Salinity-Dependent Switching of Osmolyte Strategies in a Moderately Halophilic Bacterium: Glutamate Induces Proline Biosynthesis in *Halobacillus halophilus*[▽]

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The moderately halophilic bacterium *Halobacillus halophilus* copes with the salinity in its environment by the production of compatible solutes. At intermediate salinities of around 1 M NaCl, cells produce glutamate and glutamine in a chloride-dependent manner (S. H. Saum, J. F. Sydow, P. Palm, F. Pfeiffer, D. Oesterhelt, and V. Müller, J. Bacteriol. 188:6808–6815, 2006). Here, we report that *H. halophilus* switches its osmolyte strategy and produces proline as the dominant solute at higher salinities (2 to 3 M NaCl). The proline biosynthesis genes *proH*, *proJ*, and *proA* were identified. They form a transcriptional unit and encode the pyrroline-5-carboxylate reductase, the glutamate-5-kinase, and the glutamate-5-semialdehyde dehydrogenase, respectively, catalyzing proline biosynthesis from glutamate. Expression of the genes was clearly salinity dependent and reached a maximum at 2.5 M NaCl, indicating that the *pro* operon is involved in salinity-induced proline biosynthesis. To address the role of anions in the process of *pro* gene activation and proline biosynthesis, we used a cell suspension system. Chloride salts lead to the highest accumulation of proline. Interestingly, chloride could be substituted to a large extent by glutamate salts. This unexpected finding was further analyzed on the transcriptional level. The cellular mRNA levels of all three *pro* genes were increased up to 90-fold in the presence of glutamate. A titration revealed that a minimal concentration of 0.2 M glutamate already stimulated *pro* gene expression. These data demonstrate that the solute glutamate is involved in the switch of osmolyte strategy from glutamate to proline as the dominant compatible solute during the transition from moderate to high salinity.

An increase in the external salt concentration poses a serious problem to any living cell. Due to the permeability of membranes for water, an increase in external salinity will lead to a loss of water, and this will finally lead to cell death if no countermeasures are taken (4). Prokaryotes have developed two strategies to cope with increasing salinities. One is to accumulate and adjust the internal concentration of inorganic ions such as K⁺ and Cl[−] to values that counteract the external osmolality. This strategy can be found in the extremely halophilic *Halobacteria* (Archaea), the halophilic, anaerobic *Haloanaerobiales* (Bacteria) (9, 33), and the recently discovered bacterium *Salinibacter ruber* (1, 20).

A different strategy is the accumulation of compatible solutes: small organic molecules that do not interfere, even at molar concentrations, with cellular metabolism (4). This strategy is widespread and evolutionarily well conserved in all three domains of life (3, 11, 24, 27). However, the spectrum of compatible solutes used comprises only a limited number of compounds; these can be divided into two major groups, the sugars and polyols and the α - and β -amino acids and their derivatives, including methylamines.

Moderately halophilic prokaryotes have the extraordinary

capability to grow over a wide range of external salinities, from 0.5 to 2.5 M NaCl, with identical growth rates (14). They also use the “compatible solute” strategy to cope with elevated salinities. In their natural environments, such as salt marshes, the salt concentrations may fall to low levels after rainfalls. However, after longer periods of sunlight, the soil will dry out and the salinity will gradually increase from very low concentrations up to concentrations at which salt begins to crystallize (high molar range). Therefore, every microbe living in such an environment has to cope with changing salinities over a very wide range, from about 0 to 3.5 M NaCl. It has often been reported that microorganisms use not just one solute but rather two or more to meet this challenge (6, 23, 33, 36), but little is known about the salinity-dependent regulation of intracellular solute composition. Since it has been reported many times in the literature that compatible solutes in general have common features but nevertheless exhibit very distinct properties in protecting proteins under different stress conditions (for a review, see reference 8), it is very likely that an organism has to switch from solute A, which is optimal at low salinities, to solute B, which serves better at high salinities, after osmotic upshock. Such a salinity-dependent switch in osmolyte strategy is observed in archaea and bacteria (17, 21, 22). This requires sensing not only of salinity but of different concentrations of salinities, as well as adjustment of different pool sizes of different compatible solutes appropriate to the external salinities. The molecular basis of salinity sensing and of the signal transduction chain leading to

