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The spectrum of compatible solutes in heterotrophic halophilic eubacteria of the family *Halomonadaceae*

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A new family, the *Halomonadaceae*, has recently been proposed for members of the genera *Deleya* and *Halomonas*. The three strains investigated, *Deleya halophila*, *Halomonas elongata* and *Flavobacterium halmephilum* (reclassified as *H. halmophila*), are aerobic heterotrophic micro-organisms exhibiting an extreme salt tolerance. The major organic osmoregulatory solutes of these organisms were examined using ^{13}C-nuclear magnetic resonance spectroscopy. The relative proportions of the solutes varied with respect to salt concentration, temperature and carbon source. The recently described amino acid ectoine was found to be a dominant solute. For the first time it could be shown for halophilic eubacteria that the intracellular concentration of solutes is sufficient to balance the osmotic pressure of the medium. Thus, there is no need to postulate a hypo-osmotic cytoplasm.

Introduction

Halotolerant or halophilic micro-organisms have been isolated from various biotopes such as salt or soda lakes, solar salterns, coastal lagoons or sea water. Such micro-organisms have been classified by a number of authors on the basis of their increased requirement for NaCl (Brown, 1976; Kushner, 1978; Imhoff, 1986; Russell, 1989). Since our investigations have revealed a complex interrelationship of salt tolerance and physiological growth conditions (Wohlfarth *et al.*, 1989*a*, *b*), mainly temperature and carbon source, we are inclined to follow the classification of Reed (1986) for the non-extreme marine environment: micro-organisms with no absolute requirement for more than 0·5 M-NaCl, but capable of growth at a salinity of 1 M-NaCl or greater, may be regarded as halotolerant. Halophiles have a specific requirement for higher salt concentrations than that (about 0·5 M-NaCl) of sea water. A more detailed subdivision (moderately and extremely halotolerant, or extremely halophilic) within these broad categories remains open to discussion.

Life in extremely saline habitats is mainly confined to unicellular algae and bacteria. The spectrum of species is diverse (for a review see Rodriguez-Valera, 1988) and halophilic and halotolerant micro-organisms are present among most of the taxonomic groups.

Their natural environment is characterized by a high ionic concentration and low water activity. Changes of salinity may occur due to evaporation or dilution by rainfall. The organisms inhabiting these biotopes have evolved different mechanisms of haloadaptation concerning modifications of protein structure, lipid composition, transport processes, gene transcription (Lanyi, 1974; Bayley & Morton, 1978; Le Rudulier *et al.*, 1984; Russell, 1989; Csonka, 1989) or accumulation of cytoplasmic osmolytes (Brown, 1976; Yancey *et al.*, 1982). Thus, osmoregulation may occur by various active processes in order to cope with the osmotic stress.

Our investigations have concentrated on the accumulation of solutes. These are termed 'compatible solutes' (Brown, 1976) as they serve to compensate the osmotic pressure and, by definition, are compatible with the metabolism of the cell. Thus the intracellular water activity can be reduced and cell volume and turgor can be adapted to the conditions set by the osmotic stress. The compatible solutes known so far belong to the following classes of compounds: inorganic ions, polyols, sugars, betaines and amino acids, and have been extensively reviewed by Mackay *et al.* (1984), Gould (1985), Imhoff (1986) and Trüper & Galinski (1986). The anaerobic heterotrophic eubacteria (Oren, 1985; Rengpipat *et al.*, 1988) and halophilic archaeobacteria accumulate inorganic ions such as K^+, Na^+ and Cl^- to achieve osmotic equilibrium across the cytoplasmic membrane. On the other hand, low M_r organic solutes have been reported from osmophilic fungi (Brown & Simpson, 1972; André *et al.*, 1988; Meikle *et al.*, 1988), unicellular algae (Avron,

1986; Wegmann, 1986), cyanobacteria (Reed *et al.*, 1986), anoxygenic phototrophic bacteria (Galinski & Trüper, 1982; Galinski *et al.*, 1985) and some aerobic non-halophilic heterotrophic eubacteria (Bernard *et al.*, 1986; Larsen *et al.*, 1987; Measures, 1975). Compared to these groups of organisms little is known about the solutes of the halophilic heterotrophic eubacteria.

