

## Osmoprotectants in *Halomonas elongata*: High-Affinity Betaine Transport System and Choline-Betaine Pathway

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**The osmoregulatory pathways of the moderately halophilic bacterium *Halomonas elongata* DSM 3043 have been investigated. This strain grew optimally at 1.5 to 2 M NaCl in M63 glucose-defined medium. It required at least 0.5 M NaCl for growth, which is a higher concentration than that exhibited by the *H. elongata* type strain ATCC 33173. Externally provided betaine, choline, or choline-O-sulfate (but not proline, ectoine, or proline betaine) enhanced the growth of *H. elongata* on 3 M NaCl–glucose–M63 plates, demonstrating the utilization of these compounds as osmoprotectants. Moreover, betaine and choline stimulated the growth of *H. elongata* DSM 3043 over the entire range of salinity, although betaine was more effective than choline at salinities below and above the optimum. We found that *H. elongata* DSM 3043 has at least one high-affinity transport system for betaine ( $K_m = 3.06 \mu\text{M}$  and  $V_{\max} = 9.96 \text{ nmol of betaine min}^{-1} \text{ mg of protein}^{-1}$ ). Competition assays demonstrated that proline betaine and ectoine, but not proline, choline, or choline-O-sulfate, are also transported by the betaine permease. Finally, thin-layer chromatography and <sup>13</sup>C-nuclear magnetic resonance analysis showed that exogenous choline was taken up and transformed to betaine by *H. elongata*, demonstrating the existence of a choline-glycine betaine pathway in this moderately halophilic bacterium.**

Halophiles are organisms that have a requirement of salt for growth (14). Among halophilic microorganisms, halobacteria (extremely halophilic aerobic archaea) and moderately halophilic bacteria (which grow best in media with 0.5 to 2.5 M NaCl) (15, 23) predominate and have an important ecological role in hypersaline environments (14, 20). Besides, the moderate halophiles have recently gained a great biotechnological interest (24). Although nonhalophilic bacteria, including *Escherichia coli* (22), *Salmonella typhimurium* (4), *Rhizobium meliloti* (21), *Bacillus subtilis* (30), and *Staphylococcus aureus* (10), can grow at moderate osmolarity, unlike the halophiles, they do not have a strict sodium requirement. Both halophilic and nonhalophilic bacteria have evolved mechanisms that enable them to adapt to high salinity. One of these approaches, preferred by members of the *Halobacteriaceae*, is maintenance of a high cytoplasmic potassium chloride concentration, similar to the salinity of the surrounding medium (6). A second strategy, found in a wide variety of bacteria, is the accumulation of high concentrations of compounds, named compatible solutes or osmoprotectants. Compatible solutes, which are mainly amino acids and derivatives, sugars, and polyols, can be synthesized de novo or, when present externally, accumulated by transport (9). Glycine betaine (named betaine hereafter), which is one of the most powerful osmoprotectants, cannot be synthesized de novo by most bacteria (5).

Osmoregulation has been studied most extensively at the genetic level in the nonhalophilic bacteria *E. coli* and *S. typhimurium* (5, 8). In these organisms, osmoprotectants are taken up by two osmotically regulated transport systems, ProP and ProU (8). First described as transport systems for proline, ProP and ProU were found to be able to take up betaine and other osmoprotectants, including proline betaine and ectoine (11,

12). In addition, *E. coli*, *B. subtilis*, and *R. meliloti*, but not *S. typhimurium*, have an osmoregulated choline-betaine pathway which consists of a transport protein for choline and two enzymes mediating its oxidation to betaine (1, 8).

Among the moderate halophiles, organisms belonging to the family *Halomonadaceae* display one of the widest ranges of salt tolerance found in prokaryotes (25). Therefore, they are excellent models to study bacterial osmoadaptation. Wohlfarth et al. (31) found that members of this family accumulated high concentrations of ectoine or hydroxyectoine in glucose-mineral medium. In complex media containing yeast extract, these bacteria also accumulated betaine as a compatible solute.

