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**EXPERIMENTAL ARTICLES**

# Osmoadaptation in Representatives of Haloalkaliphilic Bacteria from Soda Lakes

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**Abstract**—The adaptation of microorganisms to life in brines allows two strategies: the accumulation of organic osmoregulators in the cell (as in many moderate halophiles, halomonads in particular) or the accumulation of inorganic ions at extremely high intracellular concentrations (as, for example, in haloanaerobes). To reveal the regularities of osmoregulation in haloalkaliphiles developing in soda lakes, *Halomonas campisalis* Z-7398-2 and *Halomonas* sp. AIR-2 were chosen as representatives of halomonads, and *Natroniella acetigena*, as a representative of haloanaerobes. It was established that, in alkaliphilic halomonads, the intracellular concentrations of inorganic ions are insufficient for counterbalancing the environmental osmotic pressure and balance is attained due to the accumulation of organic osmoregulators, such as ectoine and betaine. On the contrary, the alkaliphilic haloanaerobe *N. acetigena* employs $K^+$, $Na^+$, and $Cl^-$ ions for osmoregulation. High intracellular salt concentrations increasing with the content of $Na^+$ in the medium were revealed in this organism. At a concentration of 1.91 M $Na^+$ in the medium, *N. acetigena* accumulated 0.83 M $K^+$, 0.91 M $Na^+$, and 0.29 M $Cl^-$ in cells, and, with an increase in the $Na^+$ content in the medium to 2.59 M, it accumulated 0.94 M $K^+$, 1.98 M $Na^+$, and 0.89 M $Cl^-$, which counterbalanced the external osmotic pressure and provided for cell turgor. Thus, it was shown that alkaliphilic microorganisms use osmoregulation strategies similar to those of halophiles and these mechanisms are independent of the mechanism of pH homeostasis.

*Key words*: soda lakes, haloalkaliphiles, osmoregulators, intracellular ion concentrations.

Soda lakes are highly concentrated carbonate brines of natural origin. Microorganisms inhabiting such ecosystems must have mechanisms allowing the problem of osmotic stress to be solved. In the microbial world, there exist two principally different strategies of osmoadaptation [1–3]. One of them implies equilibration of salt concentrations in the cytoplasm and in the environment at the expense of the accumulation of inorganic ions in the cell and, as a consequence, modification of intracellular proteins and other macromolecules so that they are able to function in solutions with a high ionic strength (the "salt-in" strategy). The main cation is $K^+$, usually equilibrated by the chloride anion. The advantage of the $K^+$ ion over $Na^+$ is that the potassium ion binds water to a considerably lesser degree [4]. The second strategy consists in maximum removal of salts from the cytoplasm and accumulation of small water-soluble organic molecules compatible with the cell metabolism and providing for maintenance of the osmotic balance (compatible osmoregulators). They are either synthesized by the cell itself or enter it from the outside, which is more advantageous in terms of energy expenditure. The cell concentration of osmolytes exceeds 0.5 M [2] and increases in proportion to the environmental osmotic pressure. Uncharged and zwitterionic compounds are preferable since they are more favorable for protein stabilization than ionic compounds [5]. That is why, along with the accumulation of neutral osmolytes, isosmotic displacement of electrically charged compounds (e.g., potassium ions) also occurs.

The salt accumulation strategy is used by two groups of microorganisms: aerobic extremely halophilic archaea of the order *Halobacteriales* and anaerobic halophilic bacteria of the order *Halanaerobiales.* In most other halophilic and halotolerant microorganisms, the environmental osmotic pressure is counterbalanced by organic osmoregulators. In addition, cases of combination of the two strategies are also known: the cells may contain a certain amount of $Na^+$, $K^+$, and $Cl^-$ ions, but it is insufficient to maintain osmotic equilibrium and balance is achieved by simultaneous accumulation of organic osmoregulators [6]. At present, the mechanisms of osmoadaptation are being actively studied, which is owing to, among other reasons, the possibility of the biotechnological application of halophilic micro-

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organisms and their enzymes. However, virtually no studies of microorganisms experiencing osmotic stress under high carbonate alkalinity conditions have been conducted. Meanwhile, it is possible that osmoadaptation mechanisms revealed in such microorganisms may exhibit certain specific features.