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TABLE 1. Primers used in this study

Primer	Sequence	Use
proA/proJ-for	5' CTCAAGTTCAGCTACATC 3'	Bridge between <i>proA</i> and <i>proJ</i> (Fig. 2)
proA/proJ-rev	5' TATGGCTTCCGGTATATC 3'	Bridge between <i>proA</i> and <i>proJ</i> (Fig. 2)
proJ/proH-for	5' CTGCGTGTGATTAATATCTG 3'	Bridge between <i>proJ</i> and <i>proH</i> (Fig. 2)
proJ/proH-rev	5' GATATTGATGCCGTACTTG 3'	Bridge between <i>proJ</i> and <i>proH</i> (Fig. 2)
RT-proH-for	5' GCCAGAATAAGTTGATCC 3'	Quantification of <i>proH</i> transcripts
RT-proH-rev	5' GAACATTTCAGCAGATCAG 3'	Quantification of <i>proH</i> transcripts
RT-proJ-for	5' CAGGGCTACGTCTAAATG 3'	Quantification of <i>proJ</i> transcripts
RT-proJ-rev	5' AGAAGGCGAAGAGATTGG 3'	Quantification of <i>proJ</i> transcripts
RT-proA-for	5' CTGCCTGAATCAGTTTACC 3'	Quantification of <i>proA</i> transcripts
RT-proA-rev	5' GGCAATCGTAGAAGTTATCC 3'	Quantification of <i>proA</i> transcripts

altered gene expression and protein activation in moderate halophiles is completely obscure.

Recently, we demonstrated that the moderately halophilic bacterium *Halobacillus halophilus* requires chloride for growth (28). When grown at a moderate salinity of 1 M NaCl, *H. halophilus* accumulates glutamate and glutamine as major compatible solutes, and chloride was shown to regulate the synthesis and action of a key enzyme in glutamate biosynthesis, the glutamine synthetase (30). Moreover, the chloride dependence of growth could be overcome by high external glutamate concentrations (29). These data demonstrated that *H. halophilus* uses glutamate and glutamine as osmolytes at intermediate salinities and that chloride is involved in signaling the external NaCl concentration. An open question is whether the same strategy is used by *H. halophilus* when grown at higher salinities. Here, we report that *H. halophilus* switches the osmolyte strategy at higher salt concentrations and accumulates proline in a glutamate-dependent manner. This is the first example of a solute produced at intermediate salinities being the inducer for the solute preferred at higher salinities. The molecular basis for this step-wise regulation in osmoadaptation is addressed.

MATERIALS AND METHODS

Organism and cultivation. *H. halophilus* (DSMZ 2266T) was grown in glucose mineral salt medium (G10) containing 1% glucose, NH₄Cl (2 g/liter), FeSO₄ · 7H₂O (10 mg/liter), Tris base (12 g/liter), K₂HPO₄ (0.49 g/liter), yeast extract (0.1 g/liter), DSM 141 vitamin solution (1 ml/liter), and DSM 79 artificial seawater (250 ml/liter). Sodium glutamate, NaNO₃, or sodium gluconate was added to a final concentration of 1.0 M. The final concentration of NaCl varied depending on the assay conditions (values are given below). The pH was adjusted to 7.8 with H₂SO₄. *H. halophilus* was cultivated aerobically and shaken on a rotary shaker at 30°C.

Preparation of concentrated cell suspensions. For preparation of concentrated cell suspensions, *H. halophilus* cultures were grown in mineral salt medium (G10) in the presence of 0.8 M NaCl to an optical density at 578 nm (OD₅₇₈) of about 0.8 to 0.9. After harvest of the cells, they were incubated in mineral medium containing 0.4 M NaCl in order to expel accumulated compatible solutes. After a wash, the cells were resuspended in mineral salt medium (G10) containing 2 M osmolyte as indicated below to an optical density at 578 nm of about 9. The cell suspensions were incubated on a rotary shaker at 30°C, and samples were taken after several points in time.