The fact that moderate halophiles can grow at high salinity raises several intriguing questions concerning the physiology of these organisms. Do they have any unique mechanism that enable them to grow in the presence of high concentrations of NaCl inhibitory to most nonhalophiles? Is there a casual connection between their requirement for a minimum salinity and their exceptional salt tolerance? What responses do these organisms display to adapt to changes in the external osmotic or ionic strength? In order to gain insights into these questions, we have initiated a physiological study of the osmoregulatory responses of the moderately halophilic bacterium *Halomonas elongata*. In this work, we report the first evidence of a high-affinity betaine transport system and the existence of a choline-betaine pathway in this halophile.

### MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** *H. elongata* DSM 3043 was grown in the defined medium M63 (3). Carbon sources were present at 20 mM. Unless otherwise stated, glucose was used as the carbon source. Osmotic strength of the medium was increased by the addition of 0.5 to 4 M NaCl, or 1.5 M NaCl together with 1.5 M KCl or 1.5 M NH<sub>4</sub>Cl. When used, 1 mM of the osmoprotectants betaine or choline were added. The pH of the medium was adjusted to 7.2 to 7.4 with NaOH. Solid medium contained an additional 20 g of Bacto-agar (Difco) per liter. Cultures were incubated at 37°C in an orbital shaker at 200 rpm. Growth was monitored by measuring the optical density of the culture at 600 nm with a Shimadzu UV160U UV-visible spectrophotometer.

**Screening of osmoprotectants.** The ability of *H. elongata* DSM 3043 to use a

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number of compatible solutes was determined qualitatively on solid media. Overnight cultures (50  $\mu$ l) were spread on M63 plates containing 3 M NaCl, and filter disks containing 10- $\mu$ l aliquots of a 0.1 M solution of betaine, choline, choline-O-sulfate, ectoine, proline, or proline betaine were placed on each plate. The efficacy of these solutes to act as osmoprotectants was estimated from the density of growth around the filter disks.

**Determination of the intracellular betaine levels.** Cells grown overnight in M63–0.5 M NaCl were subcultured at a 1:20 dilution in M63 containing 0.5 to 3 M NaCl, 10 mM [ $^{14}$ C]betaine (10,000 cpm), and 20 mM [ $^3$ H]glucose (10,000 cpm). When cultures reached stationary phase, 0.5 ml of cell cultures was harvested by centrifugation and washed twice with carbon-free M63 medium containing the same NaCl concentration as in the growth media. Cells were resuspended in 50  $\mu$ l of the same medium, and their [ $^{14}$ C] and [ $^3$ H] content was determined with a Beckman LS6000C scintillation counter. The intracellular level of betaine was expressed as nanomoles of [ $^{14}$ C]betaine accumulated per nanomoles of [ $^3$ H]glucose incorporated.

**Determination of betaine transport rate.** Cells grown overnight in M63 plus 0.5 M NaCl were subcultured at a 1:20 dilution in M63 containing 1.0, 2.0, or 3.0 M NaCl and grown to early exponential phase (optical density at 600 nm, 0.2). Transport was carried by adding [ $^{14}$ C]betaine (10,000 cpm; final concentration, 30  $\mu$ M) to 0.5 ml of the cell cultures and incubating the cultures at room temperature (25°C). At 10-s intervals, 100- $\mu$ l samples were withdrawn and filtered through a Metrical 0.45- $\mu$ m-pore-size membrane filter (Gelman Sciences). The filters were rapidly (<5 s) washed with 4 ml of carbon-free M63 containing NaCl at the same concentration as in the respective media used for the growth of the cells. The radioactive betaine accumulated by the cells was determined by scintillation counting. Each experiment was carried in at least two independent cell cultures, and each datum point was determined in duplicate. The protein content of cell suspensions was measured routinely with the Pierce BCA Protein assay kit, according to the specifications of the supplier. Transport rates were expressed as nmoles taken up per time unit per milligram of protein. To determine the kinetic constants of the betaine transport system, the above method was used to measure the rate of betaine uptake as a function of betaine concentration in the range of 5 to 105  $\mu$ M, in cells grown in M63 plus 2.0 M NaCl.

Competition assays for transport of [ $^{14}$ C]betaine and other osmolytes were performed in M63 plus 2 M NaCl containing 10  $\mu$ M [ $^{14}$ C]betaine (30,000 cpm) and 100  $\mu$ M unlabeled betaine, choline, choline-O-sulfate, ectoine, proline, or proline betaine, and the rate of betaine uptake was measured as described above.

