Chloride dependence of glycine betaine transport in *Halobacillus halophilus*

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Abstract Growth of *Halobacillus halophilus* is strictly chloride-dependent but the physiological basis for the chloride dependence remains to be elucidated. To address the function of Cl^- in *H. halophilus*, a physiological study was performed. It was found that uptake of the compatible solute glycine betaine under isoosmotic conditions was stimulated by increasing salt concentrations. Uptake of glycine betaine required both, Na^+ and Cl^-. Cl^- could be substituted by nitrate and bromide, but not by sulfate. Glycine betaine transport was optimal at around 0.7 M Cl^-. Cells responded to an osmotic upshock by accumulating glycine betaine, but only in the presence of chloride. These studies revealed the first chloride-dependent glycine betaine transporter in a prokaryote.

Key words: Chloride; Glycine betaine; Transport; Moderately halophile; *Halobacillus halophilus*

1. Introduction

Halobacillus halophilus (formerly *Sporosarcina halophila*) is a moderately halophilic bacterium isolated from salt marsh at the North Sea coast of Germany [1]. In the absence of NaCl growth is impaired but it increases gradually with increasing NaCl concentrations. Highest growth rates were obtained between 0.5 and 2.0 M NaCl, but even concentrations as high as 2.5 and 3.0 M were tolerated with growth rates of 50 and 38%, respectively. Not only the growth rate but also the final yield was dependent on the salt concentration. Most interestingly, growth as well as the final yield were also strictly dependent on the Cl^- concentration. Highest growth rates were obtained between 0.5 and 2.0 M Cl^-. Chloride could be substituted by Br^- and, after an adaptation time of a few hours, by NO_3^-, but not by SO_4^{2-} or other anions [2].

Osmotic work is an energy-dependent, potentially chloride-dependent process. Halophilic microorganisms are faced with the problem to keep the water activity of their cytoplasm high. Two types of osmoadaptation are known in bacteria. First, the intracellular salt concentration is equal to the extracellular salt concentration. This so-called 'salt in cytoplasm' type is not found in *H. halophilus*, but in some archaea as well as halophilic bacteria [3,4]. Second, organisms synthesize or take up so-called compatible solutes which do not interfere with the central metabolism [5]. *H. halophilus*, for example, synthesizes a variety of different compatible solutes such as N_ε-acetyllysine, N_δ-acetylornithine, ectoine, glutamate, and proline during growth in mineral media [6]. When compatible solutes are available, as in nutrient broth (NB), uptake of compatible solutes from the medium is preferred over de novo synthesis. Here, we will provide evidence that the intracellular concentration of the compatible solute glycine betaine, taken up by *H. halophilus* from the medium, is dependent on the external salt concentration. Most interestingly, uptake of glycine betaine under steady state conditions and after osmotic upshock is Cl^--dependent. This is the first report on a Cl^--dependent glycine betaine transport system in a prokaryote.

2. Materials and methods

2.1. Organism and cultivation

H. halophilus (DSMZ 2266) was obtained from the 'Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ)', Braunschweig, Germany and maintained on NB, which contained per liter 5 g peptone and 3 g meat extract (Difco laboratories, Augsburg, Germany), supplemented with 1 M NaCl and 0.05 M $MgSO_4$. The pH was adjusted to pH 7.5.

2.2. Preparation of concentrated cell suspensions

For preparation of concentrated cell suspensions, NB was inoculated (1%) from cultures maintained in the same medium. Fresh cell suspensions were prepared for each experiment. Cells in the late logarithmic growth phase were harvested by centrifugation and washed once with 0.05 M Tris–HCl, pH 7.5, containing 0.66 M Na_2SO_4 and 0.05 M $MgSO_4$. The cell pellet was resuspended in the same buffer to a protein concentration of 12–18 mg ml^{-1} and stored on ice until use. The protein concentration of the cell suspension was determined as described [7], with bovine serum albumin as a standard. For upshock experiments, the NaCl concentration of the growth medium and the buffers was reduced to 0.5 M.

