Extremophiles (2002) 6:453–462
Digital Object Identifier (DOI) 10.1007/s00792-002-0277-4

ORIGINAL PAPER

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Novel insights into the role of potassium for osmoregulation in *Halomonas elongata*

Received: May 5, 2002 / Accepted: May 10, 2002 / Published online: July 13, 2002

Abstract The role of K^+ in osmoregulation of the halophilic bacterium *Halomonas elongata* was investigated. At lower salinities (0.51 M NaCl), K^+ was the predominant cytoplasmic solute (1.25 µmol mg protein^{-1}). At higher salinities (1.03 M NaCl) ectoine became the main cytoplasmic solute (1.57 µmol mg protein^{-1}), while the K^+ content remained unchanged. In response to osmotic upshock, cells of *H. elongata* simultaneously accumulated ectoine and K^+ glutamate. The ectoine and K^+ glutamate levels in osmotically stressed cells exceeded the level of cells adapted to high salinities. The increase in K^+ glutamate was long lasting (>120 min) and not transient, as described for non-halophiles. Regulation of the synthesis of ectoine and glutamate was proven to occur mainly at the level of enzyme activity. Limitation of K^+ inhibited the growth of salt-adapted *H. elongata* cells, especially at high salinities, and caused a decrease of the intracellular organic solute content, inhibition of respiration, and an abolition of the cell's ability to respond to osmotic stress. The saturation constant K_S for K^+ was estimated to be 105 µM at a salinity of 0.51 M NaCl, indicating that an uptake system of medium affinity is responsible for K^+ accumulation in *H. elongata*.

Key words Compatible solute · Ectoine synthesis · *Halomonas elongata* · Osmolyte · Osmoregulation · Potassium · Respiration

Communicated by W. Grant

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Introduction

Halophiles have developed two different basic strategies of osmoregulatory solute accumulation to cope with water stress caused by high salt concentrations in their environment: the "salt-in-cytoplasm mechanism" and the "organic-osmolyte mechanism." The "salt-in-cytoplasm mechanism," first discovered in halobacteria, is considered the typical archaeal strategy of osmoadaptation. Halobacteria and related microorganisms accumulate K^+ in molar concentrations to cope with high external osmolality (Larsen 1973; Eisenberg and Wachtel 1987). However, some anaerobic halophilic bacteria are known to employ this strategy as well and accumulate either K^+ or Na^+ depending on the growth phase (Rengpipat et al. 1988; Oren et al. 1997). The recently discovered genus *Salinibacter*, as a member of the *Cytophaga–Flavobacterium–Bacteroides* phylum also accumulates K^+ within the cytoplasm and displays a salt tolerance comparable to that of extremely halophilic Archaea (Antón et al. 2000; Oren et al. 2001). In contrast to these organisms, methanogenic Archaea (i.e., *Methanohalophilus*) as well as phototrophic and aerobic chemoheterotrophic bacteria employ the organic osmolyte mechanism and accumulate polar or zwitterionic, highly water-soluble, organic molecules called compatible solutes (Galinski et al. 1985; Lai et al. 1991; Severin et al. 1992). Despite the abundance of inorganic ions such as Na^+ and K^+, respectively, in high-salt growth media, these ions are largely excluded from the cytoplasm of microorganisms of the osmolyte strategy (Matheson et al. 1976; Sadler et al. 1980; Ventosa et al. 1998). For a variety of non-halotolerant and halotolerant bacteria it was shown that this state is altered only after a sudden increase in salinity, resulting in a transient accumulation of potassium. This inorganic cation accumulates on a short-term basis after a sudden increase in salinity. It transiently acts as an osmolyte to restore cell volume and turgor pressure until it is replaced by compatible solutes (Reed et al. 1985; Dinnbier et al. 1988; Whatmore et al. 1990; Welsh and Herbert 1993). Additionally, K^+ is thought to serve as a cellular messenger in the regulation of enzyme activity and gene expression (Sutherland et al. 1986;

Higgins et al. 1987; Booth and Higgins 1990). It is also known that the accumulation of K^+ supports the respiration of cells exposed to an osmotic upshock (Meury 1994). K^+ influences the generation of a proton gradient [ΔpH; pH difference across the cytoplasmic membrane ($pH_{in} - pH_{out}$)] as a component of the proton electrochemical gradient across cytoplasmic membranes (Padan et al. 1976; Tokuda et al. 1981). K^+ is also required for the generation of a Na^+ electrochemical gradient in the uptake of organic acids (Tokuda et al. 1982).

Since the role of potassium in the osmoregulation of halophilic bacteria is still unknown and, in order to prove whether *Halomonas elongata* is an exception in respect to its cytoplasmic K^+ concentration, we analyzed the accumulation of inorganic and organic solutes of *Halomonas elongata* DSM 2581^T in response to hyperosmotic shock. *H. elongata* belongs to the γ-subdivision of the proteobacteria, displays wide salt tolerance (0.009–4.3 M NaCl) and synthesizes ectoine as its main compatible solute (Severin et al. 1992).

Materials and methods

Bacterial strain and growth conditions

Type strain *Halomonas elongata* DSM 2581^T (Vreeland et al. 1980) was used for all experiments. Cells were grown in Na-MM63 medium modified from MM63 medium (Larsen et al. 1987) to diminish the initial K^+ concentration. Na-MM63 medium contained 70.4 mM Na_2HPO_4, 1 mM $MgSO_4 \cdot 7H_2O$, 15 mM $(NH_4)_2SO_4$, 4 µM $FeSO_4 \cdot 7H_2O$, 5 mM KCl and 25 mM glucose. The pH was adjusted to pH 7.0 with 1 M HCl. NaCl concentrations varied from 0.09 to 2.05 M NaCl.

For K^+ depletion experiments, the medium was prepared using highly pure chemicals (Merck Suprapur: Merck, Darmstadt, Germany) and double deionized water (conductivity 0.06 µS cm^{-1}). The pH of this medium was determined by the use of indicator strips to avoid potassium contamination from the pH electrode. Prior to use, glassware was rinsed with double deionized water. Although highly pure chemicals were used to prepare K^+ limitation media, media were not totally free of K^+ as determined by atomic absorption spectrometry (AAS) measurements. NaCl was the main contributor to the K^+ contamination of the medium. Therefore, the minimal concentration of K^+ in the medium was adjusted by the amount of NaCl added. At concentrations of 0.51, 1.03, and 2.05 M NaCl, the lowest obtainable K^+ concentrations were 11, 15, and 22 µM, respectively. To adjust potassium to higher concentrations, K^+ from 0.1 or 1 M KCl stock solutions was added to the medium.