**Chromatographic identification of intracellular betaine.** Cells grown overnight in M63–0.5 M NaCl were subcultured in M63–2 M NaCl containing 10  $\mu$ M [ $^{14}$ C]choline or 10  $\mu$ M [ $^{14}$ C]betaine (100,000 cpm) and grown to stationary phase. Cells from 0.5-ml samples were sedimented by centrifugation, washed twice with carbon-free M63–0.5 M NaCl, and extracted with 100  $\mu$ l of 70% (vol/vol) methanol/H<sub>2</sub>O. The radioactive metabolites in the supernatant were analyzed by silica-gel thin-layer chromatography, with 60:20:20 (vol/vol/vol) *n*-butanol:acetic acid:water. The radioactive metabolites were visualized by autoradiography.

**Analysis of intracellular osmolytes with  $^{13}$ C-NMR.** Samples (500 ml) of mid-exponential phase cell cultures, grown in M63–2 M NaCl were harvested and washed twice in the growth medium without any carbon source. Cells were resuspended in 1 ml of double-distilled water and lysed with a Labsonic 2000 sonicator. Natural abundance  $^{13}$ C-nuclear magnetic resonance (NMR) spectra were obtained from 0.5 ml aliquots of the suspension, to which 50  $\mu$ l of D<sub>2</sub>O and 7  $\mu$ l of dimethyl sulfoxide-*d*<sub>6</sub> were added for internal reference.  $^1$ H-decoupled  $^{13}$ C-NMR spectra were recorded on a Bruker ac200 spectrometer at 50 MHz with probe temperature of 20 to 22°C. Solutes were identified by comparison of chemical shifts with published values (7, 31).

## RESULTS

**Salinity range of *H. elongata*.** *H. elongata* DSM 3043 (formerly strain 1H11) was shown to be able to grow in the range of salinity 0.6 to 5.5 M NaCl in complex medium (26). Since the NaCl requirement and tolerance of this organism has been described only in complex medium, we determined its salinity range in the minimal medium M63. *H. elongata* exhibited a wide salt growth range (from 0.5 to 3.0 M NaCl) in M63 also. Most rapid growth, with a generation time (g) of 3.5 h, was at 1.5 M NaCl. Growth was progressively slower at 2.5 M NaCl (g = 6.5 h) and 3.0 M NaCl (g = 16.5 h) and completely inhibited by 4 M NaCl. Thus, *H. elongata* DSM 3043 appears to be more sensitive to very high salinity in minimal than in complex medium, because it was reported to be able to grow up to 5.5 M NaCl in complex medium (26). Growth was also suboptimal at NaCl concentrations below 1 M, and at least 0.5 M NaCl was required for any growth at all. At NaCl concentrations around 1 M, the cells formed large clumps, which

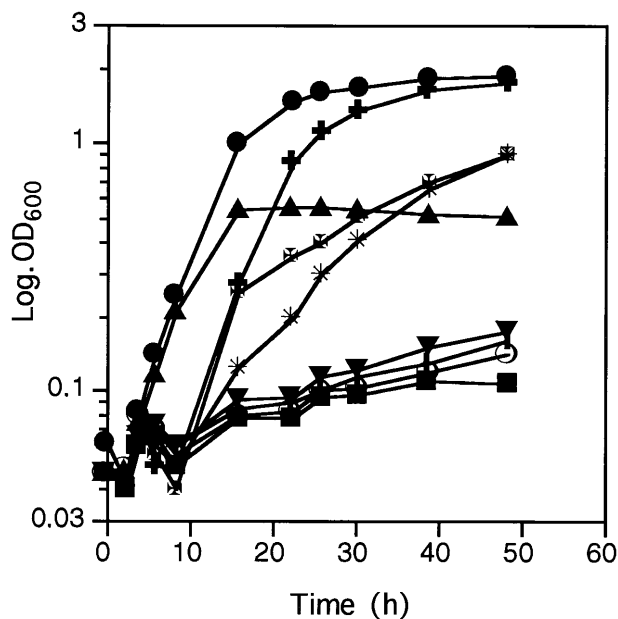


FIG. 1. Growth of *H. elongata* in 1.5 M NaCl–M63 containing different carbon sources (20 mM). Symbols: ●, glucose; ○, glutamate; ■, glycerol; ▲, citrate; ▼, succinate; I, sorbitol; ⊕, proline; ⋆, choline; ⊞, betaine.

interfered with the accurate determination of the generation time. The facts that *H. elongata* requires at least 0.5 M NaCl and grows optimally at 1.5 M NaCl document that it is a true halophile.