2.3. Glycine betaine uptake studies

100 μl of the concentrated cell suspension were centrifuged in Eppendorf tubes and resuspended in assay buffer (0.05 M Tris–HCl buffer, pH 7.5, containing 0.05 M $MgSO_4$, salts as indicated, and 1 mM [^{14}C]glycine betaine (0.3 μCi, specific activity: 60 μCi μmol^{-1}). The experiment was started by resuspending the cells in the assay buffer. 50-μl samples were taken at the time points indicated, filtered through nitrocellulose filters (25 mm in diameter; pore size, 0.45 μm; Sartorius, Göttingen, Germany), and washed three times with the corresponding buffer. Sampling and washing were done in less than 15 s. All values were corrected for unspecific binding of glycine betaine to the filters. The filters were air-dried, and radioactivity was determined in a liquid scintillation counter type 2100 TR (Packard, Dreieich, Germany) using Rotiszint eco plus (Roth, Karlsruhe, Germany) as the scintillation cocktail. The internal betaine concentration was calculated on the basis of the internal volume published earlier [2].

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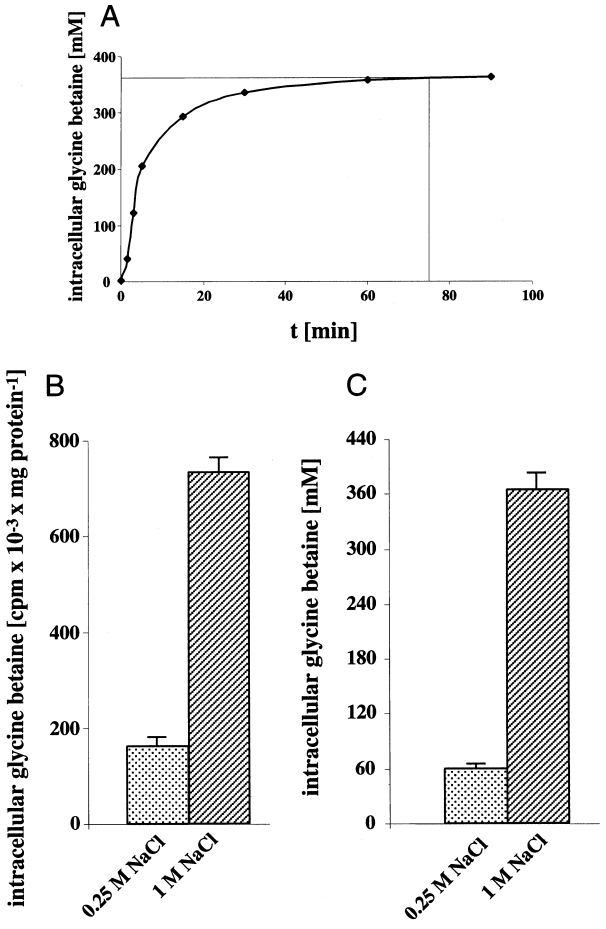

Fig. 1. Internal glycine betaine concentrations in *H. halophilus*. A: Time course of the glycine betaine uptake at 1 M NaCl. Cells were suspended in NB containing carrier-free [^{14}C]glycine betaine (B) or in assay buffer (containing 1 mM [^{14}C]glycine betaine) (C). The NaCl concentrations were adjusted to 0.25 M and 1 M, respectively. Internal glycine betaine concentrations were determined in the steady state, i.e. 75 min after glycine betaine addition, under isoosmotic conditions. The protein concentration was 1.2–1.8 mg ml^{-1}. The data presented reflect the means of at least three experiments; standard deviations are indicated by the error bars.