Earlier, the extremely haloalkaliphilic anaerobic acetogenic bacterium *Natroniella acetigena* [7], attributed to the order *Halanaerobiales*, as well as several alkaliphilic denitrifying halomonads—the moderately halophilic *Halomonas campisalis* Z-7398-2 [8] and the facultatively halophilic representative of the same genus strain AIR-2—were isolated from soda lakes. *N. acetigena* grows at a $Na^+$ content of the medium of 1.7–4.44 M (the optimum is 2–2.56 M) and pH 8.1–10.7. The growth of *H. campisalis* is possible in the $Na^+$ range 0.16–3.2 M (the optimum is 1.0 M) at pH 7.5–10.4; the growth of strain AIR-2 occurs in the $Na^+$ range 0.04–2.2 M (the optimum is 0.5–1.2 M) at pH 7.5–10.6. The aim of the present work was to investigate the strategies of osmoadaptation of these haloalkaliphilic bacteria.

## MATERIALS AND METHODS

**Media and cultivation conditions.** Medium with acetate (2 g/l) of the following composition (g/l) was used for the cultivation of *Halomonas campisalis* Z-7398-2 and *Halomonas* sp. AIR-2: $Na_2CO_3$, 6.5; $NaHCO_3$, 3.5; $KH_2PO_4$, 0.2; $MgCl_2 \cdot 6H_2O$, 0.05; $NaNO_3$, 0.5; yeast extract, 0.1; trace element solution, 1 ml; medium pH 9.3–9.6. The salinity level was varied by adding NaCl: 3 and 10% for *H. campisalis* and 2 and 8% for strain AIR-2. In the media for *H. campisalis*, the total $Na^+$ ion content was calculated to be 0.70 and 1.90 M and the total content of the $Cl^-$ ion, 0.51 and 1.70 M. The media for *Halomonas* sp. AIR-2 contained 0.53 and 1.56 M $Na^+$ and 0.34 and 1.37 M $Cl^-$. The $K^+$ concentration was the same in all cases and constituted 0.015 M.

*Natroniella acetigena* Z-7937$^T$ was cultivated with lactate (5 g/l) at pH 9.7 in medium of the following composition (g/l): $Na_2CO_3$, 68.3; $NaHCO_3$, 38.3; $KH_2PO_4$, 0.2; $MgCl_2 \cdot 6H_2O$, 0.1; $NH_4Cl$, 1.0; KCl, 0.2; yeast extract, 0.2; $Na_2S \cdot 9H_2O$, 1.0; trace element solution, 1 ml; 0.04% resazurin, 2 ml; vitamin solution according to Wolin, 2 ml. The medium contained 1 or 5% NaCl. The total $Na^+$ content in the media was calculated to be 1.91 and 2.59 M; that of $Cl^-$, 0.17 and 0.85 M. The $K^+$ concentration in the media was 0.013 M.

**Preparation of cell-free extracts.** The cells were precipitated by centrifugation at 16000× *g* for 30 min and subjected to aerobic ultrasound disruption in a UZDN-1 apparatus at 0.4 mA three times for 1 min each. The destroyed cells were centrifuged at 10000× *g* to remove cell fragments. The supernatant was used to determine the intracellular ion concentrations.

**Determination of the intracellular concentrations of $Na^+$, $K^+$, and $Cl^-$ ions.** The concentration of inorganic ions in cell-free extracts was determined by high-performance liquid chromatography (HPLC) on a Staier chromatograph (ZAO Akvilon, Russia) with a conductometric detector. The determination of chloride ion was carried out in 3 mM carbonate buffer in a BTX An separation column (4.6 × 100 mm). The elution rate was 1.5 ml/min; the volume of the sample introduced was 20 µl. The determination of sodium and potassium ions was performed in a 4 mM nitric acid solution in a CIP separation column (4 × 100 mm). The elution rate was 1.5 ml/min; the volume of the sample introduced was 20 µl. The volume of intercellular water was taken into account with the use of blue dextran 2000 T [9]. The intracellular volume was calculated based on the geometric sizes of the cells [10].