**Characterization of carbon sources.** We tested a number of compounds as potential sole carbon sources for *H. elongata* in M63–1.5 M NaCl (Fig. 1). Of the compounds examined, glucose supported the most rapid growth rate. Betaine, choline, and proline could also be utilized as the sole carbon sources, although the growth was slower than with glucose. Citrate was able to support the growth, although at a substantially lower molar growth yield than that observed with the other carbon sources. Under the test conditions, *H. elongata* was unable to utilize sorbitol, glutamate, succinate, or glycerol as the sole carbon source.

**Screening of compatible solutes. (i) Stimulation of growth of *H. elongata* by betaine and choline.** *H. elongata* has been reported to accumulate two compounds as compatible solutes: ectoine, which is produced by de novo synthesis, and betaine, which is accumulated by transport from the medium (31). We screened qualitatively the osmoprotectant efficacy of a number of other compounds on M63–3 M NaCl plates on which solutions of the test compounds were added to filter disks. Whereas growth was very slow on these plates after 3 days in the absence of any osmoprotectant, there was a pronounced stimulation of growth around the filter disks permeated with a solution of betaine (positive control), choline, and choline-O-sulfate, indicating that *H. elongata* can accumulate these compounds to sufficiently high levels so that they (or perhaps some metabolites derived from them) can function as compatible solutes. In contrast, no stimulation of growth was detected around the disks containing proline, proline betaine, or ectoine.

In *E. coli* and *S. typhimurium*, osmoprotectants result in growth stimulation only in media whose osmolarity is high enough to inhibit the growth rate (2, 4, 12). In contrast, betaine and choline resulted in a pronounced stimulation of growth for

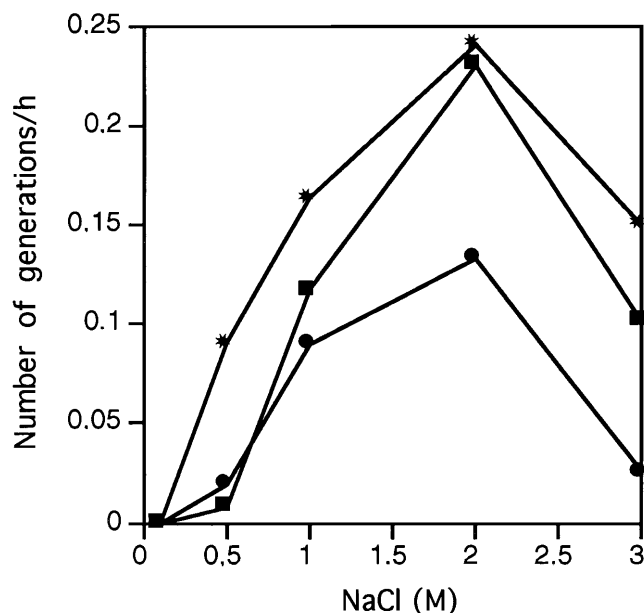


FIG. 2. Enhancement of growth of *H. elongata* by exogenous choline or betaine in media of various osmotic strength. Cells were grown in M63–glucose–minimal medium in absence of choline or betaine (●), in the presence of 1 mM choline (■) or 1 mM betaine (\*). The osmolarity of the medium was increased by the indicated concentrations of NaCl.

*H. elongata* over the entire range of salinity (Fig. 2). At the optimal salt concentration (1.5 to 2 M NaCl), both betaine and choline led to the maximum growth rate. However, at salinities below and above the optimum, betaine was more effective than choline in stimulating the growth rate of *H. elongata*.

(ii) Effect of osmotic stress on the accumulation of betaine.

As described above, betaine can be used as sole carbon source by *H. elongata*. A retention of approximately 10 or 90% of radioactivity after 70% methanol extraction was found when cells were grown in M63–2 M NaCl in the presence or absence of glucose, respectively. This indicates that only when betaine was provided as a sole carbon source it was primarily incorporated into cellular components. In order to study the osmotic regulation of betaine uptake in *H. elongata*, we determined the effect of osmolarity on the steady-state intracellular betaine levels. The intracellular level of betaine increased with increasing salinity in the range of 0.5 to 2.5 M NaCl, in cells grown in M63 (Fig. 3). However, there was a decrease in betaine accumulation at 3 M NaCl, suggesting that the uptake or retention of betaine is less efficient at this high osmolarity.