3. Results

3.1. The intracellular glycine betaine concentration is dependent on the extracellular NaCl concentration

As mentioned above, osmoadaptation in *H. halophilus* grown in complex media relies on the uptake of compatible solutes, preferably glycine betaine [6]. The intracellular concentration of glycine betaine in cells washed and resuspended in growth medium (with unknown glycine betaine content) containing carrier-free [^{14}C]glycine betaine was strictly dependent on the salt concentration (Fig. 1). The intracellular glycine betaine concentration was quantified with cells suspended in assay buffer which contained a defined glycine betaine concentration (1 mM [^{14}C]glycine betaine). Again, the intracellular glycine betaine concentration was dependent on the extracellular NaCl concentration. The intracellular concentration was calculated using the intracellular volume as determined earlier [2]. At 250 mM NaCl the internal glycine betaine concentration was 60 mM, but it increased to 365 mM at 1.0 M NaCl. These values correlate well with previously determined values [6].

3.2. Chloride dependence of glycine betaine transport under isoosmotic conditions

To determine whether Cl^- affects the uptake of glycine betaine from the medium, we determined the anion dependence of glycine betaine uptake using resting cells incubated under isoosmotic conditions (osmolarity of the growth medium and the assay buffer was identical). [^{14}C]Glycine betaine was used at a concentration of 1 mM which is well above the K_m values for all glycine betaine transporters known in bacteria to date. Cells were suspended in assay buffer containing various salts, and the glycine betaine content was determined after 75 min in the steady state (Fig. 2). $NaNO_3$ or NaBr could fully substitute for NaCl. This observation is in accordance with the fact that the chloride requirement for growth of *H. halophilus* is satisfied also by NO_3 and Br^-. Neither did Na_2SO_4 stimulate glycine betaine transport nor was it able to support growth. In the presence of sucrose, KCl, Na-gluconate or sorbitol, glycine betaine transport was only 44%. In the presence of $CaCl_2$ or $MgCl_2$, transport activities were 59–67%. The transport activities measured may result from the action of different devices with different ion specificities. However, the results indicate that, in any case, both Na^+ and Cl^- (or its substituent Br^- or NO_3^-) are required for optimal glycine betaine transport in *H. halophilus*, which is also true for several amino acid and glycine betaine transporters from eukaryotes [8,9].

To analyze the effect of Cl^- independent of the Na^+ concentration, cells were incubated in buffer containing increasing Cl^- concentrations (supplied as NaCl) but a constant osmolarity equivalent to 1 M NaCl. The osmolarity was kept constant by appropriate addition of Na_2SO_4, thus, the only varying parameter was the Cl^- concentration. In the absence of Cl^- there was only little glycine betaine transport (Fig. 3).

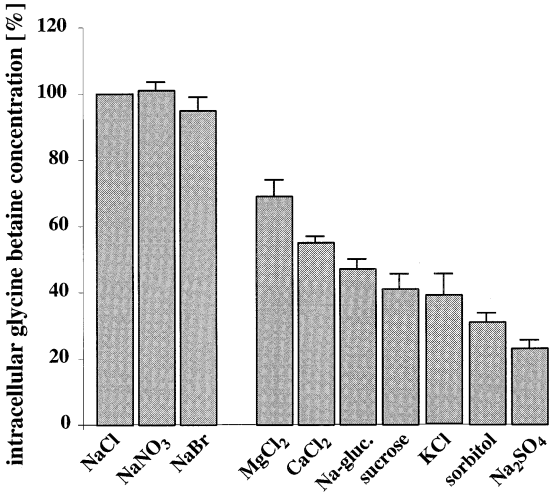

Fig. 2. Ion-dependence of glycine betaine uptake in *H. halophilus*. Glycine betaine uptake was determined in assay buffer containing the osmolutes indicated. $MgCl_2$, $CaCl_2$, and Na_2SO_4 were used at a concentration of 0.66 M, all other osmolutes at 1.0 M. The intracellular concentration was determined at steady state, i.e. 75 min after glycine betaine addition. 100% corresponds to 365 mM glycine betaine. The protein concentration was 1.2–1.8 mg ml^{-1}. The data presented reflect the means of at least three experiments; standard deviations are indicated by the error bars.