Studies on the cellular ion content of halotolerant and halophilic aerobic heterotrophic micro-organisms have revealed that the intracellular ion concentration is too low to balance the osmotic pressure, as shown for *Pseudomonas halosaccharolytica* (Matheson *et al.*, 1976), *Halomonas elongata* (Vreeland *et al.*, 1983) and *Paracoccus halodenitrificans* (Sadler *et al.*, 1980). As no organic solutes have been detected in concentrations sufficiently high to account for a function as compatible solutes, the osmoadaptive mechanism remains obscure.

A relationship between the salt concentration of the medium and the intracellular content of betaines was reported by Imhoff & Rodriguez-Valera (1984) for various organisms grown in a yeast extract medium. Vreeland *et al.* (1983) showed a similar correlation for the amino acid pool of *H. elongata* grown on a defined medium with alanine, but the values obtained were too low to achieve osmotic equilibrium. Thus the mechanism of osmotic response to high external ion concentrations was still unclear, as discussed in recent reviews (Vreeland, 1987; Kushner, 1988; Reed, 1986; Csonka, 1989). As a series of investigations into the osmoadaptive mechanism of *H. elongata* and other halophiles were unable to detect the 'major' compatible solute, a hypoosmotic cytoplasm was proposed by Vreeland (1987).

The present uncertainty about osmoregulation in halophilic heterotrophic eubacteria has led us to focus our investigations on this group of organisms. Micro-organisms like *Deleya halophila*, *Flavobacterium halmephilum* and *H. elongata*, now proposed as members of the new family *Halomonadaceae* (Franzmann *et al.*, 1988; P. D. Franzmann & B. J. Tindall, personal communication), exhibit a broad salt tolerance and therefore seemed to be suitable for our investigations.

The aim of our work was to identify and to quantify the intracellular amount of the compatible solutes of the *Halomonadaceae* and to monitor the influence of environmental parameters on the solute accumulation and *de novo* synthesis. As nuclear magnetic resonance spectroscopy (NMR) has previously been shown to be a useful method for the identification of organic osmolytes in phototrophic bacteria (Borowitzka *et al.*, 1980; Galinski & Trüper, 1982; Hocking & Norton, 1983), we have used this method, combined with chromatographic methods, to solve the uncertainties about compatible solutes in halophilic eubacteria of the family *Halomonadaceae*.

Methods

Organisms and growth media. The organisms used in these experiments were *Halomonas elongata* (ATCC 33173^T), *Deleya halophila* (CCM 3662^T) and *Flavobacterium halmephilum* (CCM 2833^T). Most of the growth experiments were conducted with *H. elongata*, but final results were also confirmed for *D. halophila* and *F. halmephilum*.

Cells were grown either on a complex yeast extract medium or on a defined glucose/mineral salt medium, which was used to demonstrate the *de novo* synthesis of the osmotica as an accumulation of solutes from the growth medium was not possible.

The defined medium contained (l^{-1}): trace element solution (Claus *et al.*, 1983), 1 ml; NaBr, 0·1 g (0·97 mmol); $FeSO_4(NH_4)_2(SO_4).6H_2O$, 0·1 g (0·25 mmol); NH_4Cl, 2·0 g (37·4 mmol); yeast extract, 0·1 g; 1 M-Tris/maleate buffer (pH 7·4), 50 ml; KCl, 1·0 g (13·4 mmol); $MgSO_4.7H_2O$, 10·0 g (40·57 mmol); NaCl, 0–250 g (0–4·27 mol); K_2HPO_4/KH_2PO_4, 0·5 g/0·3 g (2·87/2·2 mmol); glucose.H_2O, 20 g (100·92 mmol). Glucose and phosphate solutions were autoclaved separately.

The complex medium contained (l^{-1}): yeast extract, 20 g; 1 M-Tris/maleate buffer (pH 7·4), 50 ml; KCl, 1·0 g; $MgSO_4.7H_2O$, 10·0 g; NaCl, 0–250 g. The pH of both media was adjusted to pH 7·4 before adding salts. All chemicals (analytical quality) were obtained from Merck except for yeast extract, which was purchased from Difco.