Added to the medium were: chloramphenicol (Cm) (to test whether ectoine synthesis is regulated at the level of enzyme activity or at the level of gene expression), from a 100 mg ml^{-1} stock solution to a final concentration of 100 µg ml^{-1}; betaine, from a 1 M stock solution to a final concentration of 5 mM; NaCl from a 5.1 M stock solution or in crystalline form; and KCN, from a 100 mM stock solution to a final concentration of 1 mM.

Cells were grown aerobically at 30°C in 100 ml liquid medium contained in 250-ml flasks. Precultures were grown overnight in Na-MM63 medium containing the same salt concentration as the main culture.

Growth experiments and K^+ depletion

K^+-depleted cultures (50 ml) were inoculated with 4% from the preculture. Prior to inoculation, precultures were harvested by centrifugation and washed once in the corresponding medium to remove traces of potassium. To determine the organic solute content accumulated by de novo synthesis, cells were harvested by centrifugation, freeze-dried, weighed and extracted according to the chromatographic method of Galinski and Herzog (1990). K^+-depleted cultures grown in the presence of external betaine were washed once in medium of the same osmolarity without betaine before harvesting.

Upshock experiments

For salt shock experiments the optical density at 600 nm (OD_{600}) of cultures was initially adjusted to an OD_{600} of 0.5 by adding preculture to fresh medium. Upshocks were carried out on exponentially growing cells at an OD_{600} of 1. The salinity of the medium was elevated from 0.51 up to 1.03 M NaCl by the addition of salt crystals. Cm was added 5 min prior to upshock in case protein synthesis had to be inhibited. Control cultures were grown under identical conditions without salt addition. During a time course of 120 min, 1 ml samples were taken for the measurement of the cellular solute and protein content.

Determination of optical density and protein content

OD_{600} was determined at 600 nm against air. During growth experiments OD_{600} was measured in side arms of the incubation-flasks. During salt shock and respiration experiments, OD_{600} was measured in glass microcuvettes at a 1:10 dilution.

Cell protein was determined by the method of Smith et al. (1985) using the 4,4′-Bichinolin-2,2′-dicarbonic acid (BCA) protein assay kit (Pierce). Cell suspensions were centrifuged at 15,800 *g* for 4 min and the supernatant was removed. Cell pellets were dried overnight at 100°C and resuspended in 1 ml of demineralized water by shaking at room temperature for 30 min. After addition of BCA reagent, absorption of samples was measured at 550 nm in an automated plate-reader and compared to protein standards containing bovine serum albumin in a concentration range of 0–500 µg ml^{-1}.

Determination of K^+

To determine the cellular K^+ content, 1 ml of the culture was centrifuged through 250 µl silicone oil (ρ = 1.07 g ml^{-1}, DC

550 fluid; Serva, Heidelberg, Germany). Cell pellets were cut out with a razorblade, transferred into 1 ml of trichloroacetate (5%, w/v) in Sarstedt tubes, vortexed, and frozen immediately. After thawing, suspensions were incubated for 10 min at 90°C. Three ml of CsCl (59.4 mM) was added and suspensions were centrifuged for 10 min at 2,710 *g* (Michels and Bakker 1987). The supernatants were transferred into clean vials and, if necessary, cell extracts and growth media were diluted in 59.4 mM CsCl solution. Measurements were carried out on a Shimadzu AA-660 atomic absorption spectrophotometer. KCl-standards contained 0–100 µM K^+.

Chromatographic methods

To determine the cytoplasmic organic solute content, cells from 1 ml culture were centrifuged at 15,800 *g* for 4 min and the supernatants were removed with a vacuum pump. Then 250 µl extraction solution (methanol:chloroform:water 10:5:4 v/v/v) were added and the microfuge tubes were incubated on a shaker (IKA Vibrax) for at least 10 min. Next 65 µl of each of chloroform and water were added and phase separation was enhanced by centrifugation at 8,000 *g* for 5 min (Bligh and Dyer 1959). Amino-reactive solutes dissolved in the aqueous phase were analyzed by gradient high performance liquid chromatography (HPLC) applying 9-fluorenylmethyl chloroformate (FMOC) pre-column derivatization, according to Kunte et al. (1992). Non-amino acid-type solutes were analyzed by isocratic HPLC on a NH_2-column (GromSil 100 Amino-1 PR, 3 µm; Grom Analytik, Herrenberg, Germany) using an UV-detector (220 nm) for ectoine detection and a refractive index detector for betaine detection, respectively (Galinski and Herzog 1990).

Respiratory rates

Respiratory rates were determined by closed-cell respirometry using Clark-electrodes (6-channel dissolved oxygen system 928; Strathkelvin Instruments, Glasgow, UK). Samples of growing cell suspensions were pipeted into the measuring cell, yielding a final volume of 1 ml and an OD_{600} of 0.5. Measurements were performed under constant stirring at 30°C. At that temperature the solubility of oxygen was taken to be 234 µmol l^{-1} in a 0.51 M NaCl solution. Respiration rates were expressed as micromole of O_2 per milligram of protein per minute. For osmotic upshocks, NaCl was added from a 5.1 M stock solution.

Results

Solute content of salt-adapted cells

The optimal salt concentration for growth of *H. elongata* in Na-MM63 medium was determined to be 0.51 M NaCl. Therefore, the cytoplasmic solute content of cells growing at 0.51 M NaCl was determined and compared to the internal solute level of cells from medium containing 1.03 M NaCl. The analysis was carried out using exponentially growing cells and comprised potassium, the organic compounds ectoine, glutamate, glutamine, and the ectoine precursor Nγ-acetyl L-2,4-diaminobutyric acid (N-Ac-DABA). At the low salt concentration of 0.51 M NaCl, potassium was the main solute. It reached a value of 1.25 µmol mg $protein^{-1}$ (Fig. 1), which is similar to the level found in enteric bacteria without osmotic stress and other halophilic bacteria growing at comparable salt concentrations (Dinnbier et al. 1988; Matheson et al. 1976). In osmotically adapted cells grown at different salinities, the amount of K^+ as well as the content of glutamate (0.52 µmol mg $protein^{-1}$) remained unchanged. In contrast with K^+ glutamate, the content of the compatible solute ectoine (0.77 µmol mg $protein^{-1}$) and its precursor N-Ac-DABA (0.04 µmol mg $protein^{-1}$) was doubled in cells growing at a salinity of 1.03 M NaCl. The total cytoplasmic solute content increased from 2.6 µmol mg $protein^{-1}$ in cells grown at 0.51 M NaCl up to 3.4 µmol mg $protein^{-1}$ in cells exposed to a salt concentration of 1.03 M NaCl, mainly due to the increase of ectoine, which became the main cytoplasmic solute (Fig. 1).