**Determination of osmolytes.** The cells at the end of the exponential growth phase were harvested by centrifugation at 16000× *g* for 30 min. Low-molecular-weight components were extracted from the cells with 70% ethanol. The supernatant obtained after centrifugation (ethanol extract) was evaporated in a vacuum rotary evaporator until a dry residue was obtained and dissolved in dimethyl sulfoxide (DMSO). The $^{13}C$ spectra of the preparation obtained were recorded on a Bruker AC300 NMR spectrometer (Germany) at a frequency of 75.64 MHz with respect to carbon. The chemical shifts were measured from the DMSO signals at room temperature.

## RESULTS AND DISCUSSION

To study the osmoadaptation strategies in representatives of different groups of haloalkaliphilic microorganisms, we determined the intracellular concentrations of sodium, potassium, and chlorine ions in two species of *Halomonas*, as well as in *N. acetigena*, at various concentrations of NaCl in the medium. The data obtained are shown in the table.

It can be seen from the table that, in *H. campisalis*, the intracellular $Na^+$ ion content constituted approximately 0.23 M and virtually did not increase with the increase of the $Na^+$ concentration in the medium from 0.70 to 1.90 M. In *Halomonas* sp. AIR-2, the intracellular $Na^+$ concentrations turned out to be twice as high as in *H. campisalis* and constituted about 0.5 M. With an increase in the total $Na^+$ concentration in the medium from 0.53 to 1.56 M, the content of this ion in the AIR-2 cells increased insignificantly. It should be noted that, when strain AIR-2 grew in the medium with 2% NaCl, the intracellular and medium $Na^+$ concentrations were close. On the contrary, the intracellular sodium ion content in *H. campisalis* was in all cases significantly lower than its content in the medium.

The chloride ion content in *H. campisalis* cells did not depend on the amount of NaCl in the medium and constituted about 0.22 M. This value virtually coincided with the intracellular $Na^+$ concentration, which gives evidence of the possibility of combined accumu-

Concentrations of $Na^+$, $K^+$, and $Cl^-$ ions in the intracellular space (cells) and in culture fluid (cf) as dependent on the medium salinity

| Microorganism and cultivation conditions* | $Na^+_{cf}$, M | $Na^+_{cells}$, M | $Na^{+**}_{cf}$, M | $K^+_{cells}$, M | $Cl^-_{cf}$, M | $Cl^-_{cells}$, Mz |
|---|---|---|---|---|---|---|
| *Halomonas campisalis* 3% NaCl (0.51 M NaCl) 0.70 M $Na^+_{tot}$ | 0.75 | 0.23 | 0.015 | 0.12 | 0.42 | 0.22 |
| *Halomonas campisalis* 10% NaCl (1.71 M NaCl) 1.90 M $Na^+_{tot}$ | 2.06 | 0.24 | 0.015 | 0.26 | 1.47 | 0.22 |
| *Halomonas* sp. AIR-2 2% NaCl (0.34 M NaCl) 0.53 M $Na^+_{tot}$ | 0.60 | 0.49 | 0.015 | 0.20 | 0.34 | 0.08 |
| *Halomonas* sp. AIR-2 8% NaCl (1.37 M NaCl) 1.56 M $Na^+_{tot}$ | 1.73 | 0.58 | 0.015 | 0.34 | 1.27 | 0.16 |
| *Natroniella acetigena* 1% NaCl (0.17 M NaCl) 1.91 M $Na^+_{tot}$ | 2.07 | 0.91 | 0.013 | 0.83 | 0.18 | 0.29 |
| *Natroniella acetigena* 5% NaCl (0.85 M NaCl) 2.59 M $Na^+_{tot}$ | 2.78 | 1.98 | 0.013 | 0.94 | 0.86 | 0.89 |

Note: Averages from no less than three experiments are presented.

* Theoretical concentrations calculated on the basis of the total content of the main three sodium salts ($Na_2CO_3$, $NaHCO_3$, and NaCl) in the medium.