Culture conditions and cell harvest. All experiments concerning the effect of carbon source, salt concentration and temperature on the solute spectrum were carried out in 250 ml conical flasks with baffles and sidearms shaken at 180 r.p.m. Pre-cultures of *H. elongata* were grown under identical conditions as in the experiments. An inoculum of 0·5–1 ml of exponential phase cells was transferred into 100 ml fresh broth. The effect of temperature was investigated at 20, 30 and 40 °C. Growth was monitored by measuring the optical density at 600 nm. Unless otherwise stated, cells were harvested in the late exponential growth phase by centrifugation for 15 min at 27000 ***g***. The cell pellet was washed once with a basal salt solution containing adequate amounts of KCl, $MgSO_4$ and NaCl and was subsequently centrifuged for 5 min in a microfuge at 15000 ***g***. The supernatant was removed and the pellet was freeze-dried prior to quantification of solutes, which was always performed in duplicate. Batch fermentations were conducted either in 5 l laboratory vessels (Biostat V, Braun Melsungen) or in a specially designed 20 l fermenter (Galinski, 1987). The cells were harvested in the late exponential growth phase either with a Westfalia separator or with a Heraeus suprafuge at 20000 ***g***.

Determination of compatible solutes. Freeze-dried cells were extracted with chloroform/methanol/water (1 : 1 : 0·8, by vol.) using a modified Bligh and Dyer technique (Bligh & Dyer, 1959). The water-soluble fraction was deproteinized, desalted and prepared for HPLC as described by Galinski (1987) and subsequently analysed on an NH_2-phase column (Nucleosil, Macherey & Nagel) using acetonitrile/water (70 : 30, v/v) as a solvent. Compounds were monitored using an LDC/Milton Roy HPLC unit with refractive index monitor (model 1109) and integrator (CI-10). Quantification of the new compatible solutes, which are not commerically available, was enabled by use of standards for ectoine and the new compound 'Y' prepared and purified in our laboratory.

Sample preparation for NMR spectroscopy. The micro-organisms were grown in a fermenter to yield the biomass necessary for natural-abundance ^{13}C-NMR spectroscopy. About 20 g wet weight was extracted with chloroform/methanol/water overnight in a separation funnel. The water-soluble fraction was further concentrated in a rotary evaporator and desalted on an ion-retardation column (Bio-Rad, AG 11 A8). The fraction containing the compatible solutes was deproteinized with perchloric acid at a final concentration of 10% (w/v). Proteins

were removed by centrifugation and the supernatant was neutralized with KOH (10 M). After complete precipitation of $KClO_4$ (4 °C) the clear supernatant was desalted once again, concentrated, and prepared for NMR spectroscopy: 1 ml of the solute fraction containing at least 100 mg of organic compounds was mixed with 0·5 ml D_2O as a lock signal and approximately 10–20 µl acetonitrile as internal reference (signals at 1·3 and 119·5 p.p.m.). The acetonitrile concentration was chosen to be equivalent to a cellular solute concentration of approximately 100 mM. This enabled us to obtain an approximate estimation of internal solute concentrations from NMR spectra. ^{1}H-decoupled ^{13}C-NMR spectra were recorded with a Bruker WH 90 spectrometer (FT mode, 10 mm vial, 22·63 MHz and 90 MHz for the ^{1}H-decoupling channel). Chemical shifts are expressed in p.p.m. downfield from tetramethylsilane. Solutes were identified by comparison of chemical shifts with published values (Galinski *et al.*, 1985; Wüthrich, 1976).

Results

Identification of organic osmolytes by ^{13}C-NMR spectroscopy

In order to determine the content of compatible solutes in *Halomonadaceae* we recorded natural-abundance ^{13}C-NMR spectra of extracts of *D. halophila*, *F. halmephilum* and *H. elongata* grown at low water activities in a glucose/mineral medium. The NMR spectra of the three organisms proved to be similar in general, but the relative proportions, especially of minor compounds, showed great variations with changing physiological parameters (Fig. 1 *a*, *b*, *c*). The major ^{13}C-NMR signals corresponded to those obtained from ectoine (Galinski *et al.*, 1985), while minor compounds were identified as glutamate, alanine, glucose (not shown) and a new compound, 'Y', which had been previously reported from a halophilic actinomycete (Galinski, 1987).

Glucose was only found in early exponential-phase cells and we suppose that it is rapidly accumulated from the medium and subsequently metabolized or incompletely removed by the washing procedure applied; the other compounds, however, must be considered to be genuine biosynthetic products as they were not supplied in the medium. In contrast to the results obtained from mineral salt media, we detected both glycine betaine and ectoine when the organisms were grown on a yeast extract medium at 10% (w/v) NaCl (Fig. 2). Thus NMR spectroscopy has enabled us to reveal the presence of several new compatible solutes, among which ectoine plays an important role.