We also determined the effect of the NaCl concentration on the rate of transport of betaine by *H. elongata* (Fig. 4A). Transport rate was maximal at 2 M, slightly slower at 1 M, and reduced dramatically at 3 M. The kinetic constants of the transport system were determined from the transport rates at various concentrations of betaine, from 5 to 105  $\mu\text{M}$ . The betaine transport system was saturable and displayed typical Michaelis-Menten-type kinetics (Fig. 4B). The Lineweaver-Burk transformation of the data suggested that there is a single, high-affinity betaine transport system in *H. elongata* ( $K_m = 3.06 \mu\text{M}$  and  $V_{\text{max}} = 9.96 \text{ nmol min}^{-1} \text{ mg of protein}^{-1}$ ).

To determine whether the betaine transport system of *H. elongata* can recognize other osmoprotectants as substrates, competition assays were performed with [ $^{14}\text{C}$ ]betaine and various unlabeled compatible solutes. In Table 1, the percentage of reduction in the velocity of [ $^{14}\text{C}$ ]betaine transport by these

osmolytes is represented. While none of the osmoprotectants competed efficiently with betaine for uptake, a 10-fold excess of proline betaine and ectoine resulted in a 33 and 17% reduction in the rate of betaine transport, respectively, suggesting that these two compounds are taken up by the betaine transport system.

(iii) Choline–betaine pathway. As demonstrated in this work, choline can be used as an effective osmoprotectant by *H. elongata*. To determine whether the utilization of choline as an osmoprotectant in *H. elongata* involves conversion of this compound to betaine, as it has been found in *E. coli* and other bacteria (16), we analyzed by thin-layer chromatography the [ $^{14}\text{C}$ ] metabolites extracted from *H. elongata* grown in M63 plus 2 M NaCl containing [ $^{14}\text{C}$ ]choline. The radioactive metabolite isolated from the cells had the same mobility as the [ $^{14}\text{C}$ ]betaine standard, suggesting that choline was transformed to betaine by *H. elongata* (data not shown). This result was confirmed by natural abundance  $^{13}\text{C}$ -NMR. Figure 5 shows the spectrum of *H. elongata* grown in 2 M NaCl M63 with choline. There was a signal at 170 ppm, arising from the carboxylic group of betaine. The signal from the methyl group of choline, expected at about 5 ppm, was absent, corroborating that in these cells choline has been oxidized to betaine. Ectoine could not be detected in strain *H. elongata* DSM 3043 grown in M63–2 M NaCl. This result contrasts with the observations reported by Wohlfarth et al. (31) made with the type strain, in which ectoine was accumulated as the major organic compatible solute in both minimal and complex media.

## DISCUSSION

*H. elongata* DSM 3043 (formerly named strain 1H11), which was isolated by Vreeland et al. (26) from a solar salt facility located in The Netherlands Antilles, appeared to be phenotypically different from the other *H. elongata* strains. In this work, we have characterized the osmoregulatory responses of this moderately halophilic bacterium. Although both *H. elongata* type strain ATCC 33173 and strain DSM 3043 showed optimal growth at approximately 1.5 to 2 M NaCl, there is an important difference in the salinity requirements of the two strains. Thus, while the type strain grew well with 0.05 M NaCl

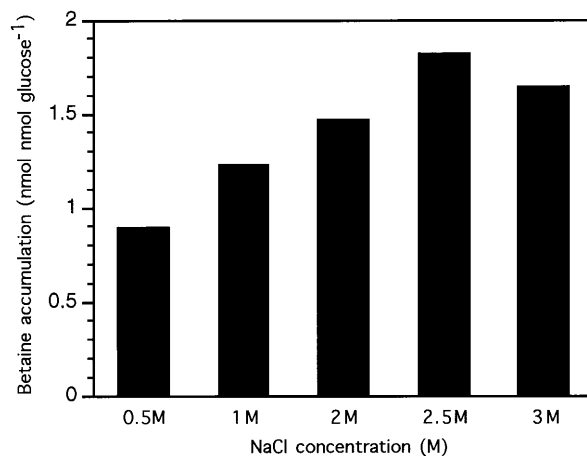


FIG. 3. Accumulation of betaine by *H. elongata* in M63 glucose medium in response to increasing osmotic strength. The measurement of the levels of [ $^{14}\text{C}$ ]betaine uptake was performed as described in Materials and Methods, in cells grown in glucose–M63 containing the indicated concentrations of NaCl. The results are expressed as the ratio of [ $^{14}\text{C}$ ]betaine per nanomoles of [ $^3\text{H}$ ]glucose incorporated into cells.