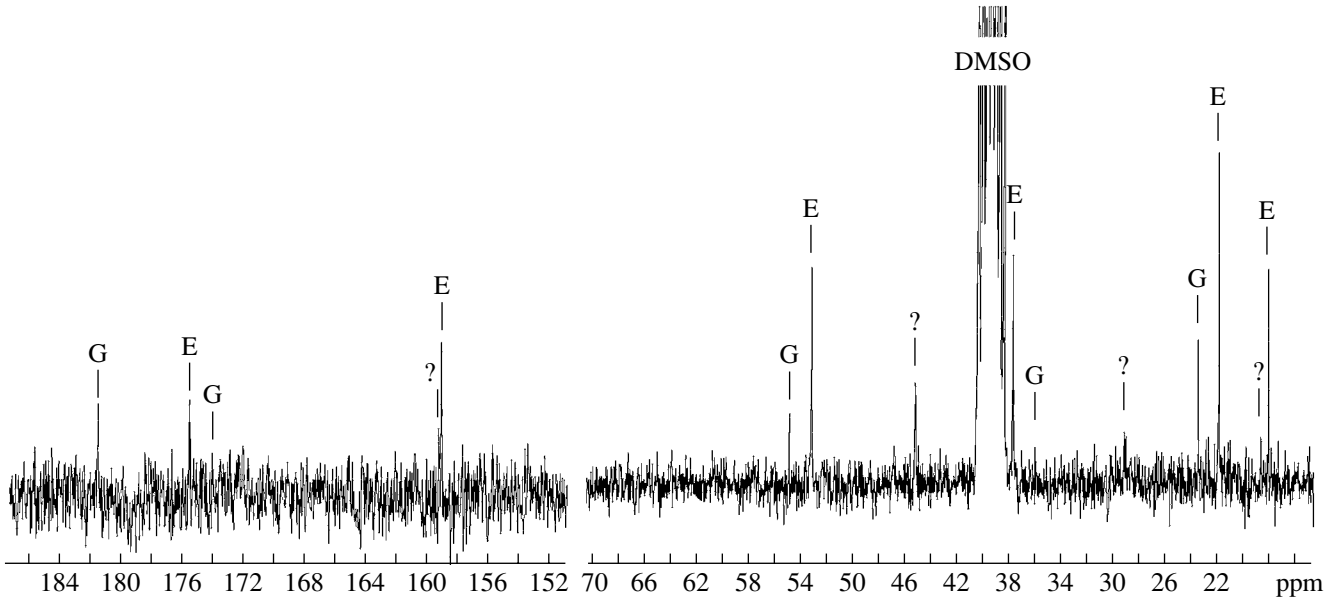
** Theoretical concentrations calculated on the basis of the total content of potassium salts ($KH_2PO_4$ and KCl) in the medium.

lation of the two ions. In strain AIR-2, the intracellular $Cl^-$ concentration increased with the NaCl content in the medium; however, it remained extremely low and did not correlate with the $Na^+$ concentration. Thus, in both cases, no accumulation of chloride ion to concentrations allowing the cell cytoplasm to be maintained in an isosmotic state with the environment was noted.

The $K^+$ ion content in the cells of both organisms considerably exceeded its concentration in the medium. The cells of *H. campisalis* contained 0.12 and 0.26 M $K^+$ at total $Na^+$ contents in the medium of 0.70 and 1.90 M $Na^+$, respectively. In strain AIR-2, the $K^+$ ion concentration in the intracellular space increased from 0.20 M at 0.53 M $Na^+$ in the medium to 0.34 M at 1.56 M $Na^+$. Thus, we can speak about the possibility of potassium accumulation by the cells of both strains. However, this accumulation showed a weak correlation with the increase in the medium salinity and was not sufficient to counterbalance the external osmotic pressure. This testifies to a subsidiary role of $K^+$ ions in the osmoadaptation of these bacteria. Apparently, potassium plays a certain role in osmoadaptation when growth occurs in media with a low mineral content. The results of our investigation are in good agreement with the data obtained earlier for this group of microorganisms. Despite the fact that the ion concentrations in the intracellular space of different halomonad species slightly differ, in all of the cases they are not high enough to counterbalance the external osmotic pressure under conditions of high salinity [6, 11].