Based on the NMR data we have also shown that a cytoplasmic pool of compatible solutes is synthesized by the *Halomonadaceae* and that the relative proportion of the solutes is greatly influenced by environmental parameters such as salt concentration, temperature and carbon source. For further quantification of these effects we continued our investigations with *H. elongata* in greater depth using chromatographic methods.

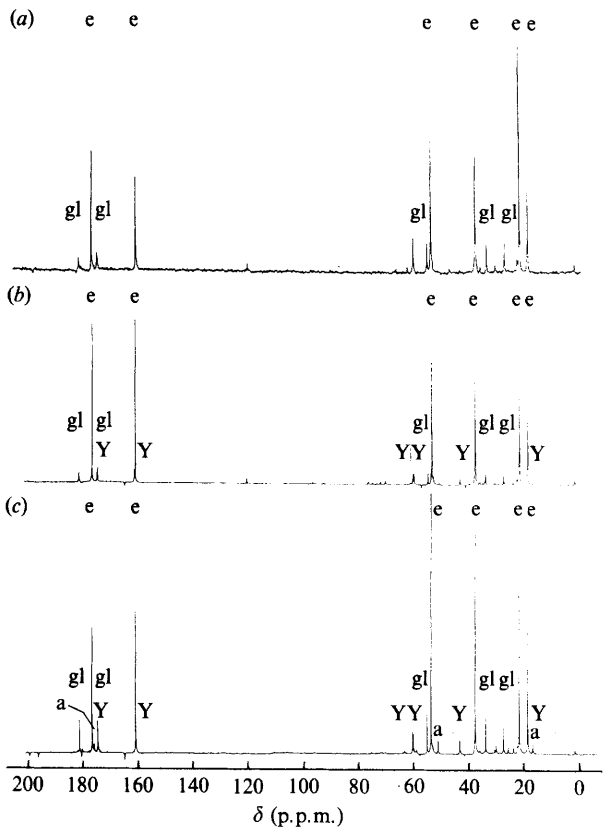

Fig. 1. Natural-abundance ^{13}C-NMR spectra of extracts of members of the *Halomonadaceae* grown on glucose/mineral medium. (*a*) *H. elongata*, 40 °C, 8% NaCl, with signals from ectoine (e) and glutamate (gl). (*b*) *D. halophila*, 30 °C, 10% NaCl, with signals from ectoine, Y and glutamate. (*c*) *F. halmephilum*, 30 °C, 10% NaCl, with signals from ectoine, Y, glutamate and alanine (a).

Influence of external parameters on the solute spectrum

The influence of environmental parameters on the solute spectrum was studied intensively with rotary flask cultures. Ectoine was the predominant compatible solute in *H. elongata* grown on a glucose/mineral medium; its concentration increased markedly with rising salinity of the medium to a maximum value of 260 µg (mg dry weight)$^{-1}$ in cells grown at 20 °C (Fig. 3*a*). Cells grown at 40 °C (Fig. 3*b*) displayed a considerably lower ectoine content [maximum 125 µg (mg dry weight)$^{-1}$]. This was reflected in the increasing concentration of compound Y and glutamate. (Glutamate was not quantified from HPLC, but was estimated by NMR as approximately 10% of total solutes.)

The solute spectrum changed markedly when *H. elongata* was grown on a yeast extract medium (Fig. 4). At low NaCl concentrations, glycine betaine was the predominant solute accumulated or synthesized from precursors. With rising salt concentration, however,