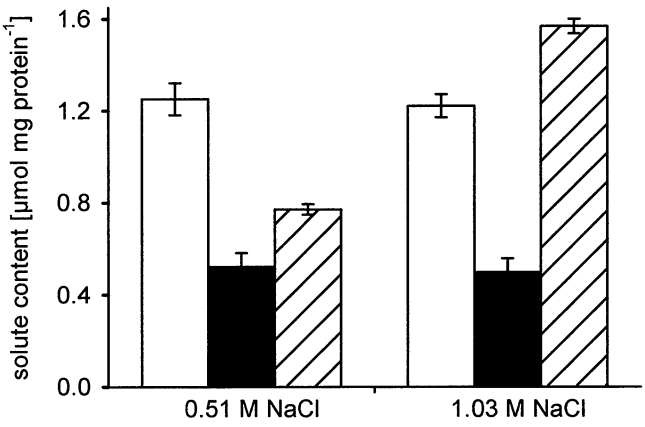

Fig. 1. Solute content of exponentially growing cells of *H. elongata* adapted to 0.51 and 1.03 M NaCl. Cells were grown in Na-MM63 medium containing 5 mM K^+ and harvested in the exponential growth phase. The solute content was analyzed by AAS (K^+), isocratic HPLC (ectoine), and gradient HPLC (amino acids). Data reflect means of at least six replicates of two independent experiments. K^+ (*open columns*), glutamate (*filled columns*), ectoine (*hatched columns*). Solutes below a level of 0.1 µmol mg $protein^{-1}$ (N-Ac-DABA and glutamine) are not shown

Osmolyte content of osmotically shocked cells exceeds the solute level of adapted cells

Exponentially growing cells of *H. elongata* were exposed to hyperosmotic shock from 0.51 M NaCl to a salt concentration of 1.03 M in medium containing 5 mM K^+. *H. elongata* responded to osmotic shock by transport of K^+ from the medium and the immediate synthesis of ectoine, which accumulated simultaneously with K^+ in the cytoplasm (Fig. 2). In contrast to enteric bacteria and other non-

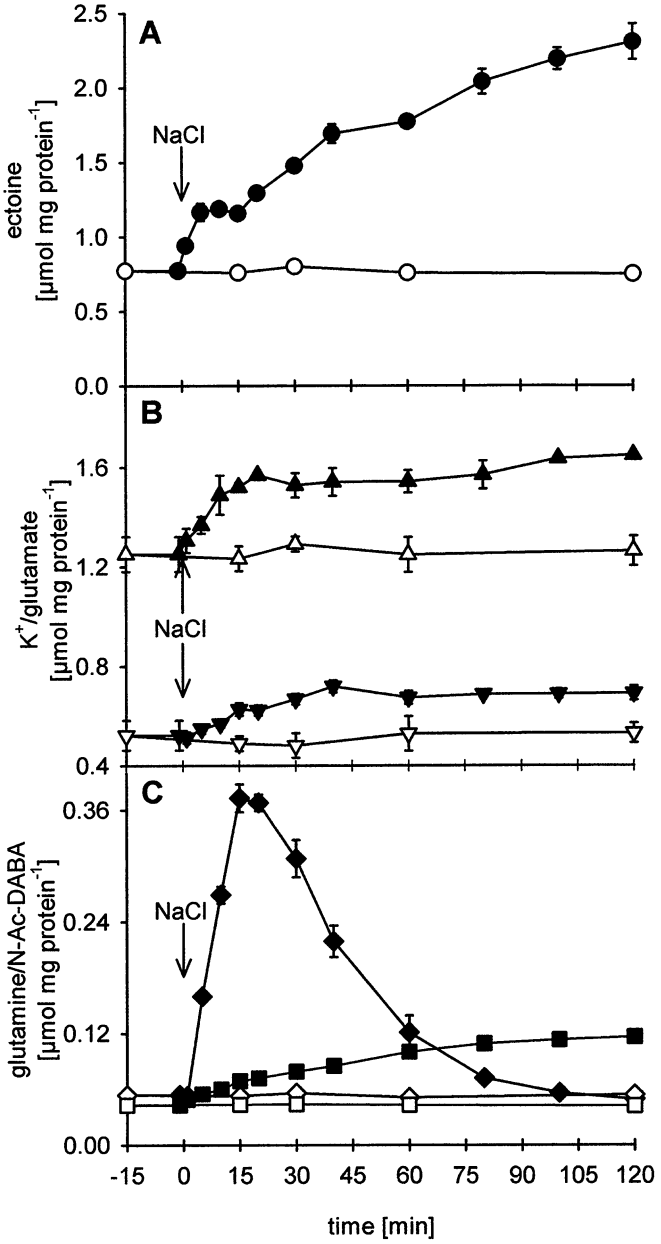

Fig. 2A–C. Effect of osmotic upshock on the cytoplasmic solute content. Cells were grown in Na-MM63 medium at 0.51 M NaCl containing 5 mM K^+. At zero time, exponentially growing cells were exposed to osmotic upshock by increasing the salinity to 1.03 M NaCl. Solutes were determined by AAS (K^+) and HPLC (organic solutes). Presented values are the means of at least three independent experiments. **A** Ectoine (*circles*), **B** K^+ (*triangles*), and glutamate (*inverted triangles*), **C** glutamine (*diamonds*), and N-Ac-DABA (*squares*). Upshocked cells (*closed symbols*), control cells without salt shock (*open symbols*). *Arrows* indicate the addition of NaCl

halophiles, where the accumulated K^+ will be replaced by organic solutes, the increased K^+ level did not decline in *H. elongata*, but remained elevated for at least 120 min after upshock. While the K^+ level remained elevated, ectoine exceeded the K^+ content after 30 min following the increase in salinity. Already 5 min after the osmotic upshock the ectoine content had already increased by a factor of 1.5. Within 120 min the ectoine content tripled to a value of 2.31 µmol mg protein^{-1} and became the main solute (Fig. 2A). Over the same time course the content of N-Ac-DABA, the direct synthesis precursor of ectoine, multiplied by 2.7 and reached a final value of 0.12 µmol mg protein^{-1} (Fig. 2C). After 5 min the content of both K^+ and glutamate had increased by a factor of 1.1 and after 120 min by a factor of 1.3 (Fig. 2B). The K^+ content was always 2.4 times greater than the amount of glutamate. The K^+ glutamate content increased mainly in the first 15 min after the hyperosmotic shift. Glutamine was the only solute whose content increased transiently over the period of 120 min after osmotic upshock (Fig. 2B); 15 min after osmotic upshock, the glutamine content reached a maximum of 0.37 µmol mg protein^{-1} and decreased to its initial value 85 min later.