The membranes of most of the halophiles studied exhibit high activities of $Na^+/H^+$ antiporters, which use the electrochemical proton gradient as a driving force for $Na^+$ extrusion from the cells [3]. Apart from maintaining the intracellular $Na^+$ concentration at a relatively low level, $Na^+/H^+$ antiporters play an important role in the regulation of intracellular pH and solute transport. In alkaliphilic halomonads, the relatively low intracellular concentrations of $Na^+$ ions at their high concentration in the medium are also likely to be maintained due to a $Na^+/H^+$ antiporter. The sodium gradient formed may be used as a driving force for certain endergonic processes in the cells.

In connection with the fact that the cells of both haloalkaliphilic representatives of the genus *Halomonas* did not show high concentrations of inorganic ions and all the representatives of this genus studied earlier are characterized by the use of organic osmolytes, it was important to assess the possibility of the accumulation of the latter in the microorganisms studied. For the determination of low-molecular-weight osmoprotectants, we used the $^{13}C$ NMR method, which does not allow exact quantitative determination of substances but makes it possible to judge their qualitative composition and approximate ratio. Extracts of *H. campisalis*




$^{13}$C NMR spectrum of low-molecular-weight intracellular substances of *H. campisalis* grown on a yeast extract–free medium. E, ectoine; G, glutamate.

biomass grown at 10% NaCl in the presence or absence of yeast extract were used for this experiment.

The studies showed that, in both cases, the cells contained ectoine and glutamate. The $^{13}$C NMR spectrum of the extract of cells grown on yeast extract–free medium is shown in the figure. The cells grown in the presence of yeast extract contained betaine in addition to ectoine and glutamate, which was evidenced by signals with chemical shifts at 54.5, 67.2, and 170.3 ppm. Other substances earlier found in the cells of different *Halomonas* species as minor osmoprotectants (e.g., glucose or alanine) were not revealed. The figure also shows several low peaks whose nature we failed to identify; however, taking into consideration the insignificant magnitude of these peaks, we can suggest that their presence is related to insufficient sample purification.

Ectoine is the most common osmolyte of aerobic chemotrophic bacteria [6, 12, 13]. This compound is widely spread among neutrophilic halomonads; thus, it can be considered that *H. campisalis* is not an exception. As a rule, several osmolytes, whose composition and ratio depend on both the species affiliation of the organism and the cultivation conditions, are simultaneously present in the cells of *Halomonas* representatives. However, in all cases, ectoine is the main component of the osmoprotectant "cocktail"; no other functions of ectoine are known. As for glutamate, here the situation is less amenable to interpretation. Glutamate is known to perform an osmoprotective function [6, 12, 14]; however, its role in the cell is significantly broader, as it may be both an end and an intermediate metabolic product. Since this substance is of an anionic nature, its accumulation depends on the presence of an equivalent amount of cations in the cytoplasm. In particular, it has been shown that the accumulation of glutamic acid (like that of other weak acids) is stimulated by potassium [15]; hence, joint accumulation of glutamic acid and potassium in the form of potassium glutamate appears to be probable. However, it is known that charged amino acids are never accumulated in cells at concentrations exceeding 0.4 M [6]; thus, the role of glutamate in the osmoadaptation of a microorganism living at considerably higher salt concentrations in the medium cannot be fundamental. Thus, it was shown by the example of *H. elongata* earlier that potassium glutamate plays the key role in osmoadaptation at a $Na^+$ content in the medium not exceeding 0.51 M; however, with increasing salinity, the concentration of potassium glutamate remains the same and ectoine becomes the main osmolyte [16]. In the case of *H. campisalis*, the regularity seems to be same. The detection of betaine in cells grown in the presence of yeast extract agrees with the literature data as well since yeast extract contains as constituents substances that are betaine precursors [17].