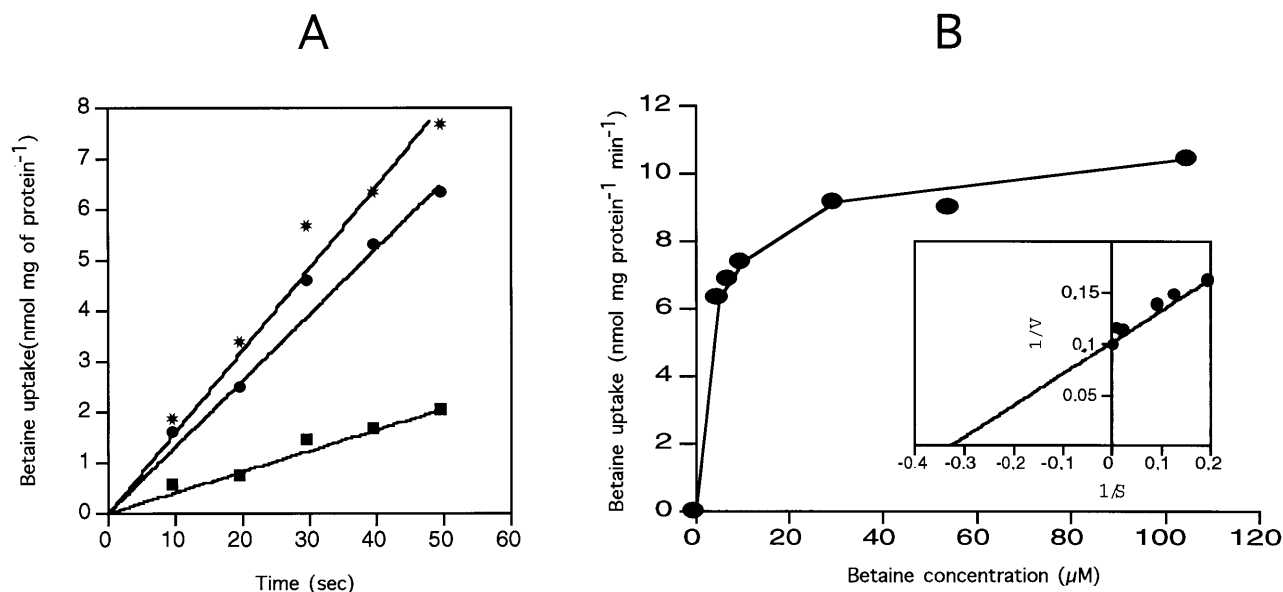


FIG. 4. Transport of betaine by *H. elongata*. (A) Effect of NaCl on the betaine transport rate, performed as described in Materials and Methods. Symbols: ●, 1 M NaCl; \*, 2 M NaCl; ■, 3 M NaCl. (B) Kinetics of betaine uptake by *H. elongata* incubated in 2 M NaCl–M63–glucose–medium containing the indicated concentrations of betaine. The inset shows the Lineweaver-Burk transformation of the data.

in a minimal medium which is similar to M63 (27), *H. elongata* DSM 3043 seems to have more stringent requirements for salt and could not grow at all in M63 unless it contained 0.5 M NaCl.

We have determined the growth rate of *H. elongata* DSM 3043 with other carbon sources not tested previously by Vreeland et al. (26), including some compatible solutes. Although glucose was the best carbon source, proline, choline, and betaine were also used by this halophile, indicating the presence of catabolic pathways for these compounds. Under the testing conditions, glycerol, succinate, sorbitol, and glutamate could not be used as sole carbon sources by *H. elongata* DSM 3043. Our result with glycerol is not consistent with the earlier observations made by Vreeland et al. (26), who reported that the strain could use this compound as a carbon source. The reason for this discrepancy could be that those authors only estimated the utilization of carbon sources qualitatively, whereas we carried out more precise growth curves.