At 5–10 min after osmotic upshock from 0.51 to 1.03 M NaCl, the total solute content equaled that of osmotically adapted cells growing at a salinity of 1.03 M NaCl. After 120 min the total solute content reached a value of 4.8 µmol mg protein^{-1} and exceeded the solute content of cells adapted to a NaCl concentration of 1.03 M by a factor of 1.4 (Fig. 2). The long-lasting increase in potassium (at least for 120 min) and the simultaneous increase and overshoot of the cytoplasmic ectoine content is a new finding in bacteria responding to osmotic stress. The overproportional increase in cytoplasmic osmolytes will result in water influx, and, as a consequence, the cell volume has to increase after osmotic upshock, a fact which is confirmed by the measurements of Miguelez and Gilmour (1994). The reason for the different response of *H. elongata* compared with non-halophiles has still to be resolved (see Discussion).

Ectoine synthesis is regulated at the level of enzyme activity

By measuring OD_{600} (data not shown) and the protein content, growth of cultures subjected to osmotic upshock in the presence of Cm and without Cm treatment was analyzed (Fig. 3). Addition of Cm to the medium stopped growth of *H. elongata* for the entire time-period of the experiment. In contrast, cells without Cm treatment were able to proliferate most of the time during the experiment and growth was stopped only briefly for 15 min after salt shock.

Although Cm turned off protein biosynthesis, *H. elongata* was able to respond to the osmotic challenge. In the presence of Cm, cells synthesized and accumulated ectoine like cells with active biosynthesis. The time course of ectoine accumulation was identical to that of untreated cells (Fig. 4) and only the final ectoine value at 120 min was lower (about 15%). The precursor of ectoine, N-Ac-DABA, as well as potassium, glutamate, and glutamine also accumulated to slightly higher levels, as known from cells without Cm treatment. These results indicate that the synthesis of the compatible solute ectoine and the other organic osmolytes is mainly regulated at the level of enzyme activity, at least under moderate upshock conditions. The response to osmotic upshock at lower salt concentrations or to a more dramatic increase in salinity is yet to be determined.

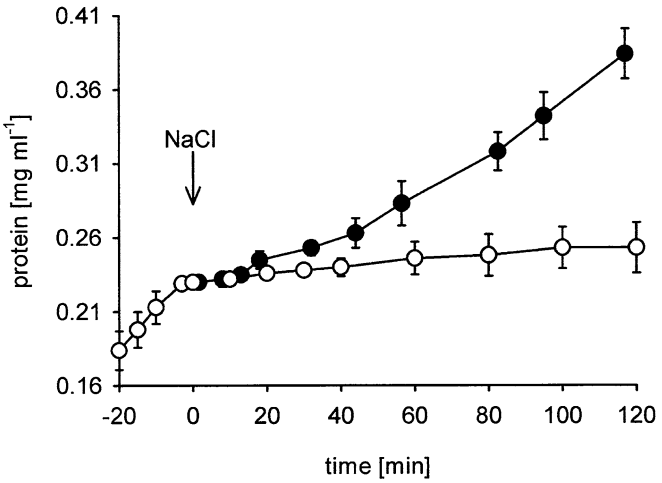

Fig. 3. Protein content of cell suspensions subjected to hyperosmotic shock from 0.51 M up to 1.03 M NaCl. The protein content was measured using the BCA protein assay kit. Chloramphenicol (Cm) was added to exponentially growing cells at $t = -5$ min, prior to NaCl at $t = 0$ (*arrow*). Values reflect means of three independent experiments. Cultures treated with Cm (*open circles*), cultures without Cm (*filled circles*)

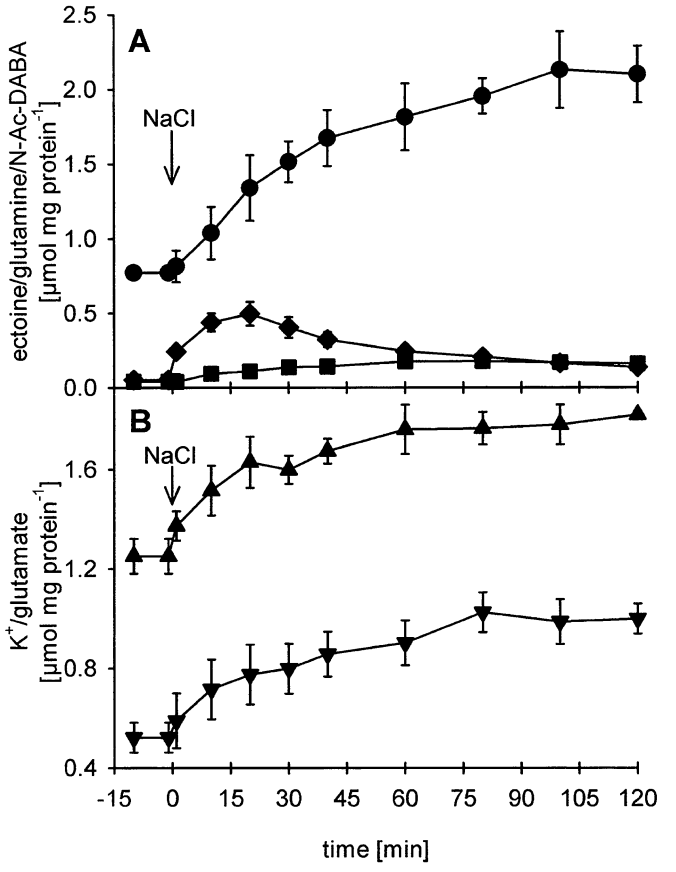

Fig. 4A, B. Solute content of exponentially growing cells subjected to osmotic upshock after addition of Cm at $t = -5$ min. At time zero salinity was suddenly elevated from 0.51 M up to 1.03 M NaCl. The cytoplasmic osmolyte level was quantified by AAS (K^+) and HPLC (organic solutes) analysis. Data reflect means of three independent experiments. **A** Ectoine (*circles*), glutamine (*diamonds*), N-Ac-DABA (*squares*), **B** K^+ (*triangles*), and glutamate (*inverted triangles*). *Arrows* indicate salt addition