Absolutely different results were obtained when we studied the extremely halophilic acetogenic bacterium *N. acetigena.* As seen from the table, the intracellular concentrations of $Na^+$, $K^+$, and $Cl^-$ ions depended on the osmotic pressure of the medium and increased from 0.91 to 1.98 M for $Na^+$, from 0.83 to 0.94 M for $K^+$, and from 0.29 to 0.89 M for $Cl^-$ with an increase in the total $Na^+$ content in the medium from 1.91 to 2.59 M. Thus, we showed that this bacterium is capable of maintaining high intracellular $Na^+$, $K^+$, and $Cl^-$ ion concentrations that allow the internal osmotic pressure to be counterbalanced with the environmental one and cell turgor to be ensured. The intracellular $K^+$ content was

almost two orders in excess of its content in the medium. The revealed intracellular concentrations of this ion (about 0.9 M) were rather high, which is indicative of the possibility that $K^+$ is involved in the osmoadaptation of *N. acetigena.* The involvement of $Cl^-$ in the osmoregulation of *N. acetigena* agrees well with the obligate dependence of the organism on the presence of this anion in the growth medium [7]. The intracellular $Na^+$, $K^+$, and $Cl^-$ ion concentrations in *N. acetigena* were close to those characteristic of other haloanaerobes. In halobacteria and haloanaerobes, the intracellular sodium ion concentration is, as a rule, slightly lower or close to the external concentration; the intracellular concentration of potassium significantly exceeds its content in the medium; and the chloride ion concentrations in the intracellular space and in the medium are counterbalanced [3, 18]. Such high intracellular salt concentrations are sufficient to counterbalance the external osmotic pressure of the medium and imply the absence of organic osmoregulators, which is characteristic of all representatives of the order *Halanaerobiales* [3]. It is known that, in most halophilic microorganisms, the electrochemical proton gradient across the cytoplasmic membrane is the primary source of energy for $Na^+$ release and $K^+$ accumulation. This gradient is formed either at the expense of electron transport in the respiratory chain or by means of the membrane-bound ATPase that uses ATP synthesized in the process of substrate phosphorylation [19]. The proton gradient is the driving force for many processes requiring energy. It was shown earlier that, in *N. acetigena*, the proton gradient is the driving force of ATP synthesis [20]. It also seems to be used for maintaining intracellular turgor. It can be seen from the data shown in the table that the intracellular concentration of chloride ion in *N. acetigena* is insufficient for neutralizing the positive charge created by cations. At the same time, no other inorganic anions (sulfate, phosphate, or nitrate) are found in the cell composition. However, the intracellular proteins of haloanaerobes are known to be rich in acidic amino acid residues, charged negatively [21], which has been confirmed for *N. acetigena* (Detkova and Boltyanskaya, unpublished data). Therefore, it is likely that high cation concentrations, which create an excessive positive charge, are required for neutralization of the charge of the proteins in order to maintain them in a metabolically active state [15, 22]. The great excess of acidic amino acids in the composition of *N. acetigena* proteins decreases their hydrophobicity and aids in preventing the protein structural collapse or aggregation in the presence of high intracellular salt concentrations. Such changes in the amino acid composition of the total cell protein of the haloalkaliphilic bacterium *N. acetigena* agree, along with the extremely high intracellular salt concentrations revealed, with the absence of an organic osmoregulator.

Thus, the investigations performed allowed us to reveal regularities of osmoadaptation of alkaliphilic halophiles similar to those established for neutrophilic halophiles. Alkaliphilic halomonads, similar to the neutrophilic representatives of this genus, use for osmoadaptation the strategy of displacement of inorganic ions from the cytoplasm and accumulation of organic osmoregulators, the main osmoregulator being ectoine, which is characteristic of all neutrophilic representatives of the genus *Halomonas* studied earlier. *N. acetigena*, an extremely haloalkaliphilic representative of the order *Halanaerobiales*, accumulates a large amount of salts in the intracellular space, which allows it to counterbalance the environmental osmotic pressure and gives evidence of the community of the mechanisms of osmoadaptation in neutrophilic and alkaliphilic haloanaerobes.

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