Increasing Cl^- concentrations led to an increase of transport rates as well as final accumulations. Above 0.5 M Cl^- uptake reached optimal rates, which is in accordance with almost optimal growth achieved at 0.5 M NaCl [2]. Since uptake in buffer containing 2 M NaCl or 1 M NaCl+1 M Na_2SO_4 was almost identical, an inhibitory effect of SO_4^{2-} on glycine betaine uptake can be ruled out. These experiments gave clear evidence that the uptake of glycine betaine in *H. halophilus* is chloride-dependent.

3.3. Chloride dependence of glycine betaine transport after osmotic upshock

The experiments described so far were performed under isoosmotic conditions. When cells are exposed to an osmotic upshock, glycine betaine transporters may become activated and new transport systems may be induced [10,11]. It was, therefore, of interest to analyze the effect of chloride on glycine betaine transport after an osmotic upshock. When cells of *H. halophilus* grown in 0.5 M NaCl were resuspended in buffer containing 0.5 M NaCl, uptake of glycine betaine occurred as described above until a steady state was reached after 40 min. Addition of NaCl to a final concentration of 1.5 M led to an increased uptake of glycine betaine (Fig. 4). Of particular importance was now the effect of Cl^-. To analyze the effect of Cl^- independent of the osmolarity, cells were shocked by adding solutions having increasing Cl^- concentrations (supplied as NaCl) but a constant osmolarity, equivalent to 1 M NaCl. The osmolarity was kept constant by appropriate addition of Na_2SO_4. As can be seen in Fig. 4, upshock in the absence of NaCl (0.66 M Na_2SO_4) led to only a negligible

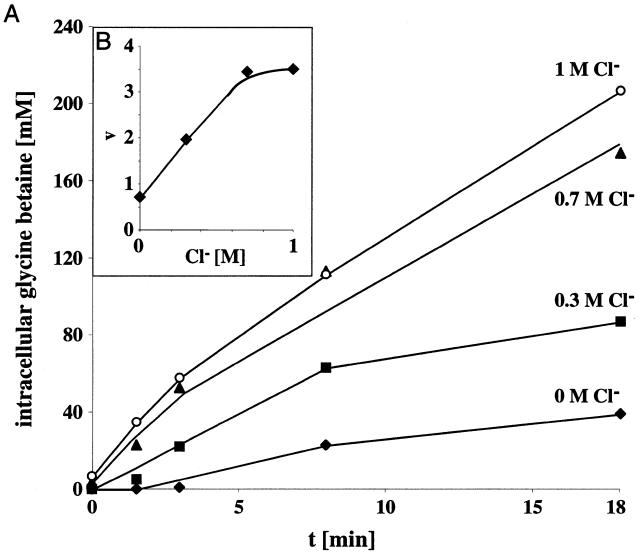

Fig. 3. Chloride dependence of glycine betaine uptake under isoosmotic conditions in *H. halophilus*. Glycine betaine uptake was determined in assay buffer containing increasing Cl^- concentrations, supplied as NaCl. The osmolarity was kept constant (equivalent to 1 M NaCl) by appropriate addition of Na_2SO_4. Chloride was present in the given concentrations. (♦) 0 M Cl^- (no NaCl+0.66 M Na_2SO_4), (■) 0.3 M Cl^- (0.3 M NaCl+0.46 M Na_2SO_4), (▲) 0.7 M Cl^- (0.7 M NaCl+0.2 M Na_2SO_4), (○) 1 M Cl^- (1 M NaCl+no Na_2SO_4) (A). Initial uptake rates v (mM glycine betaine min^{-1}) were plotted against the chloride concentration (B). The protein concentration was 1.2–1.8 mg ml^{-1}. The experiments were performed at least three times and the data presented reflect a typical result.