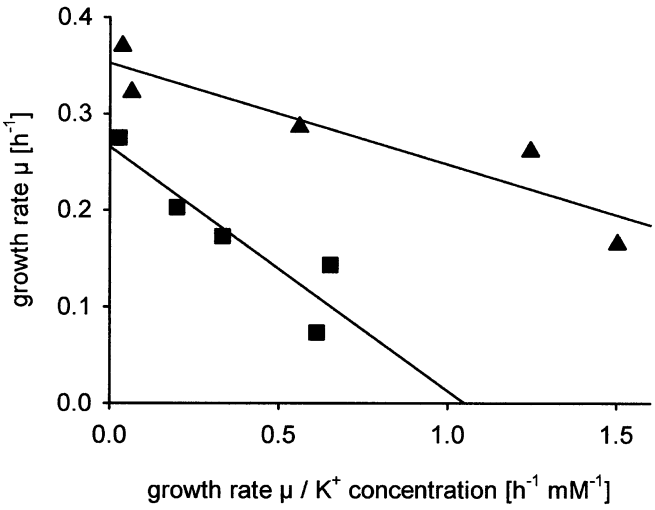

Fig. 5. Determination of the half saturation constant (K_s value) of K^+ at salinities of 0.51 and 2.05 M NaCl (Eadie-Hofstee plot). The affinity of whole cells to K^+ was determined by measurement of the growth rate of *H. elongata* in medium containing different K^+ concentrations. The slopes of the lines correlate with the K_s values. The equations were $y = 0.3527–0.1051x$ (0.51 M NaCl, *triangles*) and $y = 0.2658–0.253x$ (2.05 M NaCl, *squares*). Correlation coefficients were $R_{0.51M} = 0.922$, $R_{2.1M} = 0.914$). Means reflect data of two independent experiments

K^+ depletion inhibits the growth of *H. elongata*

To prove whether potassium is required for osmoregulation in halophiles, the response to osmotic stress of *H. elongata* was analyzed under potassium limitation in mineral salt medium containing 0.09 to 2.05 M NaCl.

The reduction of external K^+ from 10 mM down to the lowest obtainable concentration caused growth inhibition at all salt concentrations analyzed. The inhibitory effect increased with rising salinity. At a salt concentration of 0.09 M NaCl, potassium-depletion caused a reduction of the growth rate by 38%, while at a salinity of 2.05 M the growth rate was lowered by as much as 93%. Since the growth rate of *H. elongata* was limited by low potassium concentrations, the affinity of the cells (K_s) for potassium could be determined in low-potassium medium (Fig. 5). The plot of growth rate against growth rate/K^+ concentration (Eadie-Hofstee plot) showed that the cells had saturation constants of 105 µM at 0.51 M NaCl and 253 µM at 2.05 M NaCl, respectively.

K^+ depletion affects the intracellular solute content accumulated by synthesis and by transport in cells of *H. elongata*

The effect of K^+ reduction on both the intracellular solute content and the ability of *H. elongata* to cope with osmotic stress was investigated. The cytoplasmic solute content accumulated by synthesis of cells grown at a K^+ concentration of 11 µM (data not shown) and 110 µM K^+ (Fig. 6), respectively, was measured before and after cells were

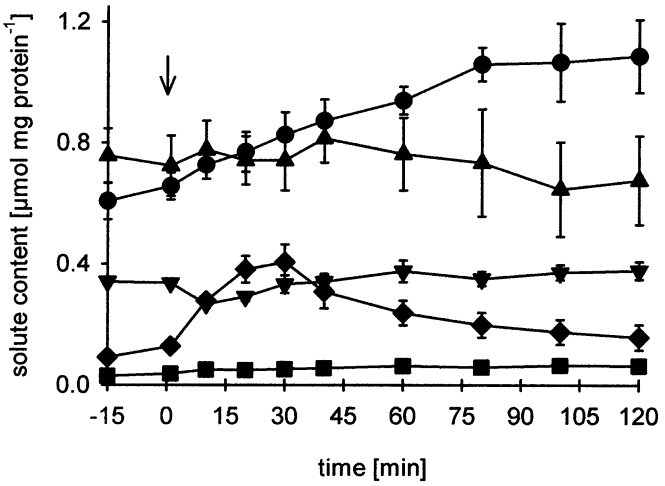

Fig. 6. Solute content of growing cells subjected to osmotic upshock under K^+ depletion (110 µM). At $t = 0$ salinity was raised from 0.51 up to 1.03 M NaCl. Changes in the intracellular solute content were investigated by AAS (K^+) and HPLC analysis (organic solutes). Means reflect data of three independent experiments. Ectoine (*circles*), K^+ (*triangles*), glutamate (*inverted triangles*), glutamine (*diamonds*), and N-Ac-DABA (*squares*). *Arrow* indicates the addition of NaCl

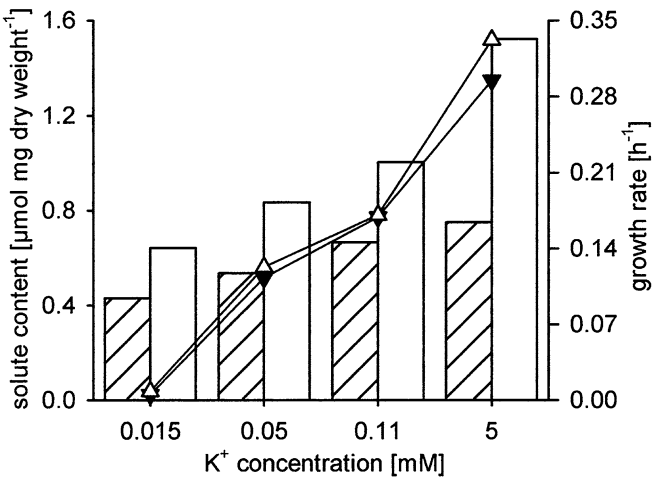

Fig. 7. Growth and solute content of cells grown at different K^+ concentrations in presence or absence of betaine. Cells were grown at a salinity of 1.03 M NaCl. Means reflect data of two parallel experiments. Columns represent the cytoplasmic content of ectoine (*hatched column*) and betaine (*open column*). Symbols show the growth rates of cells grown in absence (*filled diamonds*) or presence (*open diamonds*) of betaine

