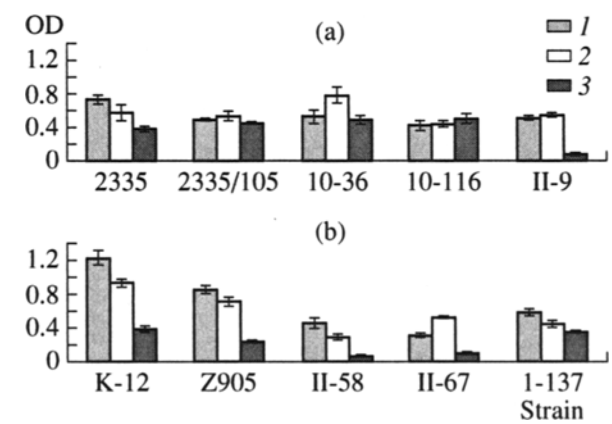

Fig. 3. Increase in the biomass of (a) *B. subtilis* 2335/105 and (b) *E. coli* Z905 (and also of plasmidless strains) during 12-h batch cultivation in the presence of different concentrations of NaCl (%): (*1*) 0.05; (*2*) 2; and (*3*) 5. Biomass is expressed in units of optical density measured at 540 nm (OD_{540}).

The majority of microorganisms isolated from the slightly saline water of Lake Shira turned out to be osmotolerant and could grow in the presence of 5–10% NaCl. Some isolates could tolerate even 30% NaCl. At the same time, the isolates obtained from samples taken near the Lake Shira resort were unable to grow in the presence of high concentrations of NaCl (Fig. 2, isolate 49), indicating that they obviously belonged to the allochthonous microflora. The isolates from the central part of Lake Shira were mainly salt tolerant (Fig. 2, isolates 3, 9, 10, 173). The absence of allochthonous microorganisms in saline water was probably for osmotic reasons.

Effect of NaCl on variants of the recombinant strains of *E. coli* and *B. subtilis*. The variants of the recombinant strain *B. subtilis* 2335/105 were tested for their ability to grow at high concentrations of NaCl (Fig. 3). The tolerance of variants to this salt differed from that of both the recombinant and the wild-type strains. The variants that lost the recombinant plasmid (e.g., variant II-9) exhibited decreased resistance to high concentrations of NaCl (Figs. 3a and 4a). At the same time, low-plasmid-number variants (e.g., variant 10-116) were tolerant to the salt concentrations tested. The optimal concentration of NaCl for plasmid-harboring variants (e.g., variant 10-36) was 2%. In saline habitats, such variants may be more competitive than the recombinant or the wild-type strain.

Strain *E. coli* Z905 was less resistant to 5% NaCl than *E. coli* K-12 (Fig. 3b). Some variants of the former strain exhibited enhanced salt resistance, although we were unable to reveal a correlation between the number of plasmid copies in cells and their salt resistance (Figs. 3b and 4b). Low-plasmid-number variants differed in growth rate and salt tolerance.

In conclusion, a proper estimation of the survival rate and competitiveness of GMMs in nature requires the knowledge of the key ecological factors of various ecosystems. For saline lake ecosystems, such as Lake Shira, of much importance is not only the total salinity

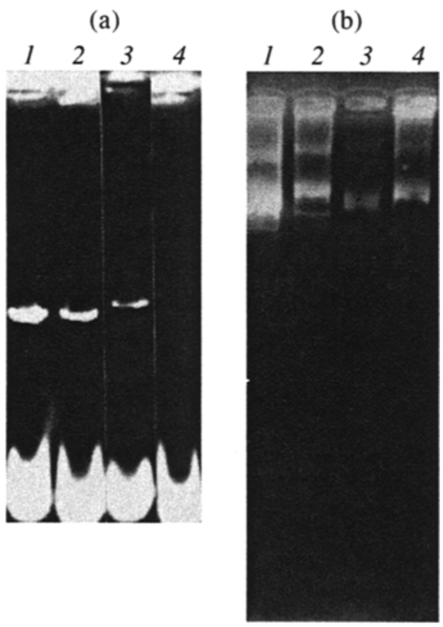

Fig. 4. Electrophoresis in 0.8% agarose gel of the plasmid DNA isolated from recombinant strains and their variants. Panel a: (*1*) *B. subtilis* 2335/105; (*2*) *B. subtilis* 10-36; (*3*) *B. subtilis* 10-116; and (*4*) *B. subtilis* II-9. Panel b: (*1*) *E. coli* Z905; (*2*) *E. coli* II-58; (*3*) *E. coli* II-67; and (*4*) *E. coli* 1-137.

of the water, but also its ionic composition. For instance, magnesium and sodium salts can adversely affect the survival of allochthonous microflora. The presence of plasmids in microorganisms can promote their adaptation to unfavorable conditions, which calls for further investigation of this effect.

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