Determination of compatible solutes. Cells were grown to an OD₅₇₈ of about 0.8, harvested, and freeze-dried, and solutes were isolated and analyzed as described previously (13, 30).

Real-time PCR analysis. For real-time PCR analysis, *H. halophilus* cells were harvested in the early exponential growth phase (OD₅₇₈, 0.15 to 0.3). Isolation and quantitative PCR (qPCR) were done as described previously (30). Amplification of *proH*, *proJ*, and *proA* fragments was achieved with the primers RT-proH-for, RT-proH-rev, RT-proJ-for, RT-proJ-rev, RT-proA-for, and RT-proA-rev (Table 1). The fragments amplified comprised 137, 146, and 166 bp,

respectively. Data analysis was accomplished by the $2^{-\Delta\Delta CT}$ method (16). Real-time PCR analysis was done with three independent physiological parallels to ensure statistical relevance. Two open reading frames, encoding a malate dehydrogenase and a glycerate dehydrogenase, respectively, served as internal normalizers. The expression of these two genes did not change with the salinity of the medium.

Cloning and DNA sequence determination. DNA sequences were retrieved from the genome sequence of *H. halophilus*, which will be described elsewhere.

Nucleotide sequence accession numbers. DNA sequences were deposited in the GenBank database under accession numbers EF617348 (*proH*), EF617347 (*proJ*), and EF617346 (*proA*).

RESULTS

Proline is the main compatible solute at high salinities.

From previous studies it was known that *Halobacillus halophilus* employs a mixture of different solutes when grown at 1.5 M NaCl, with glutamate and glutamine being the major solutes (30, 31). To unravel a possible salinity-dependent switch in the osmolyte strategy, *H. halophilus* was cultivated in mineral salt medium in the presence of salinities ranging from 0.4 to 3.0 M NaCl. Cells were harvested in the late exponential growth phase (OD₅₇₈, 0.7 to 0.8), and compatible solutes were extracted and analyzed by high-performance liquid chromatography (HPLC). As seen before, glutamate and glutamine were dominant at intermediate salinities (Fig. 1). At higher salinities, proline became dominant. In the presence of 0.4 M NaCl, cells accumulated proline to a maximal concentration of 0.26 ± 0.04 μ mol/mg of protein, but increasing salinities led to a gradual increase in intracellular proline concentrations, with a maximum of 6.18 ± 0.44 μ mol/mg of protein at 3.0 M NaCl

FIG. 1. Accumulation of glutamine, glutamate, and proline is dependent on the NaCl concentration of the medium. Cells of *Halobacillus halophilus* were cultivated in mineral salt medium (G10) in the presence of the NaCl concentrations indicated. They were harvested in the exponential growth phase (OD₅₇₈, 0.6 to 0.8), compatible solutes were extracted, and the concentrations of glutamine (white), glutamate (gray), and proline (black) were measured by HPLC.

FIG. 2. (A) The proline operon of *H. halophilus*. The proline operon of *H. halophilus* comprises three genes encoding a pyrroline-5-carboxylate reductase (*proH*), a glutamate-5-kinase (*proJ*), and a glutamate-5-semialdehyde dehydrogenase (*proA*). (B) To examine the transcriptional organization, RNA was isolated and transcribed into cDNA. The cDNA was then used as a template in a PCR. The primers and expected fragments are indicated. RNA was used as a negative control; chromosomal DNA was used as a positive control. The resulting DNA fragments were separated on a 0.8% agarose gel.

(Fig. 1). These data clearly demonstrate a switch in osmolyte strategy from glutamate/glutamine as the dominant compatible solute at intermediate salinities to proline at higher salinities.