exposed to a sudden increase in salinity from 0.51 to 1.03 M NaCl. Prior to osmotic upshock, the total cytoplasmic solute content of cells under K^+ limitation was already reduced. In comparison to cells grown at 5 mM K^+, where the solute content was estimated to be 2.6 µmol mg protein^{-1}, cells in medium containing a low K^+ concentration of 11 µM accumulated only 42% of the solutes (1.09 µmol mg protein^{-1}). At a potassium concentration of 110 µM, the reduction of the cytoplasmic solute content was less severe, but still significant (1.83 µmol mg protein^{-1}). Out of all cytoplasmic solutes, K^+ glutamate was reduced the most by the potassium limitation, reaching only 28% at 11 µM K^+ and 44% at 110 µM K^+, respectively, compared to the cytoplasmic K^+ glutamate content measured in cells grown at 5 mM potassium. When exposed to osmotic upshock, K^+ depletion severely affected the ability of *H. elongata* to accumulate solutes. After increasing the salinity of the medium with limited potassium content, cells neither took up additional K^+ nor synthesized more glutamate. At 120 min after upshock, the total cytoplasmic solute content was only 1.18 µmol mg protein^{-1} in cells grown at a K^+ concentration of 11 µM and 2.36 µmol mg protein^{-1} at an external concentration of 110 µM K^+, respectively. Hence, in cells grown in low potassium medium, the cytoplasmic solute content was reduced down to 25% compared with cells grown at 5 mM K^+.

When glycine betaine was supplied with the medium at a concentration of 5 mM, it was transported from the medium and totally replaced the intracellular ectoine. Although betaine accumulated in the cytoplasm at all K^+ concentrations tested, it could not restore growth of *H. elongata* under K^+ limitation. Only the addition of potassium to the medium allowed the cells to resume growth (Fig. 7). These results indicate that K^+ does not solely act as an osmolyte, but takes over additional functions in *H. elongata* to allow growth in a saline environment. It also shows that *H. elongata* has a higher requirement for K^+ than non-halophiles, which can be explained by lacking a high affinity transporter for K^+ (see Discussion).

Despite the reduced K^+ concentration, *H. elongata* was able to accumulate either ectoine by de novo synthesis or betaine by transport. However, at lower potassium levels the content of ectoine and betaine, respectively, decreased (Fig. 7). Interestingly, the betaine content always exceeded the ectoine level regardless of the potassium concentration cells were grown at. At a concentration of 5 mM potassium, the betaine content accumulated by transport in cells growing in betaine medium was twice as high as the ectoine content accumulated by synthesis in cells grown in the absence of betaine. However, under limiting conditions (15 µM K^+) this difference was reduced to a factor of 1.5. Apparently, potassium influences the accumulation of compatible solutes in *H. elongata*, which is an energy-dependent process (Oren 1999). Since potassium is known to support respiration in *E. coli* cells exposed to osmotic stress (Meury 1994), the influence of potassium limitation on respiration of *H. elongata* was further analyzed.

Effects of osmotic upshock and K^+ depletion on the respiration of *H. elongata*

To analyze the impact of potassium limitation and osmotic stress on respiration, cells of *H. elongata* were grown in mineral salt medium containing 11 µM, 110 µM, or 5 mM potassium and exposed to osmotic upshifts from 0.51 to 1.03 M NaCl. Changes in respiration rate were determined by analysis of the oxygen consumption of cell suspensions in a closed measuring cell using a Clark electrode.

As a control, cells grown in high- and low-potassium medium or exposed to osmotic upshocks were all treated

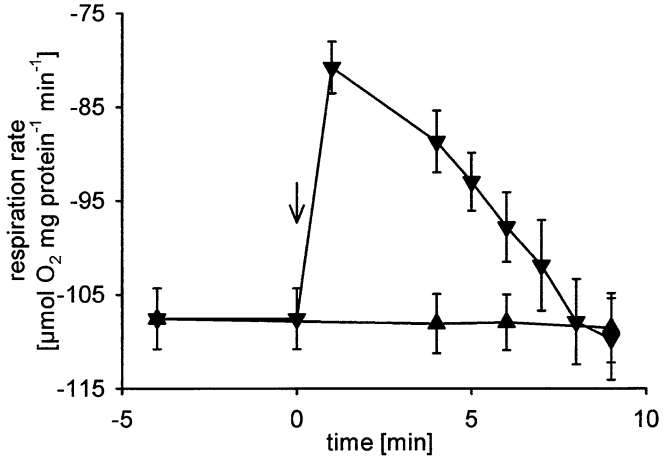

Fig. 8. Respiration rate of exponentially growing cells (5 mM K^+) subjected to hyperosmotic shock. Cells were grown in Na-MM63 medium containing 5 mM potassium. At time zero salinity was raised from 0.51 up to 1.03 M NaCl (*arrow*). The respiration rate was measured using Clark electrodes. Means reflect data of six replicates out of two independent experiments. Cells subjected to upshock (*inverted triangles*), non-treated cells (*triangles*)

with the respiration inhibitor cyanide. Adding cyanide to the cells reduced the respiration rate down to 10 µmol O_2 mg protein^{-1} min^{-1}. At the non-limiting K^+ concentration of 5 mM, the oxygen consumption was estimated to be 108 µmol O_2 mg protein^{-1} min^{-1} (Fig. 8). In contrast, cells grown in low-potassium medium showed a reduced respiration rate. In medium containing 110 µM K^+, the respiration rate decreased to 74 µmol O_2 mg protein^{-1} min^{-1} and was even further diminished to 41 µmol O_2 mg protein^{-1} min^{-1} at a potassium concentration of only 11 µM (data not shown). Only adding potassium to the medium could reverse the drop in respiration and help to enhance the respiration rate immediately. In less than 2 min the oxygen consumption increased from 41 µmol O_2 mg protein^{-1} min^{-1} to over 80 µmol O_2 mg protein^{-1} min^{-1}. In contrast, glycine-betaine added to the medium at a concentration of 5 mM did not enable the cells to restore respiration in low-potassium medium, although the compatible solute was accumulated in the cytoplasm.