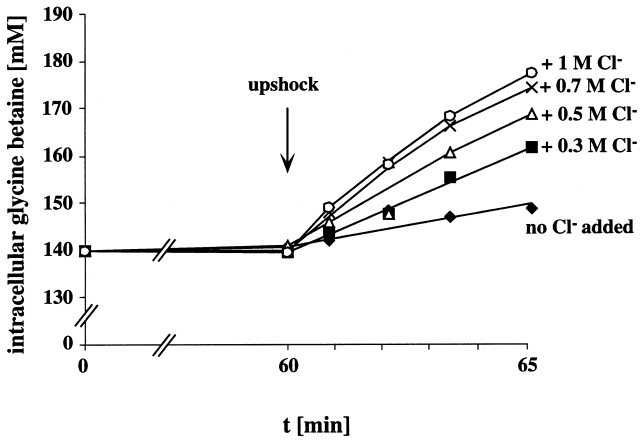

Fig. 4. Chloride dependence of glycine betaine uptake in *H. halophilus* after osmotic upshock. Glycine betaine uptake was determined in assay buffer containing 0.5 M NaCl. At the time point indicated by the arrow osmotic upshock was performed by the addition of salt solutions to a final osmolarity equivalent to 1 M NaCl. The Cl^- concentration was varied while the osmolarity was kept constant by appropriate addition of Na_2SO_4. (♦) no addition of Cl^- (no NaCl+0.66 M Na_2SO_4), (■) addition of 0.3 M Cl^- (0.3 M NaCl+0.46 M Na_2SO_4), (△) addition of 0.5 M Cl^- (0.5 M NaCl+0.33 M Na_2SO_4), (×) addition of 0.7 M Cl^- (0.7 M NaCl+0.2 M Na_2SO_4), (○) addition of 1 M Cl^- (1 M NaCl+no Na_2SO_4). The protein concentration was 1.2–1.8 mg ml^{-1}. The experiments were performed at least three times and the data presented reflect a typical result.

increase in glycine betaine concentration. However, the intracellular glycine betaine concentration increased in parallel with increasing Cl^- concentrations in the upshock buffer. Maximum activity was observed above 0.5 M Cl^-. Because SO_4^{2-} did not inhibit uptake of glycine betaine it is evident that uptake of glycine betaine by *H. halophilus* after an osmotic upshock is Cl^--dependent. Since the buffer composition is often responsible for this salt-specific effect, the same experiments were repeated in a 50 mM potassium–phosphate buffer replacing the standard assay buffer containing 50 mM Tris. Salt-specific activation occurred to the same extent, proving the significance of the results obtained (data not shown).

4. Discussion

What could be the role of chloride in the transport of glycine betaine? Generally, cells sense the external osmolarity and respond to it. There is only little information regarding sensing of and response to external salts in moderate halophiles, and the mechanisms of both sensing and response are very different in different bacteria. In *Escherichia coli*, the primary sensing is mediated by the two component system KdpE–KdpD. KdpD, a membrane-embedded protein, is thought to sense the turgor of the cells by an alteration of the physical interaction of its transmembrane helices [12]. This signal is transmitted via phosphorylation to KdpE, which then triggers cellular response, i.e. gene activation. As a result K^+ is accumulated and glutamate is synthesized as the major anion [13]. This response is transient and followed by a variety of other cellular responses, including synthesis as well as activation of transporters which take up compatible solutes. From the results described here it is evident that the activity

of the glycine betaine transporter(s) of *H. halophilus* is (are) regulated by Cl^-. Osmotic activation of transporters was studied in *Bacillus subtilis* and *E. coli*. However, in both the actual signal sensed by the transporters is unknown but a specific salt- or ion-dependence was ruled out [14,15]. Such an activating effect could also be exerted by the anions nitrate and bromide.