Identification of proline biosynthesis genes. By inspecting the genome of *H. halophilus*, a cluster of putative proline biosynthesis genes was identified. This cluster included three genes encoding, in this order, a putative pyrroline-5-carboxylate reductase (*proH*, 903 bp), a putative glutamate-5-kinase (*proJ*, 1,131 bp), and a putative glutamate-5-semialdehyde dehydrogenase (*proA*, 1,263 bp) (Fig. 2A). All three genes have the same orientation and are separated from each other by only 103 bp (*proH* and *proJ*) and 20 bp (*proJ* and *proA*). Downstream of *proA* is an open reading frame of 858 bp with an orientation opposite that of the *pro* genes. The deduced protein has no homologues in the databases. At 1,148 bp upstream of *proH* is an open reading frame of 612 bp encoding a hypothetical protein. Therefore, the open reading frame downstream of *proA* and the open reading frame upstream of *proH*

are apparently not in a functional relationship to the *pro* genes and were not investigated in more detail.

The arrangement of the *pro* genes is indicative of their organization in a transcriptional unit. To prove the existence of such a polycistronic messenger, mRNA was isolated from *H. halophilus* cells and transcribed into cDNA. This cDNA was then used as a template in a PCR using primers that bridge the intergenic regions between *proH* and *proJ* and between *proJ* and *proA*, as indicated in Fig. 2A and Table 1. Only a cDNA originating from one polycistronic mRNA is able to give products with the expected fragment sizes of 1,040 bp and 759 bp, respectively. As a negative control, the corresponding mRNA was used as a template to ensure the absence of contaminating DNA. As a positive control, chromosomal DNA of *H. halophilus* was used. As can be seen from Fig. 2B, PCR products were obtained only in the presence of cDNA and chromosomal DNA, not with the corresponding RNA. This result demonstrates that *proH*, *proJ*, and *proA* are part of one operon. The findings that several sequences upstream of *proH* resemble a σ^A -dependent promoter (58 to 83% identity to the consensus sequence of *B. subtilis*) and one sequence resembles a σ^B -dependent promoter (66% identity to *B. subtilis*), as well as the finding that a putative terminator sequence could be identified downstream of *proA*, underline the conclusion that *proHJA* are not only part of an operon but constitute an operon.

Properties of the deduced gene products. ProH is a putative pyrroline-5-carboxylate reductase, the key enzyme of the proline biosynthesis pathway. It has a length of 301 amino acids, and the protein is 57 and 55% identical (similarities, 73% and 72%) to ProH from *Bacillus licheniformis* and *Bacillus subtilis*, respectively. ProJ is a putative glutamate-5-kinase with a deduced length of 377 amino acids. It is similar to ProJ from *Bacillus licheniformis* (84% similarity, 66% identity) and *Bacillus subtilis* (80% similarity, 67% identity). ProA is a putative glutamate-5-semialdehyde dehydrogenase with a deduced length of 420 amino acids. ProA is 82% similar (68% identity) and 74% similar (54% identity) to the proteins found in *Bacillus licheniformis* and *Bacillus thuringiensis*, respectively.

Regulation of proline biosynthesis genes. Before a potential salinity-dependent regulation of the *pro* genes was addressed, we determined whether the intracellular proline level was dependent on the growth phase. Cells were grown at 2.5 M NaCl, and solutes were extracted and analyzed by HPLC. As can be seen in Fig. 3, intracellular proline levels were maximal during the early exponential growth phase and declined thereafter. Therefore, the cells for the following transcriptional analyses were harvested at early exponential growth phase.