In cells grown in high-potassium medium (5 mM) a sudden increase in salinity from 0.51 M up to 1.03 M NaCl temporarily reduced the oxygen consumption to 81 µmol O_2 mg protein^{-1} min^{-1}. Within 8 min after the salt shock, cells recovered and reached the previous respiration rate of 108 µmol O_2 mg protein^{-1} min^{-1} (Fig. 8). When cells of *H. elongata* grown in low-potassium medium (11 µM K^+) were exposed to an osmotic upshift, the already low respiration rate was further reduced. However, in contrast to cells exposed to the same osmotic stress in high-potassium medium, the respiration rate of cells in low-potassium medium was not only reduced temporarily. Instead, the respiration rate steadily declined after the salt shock and reached a final value of 21 µmol O_2 mg protein^{-1} min^{-1} after 32 min. This clearly demonstrates that potassium is required for osmoregulation, enabling cells to restore respiration while coping with osmotic stress.

Discussion

Solute content of salt-adapted cells

At a salinity of 0.51 and 1.03 M NaCl, the K^+ content in exponentially growing cells of *H. elongata* was determined to be 1.25 and 1.2 µmol mg protein^{-1}, respectively. Vreeland et al. (1983) have already investigated the cytoplasmic K^+ concentration of *H. elongata*. These authors determined the very low concentration of 15.5 mM K^+ and a potassium content of 4.34 nmol mg dry weight^{-1} in exponentially growing cells harvested from mineral salt medium containing 1.4 M NaCl. Based on the results of Vreeland et al. (1983) and assuming that cell protein contributes about 40% to the dry weight of *H. elongata*, we calculated the potassium content to be 10.85 nmol mg protein^{-1}. This value is more than 100-fold lower than the data presented in this study, which were obtained from cells grown at a similar salinity of 1.03 M NaCl. However, the values presented by Vreeland and coworkers (1983) also do not match the data published by other groups working with halophilic strains of the genus *Halomonas*. Matheson et al. (1976) measured the potassium content of exponentially growing cells of *H. canadensis* at a salinity of 0.63 M NaCl in complex medium and found it to be 1.64 µmol K^+ mg protein^{-1}, which corresponds quite well with the data presented in this study.

We used the cellular volumes of *H. elongata* calculated by Miguelez and Gilmour (1994) to estimate the cellular K^+ concentration based on our data. However, for an accurate calculation, two facts are to be considered. The cellular volumes of *H. elongata* published by Miguelez and Gilmour (1994) were determined at the end of the logarithmic growth phase, while the data presented in this study were obtained from exponentially growing cells. Since cells of *H. elongata* decrease in volume by a factor of 1.93 when entering the stationary phase (Matheson et al. 1976), a calculation based on the data of Miguelez and Gilmour (1994) would result in an overestimation of the actual cellular solute concentration. However, these volumes were estimated by the distribution of ^{14}C-dextran, measuring the total cellular water space instead of the cytoplasmic volume alone. This would lead to an underestimation of the cellular concentration by the factor of two and compensate for the decrease in the cellular size mentioned above. We therefore took the data of Miguelez and Gilmour (1994) as a rough estimation for cytoplasmic volumes of exponentially growing *H. elongata* cells. The volumes of cells grown in mineral medium at 0.51 and 1.03 M NaCl according to these authors were 2.3 and 2.1 µl mg protein^{-1}, respectively. From this we calculated potassium concentrations of 543 mM K^+ for *H. elongata* at a salt concentration of 0.51 M NaCl and 571 mM K^+ at 1.03 M NaCl. Our values are in the same order of magnitude as those reported for *H. halodenitrificans*, which accumulates potassium to a concentration of 298 mM when

grown exponentially in complex medium at a salinity of 0.63 M NaCl (Sadler et al. 1980). We therefore conclude that *H. elongata* is not an exception regarding its intracellular potassium concentration as believed, but accumulates this cation to similar levels as found in other halophilic bacteria (Ventosa et al. 1998).

Osmoregulatory response of *H. elongata* to increases in salt concentration

Following hyperosmotic shock, the accumulation of inorganic and organic solutes is initiated immediately in *H. elongata.* Uptake of K^+ and the synthesis of the predominant compatible solute ectoine occur at the same time. In contrast to the enteric bacteria, potassium will not be replaced by the compatible solute synthesized by the cell, but remains at an elevated level for at least 120 min. The intracellular solute accumulation could not be monitored for longer than 120 min because *H. elongata* stopped exponential growth when grown in 250-ml incubation flasks. The addition of fresh medium to the cultures interrupted growth and resulted in a continual decline of the OD. However, cells of *H. elongata* can be kept in the exponential growth phase for a longer period of time when grown in a fed-batch fermentation process, as shown by Sauer (1995). Sauer proved that the cytoplasmic level of the proposed counterion glutamate remained elevated for more than 6 h after the cells of *H. elongata* were exposed to osmotic upshock. Similar to *H. elongata, B. subtilis* also accumulates K^+ in response to osmotic shock and keeps the K^+ content elevated for about 12 h. However, the prolonged increase of the K^+ concentration in *B. subtilis* is explained by the rather slow synthesis of the compatible solute proline, which reaches its maximum level only after 7 h (Whatmore et al. 1990). In contrast, ectoine has already been synthesized to its maximum level in *H. elongata* after 120 min. In halophilic bacteria such as *H. elongata*, the internal compatible solute rapidly exceeds the cellular K^+ content after osmotic upshock, whereas in non-halophilic bacteria the internal solutes trehalose or proline do not accumulate to levels above 500 mM.

Corresponding to the changes in the cellular solute content, the changes in cell volume of *H. elongata* follow a similar pattern. As measured by Miguelez and Gilmour (1994), the cell volume of *H. elongata* decreased from 2.47 to 1.82 µl mg protein^{-1} within 30 min after osmotic upshock from 0.34 up to 1.37 M NaCl. Four hours after upshock the cells established a cell volume similar to cells already adapted to the high salt concentration of 1.37 M NaCl. However, the osmoregulatory volume changes are not finished after this time. Instead, the volume even exceeded the volume of adapted cells and stayed enlarged for the next 20 h. Based on these observations and our data concerning the solute accumulation, we suspect that the osmoregulatory processes in *H. elongata* will take several hours to be completed.