We found that externally provided choline, choline-O-sulfate, and betaine functioned as efficient osmoprotectants for *H. elongata* DSM 3043. However, externally provided proline, proline betaine, or ectoine, which have been described as com-

patible solutes in a number of other bacteria (8), could not be used as osmoprotectants by *H. elongata* DSM 3043. Our result with proline agrees with observations made with the type strain, in which proline was not detected neither by amino acid analysis (28) nor by <sup>13</sup>C-NMR (31). Proline betaine and ectoine seem to be taken up by the betaine transport system in strain DSM 3043, although with less affinity than betaine. Therefore, the failure of these osmolytes to serve as osmoprotectants (when provided externally) might be due to an insufficient accumulation inside the cells. However, we cannot rule out the possibility that proline betaine might be degraded by *H. elongata* DSM 3043 or that for some other reason it might not function as a good osmoprotectant even at high intracellular concentrations.

Externally provided choline-O-sulfate, choline, and betaine resulted in a strong enhancement of growth of *H. elongata* DSM 3043. We do not yet know whether choline-O-sulfate, a compound found in higher plants (19), is the osmoprotectant itself or if it is the precursor of other compounds (e.g., betaine via choline). Thin-layer chromatography and <sup>13</sup>C-NMR demonstrated that choline was converted to betaine, indicating a functional choline-betaine pathway in *H. elongata*. Choline and betaine stimulated the growth of *H. elongata* over the whole range of salinities. The enhancement of growth by the two compounds was nearly identical at optimal salinity, but choline was less efficient than betaine both below and above the optimal salinity. When cells were grown at the optimal salinity in presence of choline, only betaine could be detected by <sup>13</sup>C-NMR, but ectoine was not present at detectable levels. Therefore, it seems that betaine (synthesized from choline) was sufficient for *H. elongata* DSM 3043 to overcome the stress caused by this salinity. This differs from the type strain, which at 2 M NaCl accumulated both betaine (when present in the medium) and ectoine (31). The fact that many bacteria prefer to transport external compatible solutes rather than to carry out the energetically more expensive de novo synthesis (8) may account for the accumulation of betaine instead of ectoine in *H. elongata* DSM 3043 at 2 M NaCl.

TABLE 1. Competition assay with [<sup>14</sup>C]-betaine and various unlabeled compatible solutes for the betaine transport system of *H. elongata*

Compatible solute	% reduction <sup>a</sup>
Betaine.....	86.4
Choline.....	13.0
Choline-O-sulfate.....	8.7
Ectoine.....	17.0
Proline.....	7.1
Proline betaine.....	32.8

<sup>a</sup> The rate of [<sup>14</sup>C]betaine uptake in absence or presence of these osmolytes (10-fold in excess) was determined as described in Materials and Methods. The rate seen with [<sup>14</sup>C]betaine plus water was set to 100%, and all the data were normalized to this 100% value.