Next, we asked whether the expression of the *pro* genes was salinity dependent. To address this question, cells were grown in mineral salt medium in the presence of 0.4 to 3.0 M NaCl. Samples were taken in the early exponential growth phase (OD_{578} , about 0.3) and mRNA was prepared. The mRNA was then reverse transcribed into cDNA that was further used for a real-time qPCR analysis. We determined the relative transcript amounts compared to the amount of the 0.4 M NaCl culture, which was normalized to 1. Two open reading frames, encoding a putative malate dehydrogenase and a glycerate dehydrogenase, served as internal normalizers. As expected, the transcript levels of all three genes behaved in the same manner, again underlining our conclusion that they form an

FIG. 3. Growth phase dependence of proline accumulation in *H. halophilus*. To investigate the influence of the growth phase on the proline accumulation in *H. halophilus*, cultures were grown in the presence of 1.0 M NaCl or 2.5 M NaCl. Samples were taken as indicated in the growth curves, and the solute concentrations were determined. In the right panel, proline concentrations of the samples are given. Light gray, accumulated proline concentrations of cells grown at 1.0 M NaCl; dark gray, accumulated proline concentrations of cells grown at 2.5 M NaCl.

operon. Most important, expression of the *pro* genes was clearly salinity dependent. The mRNA levels increased steadily with increasing salinity and reached a maximum with a sixfold-increased copy number at 2.5 M NaCl (Fig. 4). These data nicely fit the increase in intracellular proline concentrations, with one difference: a slightly reduced expression at 3.0 M NaCl (whereas proline levels were highest at this concentration). In conclusion, these data are evidence that the *pro* operon is involved in salt stress protection at high salt concentrations.

Dynamics of *pro* gene transcription and proline production in cell suspensions. A goal of this study was to address the role of chloride and other anions on proline production and *pro* gene expression. Due to the fact that *H. halophilus* does not grow at salinities of 2.0 M in the absence of chloride, we asked whether concentrated cell suspensions incubated in medium are suitable for analyzing salinity-dependent gene regulation. The experimental approach is explained in Materials and Methods. As shown in Fig. 5A, we observed a dramatic increase in transcript levels as a response to the upshock that reached a maximum after 1.5 h. The amounts of transcripts were about 12 to 13 times higher than under preshock conditions. After 1.5 h, the transcript levels began to decrease again, and a constant level that was about four times higher than at the beginning of the experiment was reached after about 3 to 4 h. Interestingly, proline levels were relatively small at the time point of maximal transcript levels but reached a maximum after 6 to 10 h. Thereafter, proline concentrations steadily declined to preshock levels (Fig. 5B). To assure comparable conditions between cells at the beginning and at the end (after 20 h) of the experiment, samples were taken at 0 h and 20 h after upshock. These cells were gradually diluted up to 10^{-7} . Droplets of 10 μ l of all dilution steps were placed on solid agar medium and incubated at 30°C. The cells at the beginning, as well as the cells after 20 h of incubation, were able to form colonies up to a dilution of 10^{-5} , which clearly shows that there is no toxic effect of the osmolyte and that cells remain viable.

In conclusion, these experiments demonstrated that cell suspensions as well as growing cells accumulate proline when the salt concentration is increased from 0.8 to 2.0 M NaCl. More-

FIG. 4. Transcription of *pro* genes is dependent on the salinity of the medium. *H. halophilus* was grown in mineral salt medium in the presence of the NaCl concentrations indicated to the early exponential growth phase (OD_{578} , about 0.3). RNA was isolated and transcribed into cDNA. The cDNA was then used for real-time qPCR, and the relative copy numbers were determined with the value of the 0.4 M NaCl sample used as a reference.

FIG. 5. Dynamics of transcription of *pro* genes and production of proline in *H. halophilus*. To determine the kinetics of the response to a hyperosmotic upshock, *H. halophilus* was grown in mineral salt medium in the presence of 0.8 M NaCl. After washing the cells in medium containing 0.4 M NaCl, the cells were resuspended in mineral salt medium containing 2.0 M NaCl and incubated at 30°C on a rotary shaker. Samples were taken after several points in time to determine the mRNA levels of *proH*, *proJ*, and *proA* (A) and the proline concentration (B).

over, this treatment led to induction of the *pro* operon, an important result for quantitative analyses of transcript levels.