Furthermore, in *E. coli* and *B. subtilis* the synthesis of the compatible solutes trehalose and proline, respectively, is strongly regulated at the level of gene expression. Cm treatment of *E. coli* prior to osmotic upshock prevented the synthesis of trehalose and the secondary release of K^+ (Dinnbier et al. 1988). The synthesis of the compatible solute ectoine in *H. elongata*, however, seems to be regulated mainly at the level of enzyme activity (at least at salinities of 0.5–1 M NaCl) allowing a fast response to changes in the external osmolarity. We propose that this osmoregulatory mechanism contributes to the excellent capability of this organism to adapt to fluctuations in the external salinity. Looking at the natural habitat of *H. elongata*, which includes saltern ponds, the horizontal stratification of the water column provides adjacent layers of different salinities. Mixing water layers of different salinities, as well as vertical movement of the organism, demands rapid osmoregulatory responses.

Counterion of potassium

In many non-halophilic bacteria, glutamate was identified as the main counterion of potassium (Richey et al. 1987). In *H. elongata* the glutamate content changes in a similar way to the K^+ content, keeping the K^+ : glutamate ratio constant at a value of 2.4 : 1. In cells of *Vibrio alginolyticus* at salinities between 0.4 and 1.5 M NaCl the glutamate concentration also corresponded to about 50% of the K^+ concentration (Unemoto and Hayashi 1979).

For the following reasons we do not regard Cl^- as being the counterion for K^+. First of all, *H. elongata* has no requirement for Cl^-, which was proven by anion replacement experiments (Vreeland et al. 1980). Secondly, the cytoplasmic Cl^- concentration of halophilic bacteria has proven to be rather low. It was shown for the halophilic ectoine-producer *Ectothiorhodospira mobilis* that Cl^- appears only at a cytoplasmic concentration of 71 mM (Imhoff and Riedel 1989), a concentration too low to counterbalance potassium.

Effect of K^+ depletion on the growth and solute content of *H. elongata*

The growth inhibition caused by K^+ depletion enabled us to determine the affinity of *H. elongata* cells to K^+. We estimated the apparent K_m value to be 105 µM at a salinity of 0.51 M NaCl. This indicates that an uptake system of medium affinity mediates the accumulation of potassium in *H. elongata*. The results described here are according to the findings of Cummings et al. (1993). They analyzed the affinity of *Halomonas* strain SPC1 to potassium and measured an apparent K_m value of 115 µM. They pointed out that a system of medium affinity should supply *Halomonas* with sufficient K^+, since the natural habitats of *Halomonas* contain potassium at a concentration of at least 10 mM. Potassium transporters of medium affinity described so far are the Trk system of non-halophiles (Bakker 1993) and the inducible KtrAB transporter (K_m 0.3 mM). This latter one, which was found for the first time in the marine *Vibrio alginolyticus* (Nakamura et al. 1994), seems to be widespread, since gene homologs were identified in many different bacteria (Nakamura et al. 1998).

As yet, the mechanism by which K^+ acts on the physiology of *H. elongata* is unknown. It seems possible that the presence of K^+ is a requirement for the energy production of the cells. In *Vibrio alginolyticus,* the addition of K^+ to K^+-depleted cells also stimulates the respiration rate (Tokuda et al. 1981). It was shown that the generation of a ΔpH across the cytoplasmic membrane is dependent on K^+ as a counterion for H^+. The authors suspected that respiration is stimulated by a potassium-dependent, increased proton ejection, which is necessary to establish a ΔpH (Tokuda et al. 1981). The dependency of *H. elongata* on potassium could be also explained by the fact that potassium is required for the generation of a Na^+-electrochemical gradient, which was shown to be true for organisms like *Vibrio alginolyticus* (Tokuda et al. 1982). Another mechanism, explaining the requirement of *H. elongata* for K^+ is based on the fact that the depletion of K^+ in the growth medium is correlated to an increase in the Na^+: K^+ ratio. The excess of Na^+ might replace K^+ from usual binding sites inside and outside the cell, thereby causing a competitive inhibition of the cellular functions, including respiration and biosynthetic pathways. This was at least proven to be true for the uptake of potassium, where the apparent K_m value of *H. elongata* for K^+ increased at elevated external Na^+ concentrations, while the internal potassium level remained unchanged. This hypothesis explains the effects caused by K^+ limitation but might also explain the increased K^+ uptake following hyperosmotic shock. Reed and co-workers (1985) demonstrated that immediately after osmotic upshock, Na^+ passively enters the cells of the halotolerant cyanobacterium *Synechocystis euryhalina*. Rather than exporting the penetrated sodium and replacing it by potassium, as known from non-halotolerant and marine organisms, halophilic bacteria such as *H. elongata* might keep the cytoplasmic sodium level elevated for at least several hours. Instead of investing energy to extrude Na^+ immediately against a steep molar gradient, *H. elongata* might accumulate osmoregulatory solutes not only to achieve an osmotic equilibrium, but also to protect the cell from the negative effect of intracellular sodium. According to our model, K^+ is accumulated to compete for binding sites otherwise occupied by the penetrating sodium. In addition, the cell's interior is protected by ectoines, which are known to stabilize enzymes and even whole cells (Lippert and Galinski 1992; Knapp et al. 1999). This scenario explains the increased K^+ and ectoine levels after upshock compared with adapted cells at the same salinity. Following the primary response to osmotic shock, we postulate that the Na^+ level will be decreased passively by cell division. The "dilution" of Na^+ by cell division also lowers the cytoplasmic K^+ and ectoine levels down to a concentration determined in adapted cells. This idea is supported by the observation of Sauer (1995). According to his findings, the process reducing the solute content to a level known from adapted cells takes about 6–8 h. Considering the growth rate of cells at 6% NaCl to be $\mu = 0.28\ h^{-1}$, it takes the cells about 4 h to divide and to increase the volume by a factor of two. We therefore assume that *H. elongata* does not rely on the export of Na^+ in response to osmotic upshock, as known from non-halophilic prokaryotes. Instead, Na^+ might serve as an additional solute to achieve osmotic balance more rapidly following osmotic upshock.

Acknowledgments We thank Evert P. Bakker for the introduction to the technique of K^+ determination. We are grateful to Milan Höfer and Udo Hölcker for their kind permission for and assistance with the K^+ measurements. We thank Heide Schnabl and Kerstin Groß for their generous help in the performance of the respiration experiments. We thank Birgit Amendt and Larissa Tetsch for carrying out the K^+ limitation experiments, and Marlene Stein for expert assistance with the HPLC analysis. We also thank Erwin A. Galinski for carefully reading the manuscript and for helpful discussion. This work was supported by the Deutsche Forschungsgemeinschaft (Tr133/27–1). Annette Kraegeloh is indebted to the Graduiertenförderung des Landes Nordrhein-Westfalen for a doctoral scholarship.

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