Another possible role for Cl^- arises from studies in brush-border membrane vesicles from eukaryotic tissues like kidney and rat renal cortex. In these tissues Cl^--dependent uptake systems for taurine, β-alanine, and glycine betaine, respectively, are described, where Cl^- is cotransported with two Na^+ ions and the negatively charged substrate, leading to an electroneutral transport [8,9]. The β amino acid transport across the renal brush-border membranes is also coupled to both Na^+ and Cl^- and, as in the case of glycine betaine transport in *H. halophilus*, chloride can be substituted by bromide and nitrate [16]. Since glycine betaine transport in *H. halophilus* is stimulated by both, Cl^- and Na^+, a direct involvement of both ions is also conceivable. However, a Cl^-/Na^+/glycine betaine transport is energetically much more unfavorable than a simple Na^+/glycine betaine symport which could use both, the electrical potential and the transmembrane Na^+ gradient as driving force. Whether Cl^- is cotransported with glycine betaine is unlikely but remains to be established. It should be mentioned in this connection, that a $\Delta\mu_{Cl^-}$-based bioenergetics was found only recently in *Natronobacterium pharaonis* [17].

The molecular analysis of the glycine betaine transporter(s) from *H. halophilus* is under way; this approach will lead to the development of an experimental setup to study the role of Cl^- in this transport process.

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References

[1] Claus, D., Fahmy, F., Rolf, H.J. and Tosunoglu, N. (1983) Syst. Appl. Microbiol. 4, 496–506.
[2] Roeßler, M. and Müller, V. (1998) Appl. Environ. Microbiol. 64, 3813–3817.
[3] Galinski, E.A. and Trüper, H.G. (1994) FEMS Microbiol. Rev. 15, 95–108.
[4] Ventosa, A., Nieto, J.J. and Oren, A. (1998) Microbiol. Mol. Biol. Rev. 62, 504–544.
[5] Brown, A.D. (1976) Bacteriol. Rev. 40, 803–846.
[6] Severin, J. (1993) Ph.D. thesis, University of Bonn, Bonn.
[7] Schmidt, K., Liaanen-Jensen, S. and Schlegel, H.G. (1963) Arch. Mikrobiol. 46, 117–126.
[8] Munck, L.K. (1995) Biochim. Biophys. Acta 1241, 195–214.
[9] Yamauchi, A., Uchida, S., Kwon, H.M., Preston, A.S., Robey, R.B., Garcia-Perez, A., Burg, M.B. and Handler, J.S. (1992) J. Biol. Chem. 267, 649–652.
[10] Racher, K.I., Voegele, R.T., Marshall, E.V., Culham, D.E., Wood, J.M., Jung, H., Bacon, M., Cairns, M.T., Ferguson, S.M., Liang, W.-J., Henderson, P.J.F., White, G. and Hallett, F.R. (1999) Biochemistry 38, 1676–1684.
[11] Glaasker, E., Konings, W.N. and Poolman, B. (1996) J. Biol. Chem. 271, 10060–10065.
[12] Altendorf, K., Gassel, M., Puppe, W., Mollenkamp, T., Zeeck, A., Boddien, C., Fendler, K., Bamberg, E. and Drose, S. (1998) Acta Physiol. Scand. 643 (Suppl.), 137–146.
[13] McLaggan, D., Naprstek, J., Buurmann, A.M. and Epstein, W. (1994) J. Biol. Chem. 269, 1911–1917.
[14] Kempf, B. and Bremer, E. (1998) Arch. Microbiol. 170, 319–330.
[15] Wood, J.M. (1999) Microbiol. Mol. Biol. Rev. 63, 230–262.
[16] Turner, R.J. (1986) J. Biol. Chem. 261, 16060–16066.
[17] Avetisyan, A.V., Kaulen, A.D., Skulachev, V.P. and Feniouk, B.A. (1998) Biochem. Mosc. 63, 744–748.