Anion dependence of proline accumulation. To analyze the role of the ambient anion in salinity-dependent proline production, cell suspensions prepared as described above were incubated in medium containing 2 M NaCl, as well as another osmolyte, such as NaNO₃, Na-glutamate, Na-gluconate, sucrose, Na-tartrate, Na-succinate, glycine, or Na₂SO₄. Again, samples were taken after several points in time, and compatible solutes were extracted and analyzed by HPLC. We observed an accumulation of proline not only in the presence of chloride but also in the presence of nitrate and glutamate, but neither nitrate ($5.4 \pm 1.1 \mu\text{mol/mg}$ of protein) nor glutamate ($4.9 \pm 0.2 \mu\text{mol/mg}$ of protein) led to the same degree of accumulation as observed with chloride ($5.9 \pm 0.9 \mu\text{mol/mg}$ of protein) (Fig. 6). In the presence of gluconate, cellular proline levels were intermediate ($3.0 \pm 0.4 \mu\text{mol/mg}$ of protein), whereas there was no accumulation in the presence of the other salts. The values that are given in Fig. 6 for sucrose, Na-tartrate, Na-succinate, glycine, and Na₂SO₄ do not differ from the levels that were already present at the beginning of the experiment. These results clearly show that the accumulation of proline depends on the nature of the anion. Chloride leads to the highest accumulation of proline. Nitrate is able to replace chloride to some degree and is able to lead to a similar

rate of accumulation of proline. This is not surprising, since nitrate has already been shown to be able to replace chloride (25). An unexpected finding was the glutamate-induced proline accumulation by *H. halophilus*.

FIG. 6. The production of proline is dependent on the ambient anion. To analyze the anion dependence of proline production, *H. halophilus* cells were cultivated in mineral salt medium in the presence of 0.8 M NaCl and harvested in the late exponential growth phase (OD_{578} , 0.8 to 0.9). After washing with medium containing 0.4 M NaCl, the cells were resuspended in medium containing 2 M osmolyte and incubated on a rotary shaker at 30°C. Samples were taken after several points in time, and the concentration of proline was determined. Here the maximal values of proline accumulation that were achieved after the upshock are given.

TABLE 2. Anion dependence of the transcription of *pro* genes^a

Gene	Fold transcript copy no. $\pm$ SEM			
	Chloride	Nitrate	Gluconate	Glutamate
<i>proH</i>	11.9 $\pm$ 2.5	4.4 $\pm$ 2.4	12.5 $\pm$ 2.5	96 $\pm$ 28.4
<i>proJ</i>	13.0 $\pm$ 2.3	12.5 $\pm$ 3.5	14.5 $\pm$ 3.0	32.3 $\pm$ 14.9
<i>proA</i>	12.7 $\pm$ 3.1	11.9 $\pm$ 1.6	9.9 $\pm$ 0.9	29.3 $\pm$ 15.7

^a To elucidate the role of anions in the transcription of *pro* genes, *H. halophilus* cells (grown in the presence of 0.8 M NaCl) were resuspended in mineral medium containing 2.0 M sodium salt (chloride, nitrate, gluconate, or glutamate) and incubated at 30°C on a rotary shaker. Samples were taken after several time points. Values are maximal transcript copy numbers (compared to the values at time zero).

Anion dependence of transcription. The differences in the accumulation of proline in the presence of different anions leads to the question of which step in the process of accumulation is affected by the anions. To address this question, we used the same experimental approach with cell suspensions. Samples were taken after several points in time, and mRNA was prepared. This mRNA was reverse transcribed and used as a template in a real-time qPCR. As can be seen in Table 2, the increase in transcript levels of the *pro* genes was similar for cells incubated in the presence of NaCl, NaNO₃, or Na-gluconate. Therefore, the observed anion dependence of proline accumulation, especially in gluconate-incubated cells, must include an anion-dependent step after transcription, like translation or enzyme activity of the proline biosynthesis enzymes. This was indeed also observed before with chloride (26). Interestingly, we found dramatically increased transcript levels of all three *pro* genes when cells were incubated in the presence of 2 M glutamate. This is even more surprising since the increased mRNA levels do not find a correspondence in the proline concentration measured in these cells compared to the proline concentrations in cells incubated in the presence of chloride.