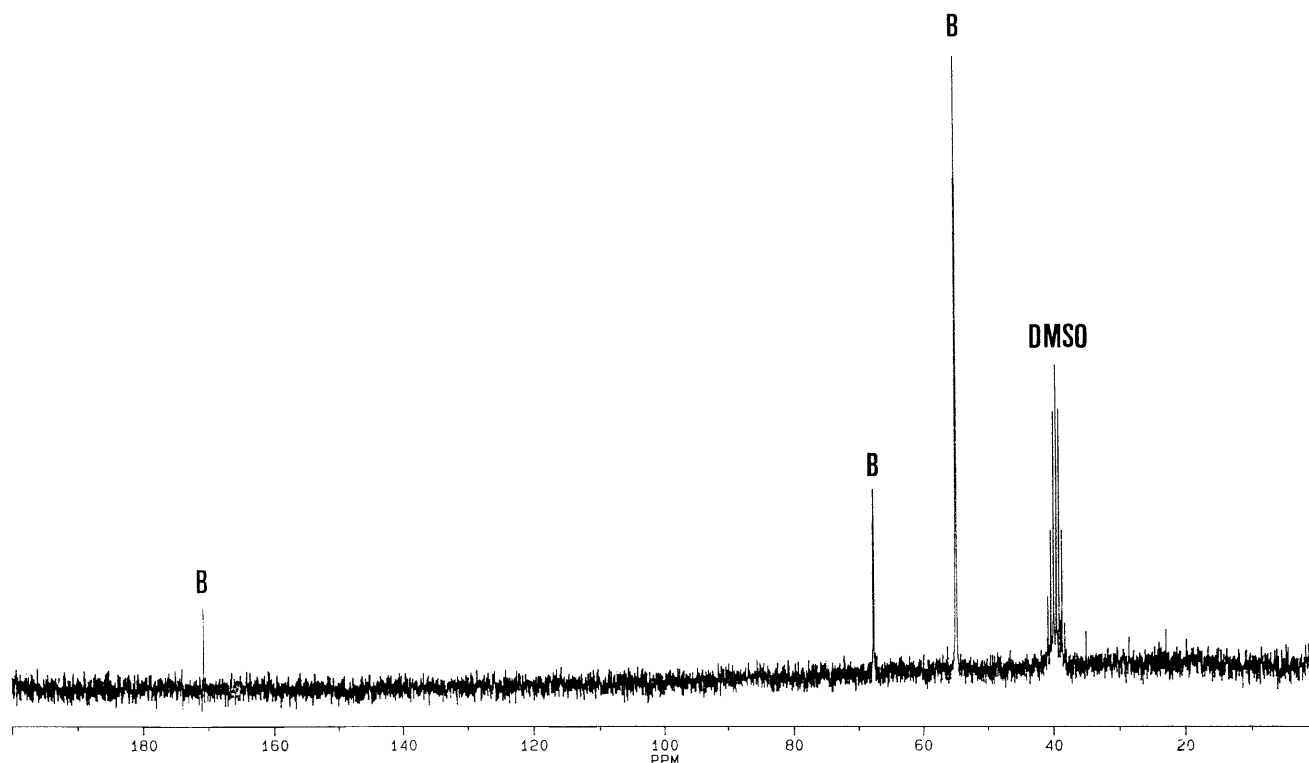


FIG. 5. Natural abundance  $^{13}\text{C}$ -NMR spectrum of major cytosolic solutes of *H. elongata* cells grown in 2 M NaCl–M63–glucose-medium containing 1 mM choline. Signals due to betaine are highlighted by the letter B. Dimethyl sulfoxide was used as a internal reference.

The studies of Wohlfarth et al. (31) showed that *H. elongata* is able to take up betaine from the medium. We have demonstrated the existence of a betaine transport system in *H. elongata* DSM 3043, whose affinity for betaine is very high, similar to the ProU system of *E. coli* and *S. typhimurium* (2, 18). The accumulation of betaine increased as a function of increasing salinity, up to 2.5 M NaCl. However, when the salinity was increased further to 3 M, there was a reduction in the rate of betaine uptake. It is noteworthy that the fourfold reduction in the rate of betaine uptake at 3M NaCl did not result in a reduction in the betaine accumulation. An efflux system for betaine, which could be repressed at high salinity, could account for the maintenance of high betaine levels at 3 M NaCl, in spite of the reduction in the uptake rate. In *E. coli* and *S. typhimurium*, efflux systems for betaine have been reported (13, 17). The mechanism for the partial inhibition of betaine transport at high salinities remains unclear, but a similar observation has been reported in the halotolerant purple sulfur bacterium *Chromatium* sp. (29).

Competition assays demonstrated that the betaine transport system of *H. elongata* is also able to uptake proline betaine and ectoine, although with less affinity than for betaine, but it does not recognize proline or choline-O-sulfate. Thus, the *H. elongata* betaine transport system differs from the *E. coli* and *S. typhimurium* ProP and ProU systems, which can recognize all these compatible solutes. To our knowledge, the transport of ectoine had not been previously demonstrated in halophilic bacteria. We found a slight reduction in the transport of betaine when ectoine was present, suggesting that the latter compound might be taken up inefficiently by the betaine transport system of *H. elongata*. Choline-O-sulfate did not compete with betaine for transport in *H. elongata*. The fact that this compound functions as a compatible solute in *H. elongata* suggests

the existence of a separate transport system for choline-O-sulfate. Finally, since proline and choline can be used as carbon sources for *H. elongata* DSM 3043, they must be transported, but as judged by the competition experiment, their transport systems are also distinct from the betaine transporter.

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