Stimulation of *pro* gene expression by glutamate. The unexpected glutamate-stimulated *pro* gene expression was analyzed quantitatively in cell suspensions. For this purpose, cell suspensions of *H. halophilus* were incubated in mineral salt medium containing glutamate at concentrations ranging from 0.0 M to 2.0 M. The osmolarity was kept constant at 2 M by adding the appropriate amount of nitrate. Samples were taken from those cultures, mRNA was prepared, and cDNA was synthesized. The cDNA was taken as a template in a qPCR. As can be seen from Fig. 7, the maximal increase of *proH* transcripts did not change from 0.0 to 0.15 M glutamate. However, in the presence of 0.2 M glutamate, the relative transcript levels increased to over 90-fold and remained at this high level from 0.2 to 2.0 M Na-glutamate. This result implies that it takes a minimal glutamate concentration of 200 mM to get a stimulation of *pro* gene expression.

DISCUSSION

In a systematic survey done by the group of Trüper, the solutes synthesized by *H. halophilus* were analyzed (8, 31). Therefore, *H. halophilus* was grown in different media at a salt concentration of 1.5 M. At that concentration, *H. halophilus* accumulated a mixture of different solutes, such as *N*^ε-acetyl

lysine, *N*^ε-acetyl ornithine, alanine, citrulline, glutamate, glutamine, ectoine, and proline. We observed the same in our studies (30). Here, we have demonstrated that the nature of the main solute changes depending on the salinity of the medium. At high salinities, *H. halophilus* prefers proline over glutamate/glutamine. Such a salinity-dependent switch in osmolyte strategy has been observed before in some archaea and bacteria. The methanogenic archaeon *Methanosarcina mazei*, for example, synthesizes glutamate as an osmolyte at 400 mM NaCl but in addition *N*^ε-acetyl-β-lysine at 800 mM NaCl (21). The diazotrophic bacterium *Azospirillum brasilense* was reported to use glutamate and proline as the main compatible solutes (17). At 300 mM NaCl, glutamate is the dominant solute, whereas proline seems to be of minor importance. When salinity is elevated to 900 mM, the proline concentration increases by almost threefold, whereas the glutamate concentration decreases by 25%. The moderately halophilic eubacterium Ba₁ was reported to use trehalose as the main solute below 600 mM NaCl and ectoine above 600 mM (22). Until now there has been no ultimate conclusion for the preferential use of different osmolytes, but it is speculated that it is due to the obvious difference in the ability to protect cellular elements against external influences such as temperature and salinity, etc. (8). It has been shown, for example, that hydroxyectoine is much more efficient in protecting rabbit muscle lactate dehydrogenase incubated at 55°C than its relative ectoine or their precursors diaminobutyrate or *N*^γ-acetyldiaminobutyrate (5). It was also found that glutamyl-tRNA synthase from *E. coli* treated with urea is more effectively protected by potassium D- or L-glutamate than by sorbitol, trimethylamine oxide, or inositol (18). The change in the solute strategy could also be explained by the observation that bacteria accumulate potassium as the first response to hypersalinity. The anion that is required to counterbalance the positive charge in the immediate response to osmotic shock in gram-positive bacteria is

FIG. 7. Concentration dependence of the stimulation of *proH* transcription by glutamate. Cells of *H. halophilus* were cultivated in mineral salt medium in the presence of 0.8 M NaCl. After washing with medium containing 0.4 M NaCl, the cells were resuspended in medium containing different concentrations of glutamate. The osmolarity was kept constant at 2 M by adding corresponding amounts of NaNO₃. The cell suspensions were incubated on a rotary shaker at 30°C, and samples were taken after several points in time. The samples were used to isolate mRNA that was then used to produce cDNA. The cDNA was then used as a template in a qPCR to determine the relative mRNA levels of *proH*. The value at the beginning of the upshock experiment was taken as the reference. Here the maximal relative accumulation of mRNA copies after upshock with different glutamate concentrations in the medium is given.

unknown. Glutamate concentrations increase only slightly (32). This is in contrast to gram-negative bacteria, where a dramatic increase in glutamate was observed (7). Nevertheless, Tempest and coworkers also reported that they found already very high concentrations of glutamate in several *Bacillus* species that were not osmotically challenged, which could argue for the possibility that previously accumulated glutamate buffers increasing potassium concentrations (32, 34, 35). Nonetheless, potassium glutamate can disturb the cellular metabolism at higher concentrations, so that accumulation of these osmolytes is limited and must be replaced by other solutes such as proline. If this is true, one must postulate a mechanism that measures potassium and/or glutamate concentrations and triggers the substitution of glutamate when concentrations increase too much.

As shown in this study, induction of proline biosynthesis is triggered by the salinity of the medium and the glutamate concentration. From a physiological point of view, this makes perfect sense. Chloride triggers the synthesis of glutamate at intermediate salinities, thereby reaching glutamate concentrations of 4.3 $\mu\text{mol/mg}$ protein at 1.0 M NaCl in the medium (30). Given an internal volume of 2 $\mu\text{l/mg}$ of protein (28), this corresponds to 2 M glutamate within the cells. Internal glutamate is then used as a "second messenger" for the induction of proline biosynthesis genes. In the group of Gralla, several studies were done demonstrating the potential of glutamate to activate or inhibit transcription (10, 15). This is in perfect correlation with our studies, where a minimal external glutamate concentration of 200 mM (at a total salinity of 2 M) effectively stimulates the transcription of the *pro* genes. This finding could give a hint of a very direct and sophisticated regulatory circuit in which glutamate is accumulated until a given concentration within the cell is reached. This concentration then directly leads to a stimulated *pro* operon transcription and finally proline production. During this process the glutamate concentration does not increase any further (data not shown), which is not unexpected, since glutamate is the precursor molecule for proline.

Inspection of the genome sequence revealed only the genes involved in proline biosynthesis described here. The gene products of this operon are sufficient to produce proline from glutamate. This is in contrast to *B. subtilis*, where two genes encoding a γ -glutamyl kinase (*proB* and *proJ*), one encoding a γ -glutamyl phosphate reductase (*proA*), and at least three pyrroline-5-carboxylate reductase genes (*proG*, *proH*, and *proI*) were found (12). It was speculated by Belitsky and coworkers that this is due to different demands, such as production of proline during proline limitation, adaptation to high salt concentrations, or degradation of toxic pyrroline-5-carboxylate (2). However, this cannot be true for *H. halophilus* and leaves us with the question of how the biosynthesis of the proteinogenic amino acid proline and the osmolyte proline is separated on a regulatory level. End-product inhibition, if present at all, cannot be the only regulatory mechanism to shut off biosynthesis. In addition, a signal reflecting the turgor of the cell must be present, but the nature of this signal is unknown. Whether glutamate is the only signal that activates proline production is presently unknown but unlikely. At 1 M NaCl, the internal glutamate concentration is already maximal, but a further increase in NaCl leads to increased production of proline. There-

fore, additional regulatory factors must be present. Whether this is the chloride concentration under physiological conditions remains to be established. However, in our cell suspension system the production of proline was similar at 2 M NaCl and 2 M Na-glutamate. This does not exclude an effect of chloride but could simply result from unphysiologically high concentrations of glutamate.

Concluding remarks. *H. halophilus* requires chloride for different cellular purposes, and it has been shown that the chloride regulon is involved in salinity perception and signal transduction (19). One output module is the production of glutamate and glutamine, and transcription of the glutamine synthetase gene, as well as the activity of the glutamine synthetase, was shown to be chloride dependent (30). Furthermore, glutamate was able to fully substitute for chloride in cells grown at 1 M sodium salts. Here, we have added another layer of regulation, glutamate-induced proline biosynthesis. Analyses of the molecular basis of this regulation are in progress.

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