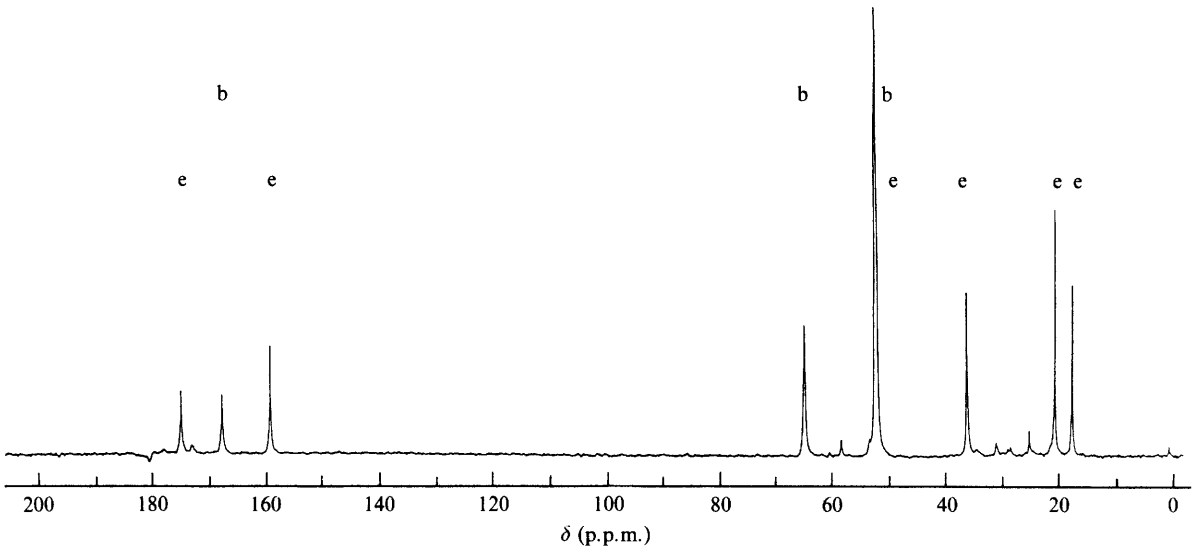

Fig. 2. Natural-abundance ^{13}C-NMR spectrum of *H. elongata* grown on a yeast extract medium at 30 °C, 10% NaCl, with signals from ectoine (e) and glycine betaine (b).

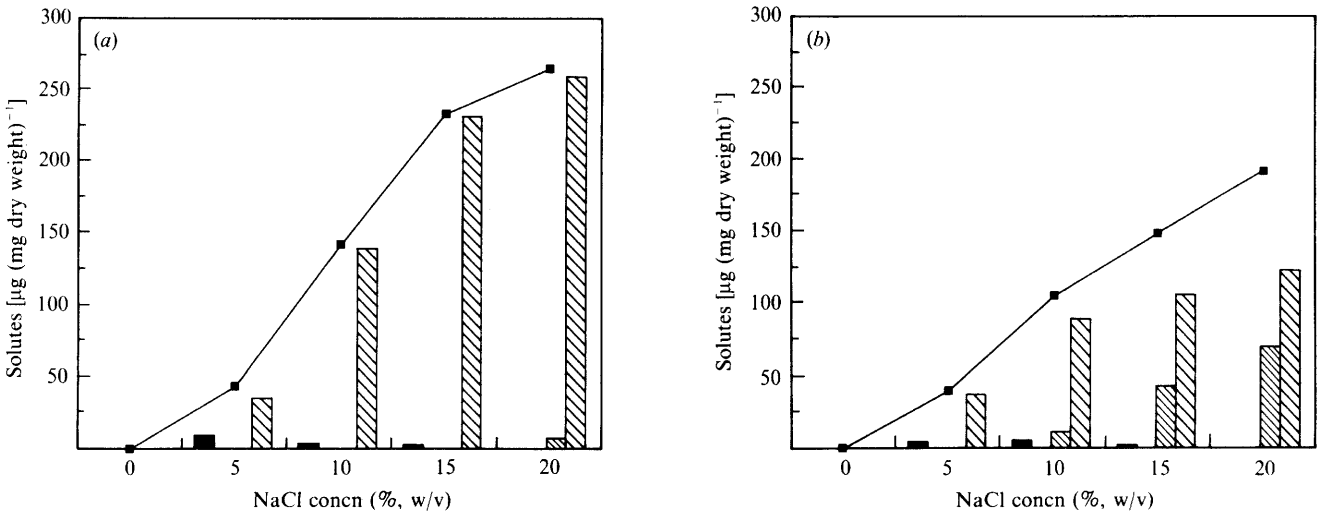

Fig. 3. Compatible solutes in *H. elongata* grown on glucose/mineral medium at (*a*) 20 °C and (*b*) 40 °C, and at various salt concentrations. ■, Unidentified sugar component, not sucrose or trehalose; ▨, Y; ▧, ectoine; ■—■, total solutes.

ectoine and the new component Y became increasingly important in the solute pool. It is clear that, in addition to the accumulation of glycine betaine, a *de novo* synthesis of ectoine takes place when cells are grown on a complex medium (Fig. 2, Fig. 4). Table 1 summarizes the results of salinity and temperature dependence of the compatible solute spectrum of cells grown on both synthetic and complex media. Betaine was found only in cells grown on yeast extract. Ectoine was also present but at a lower concentration on complex medium than on mineral medium. The compound Y was not detected in significant amounts in cells grown at 20 °C, but was found at higher concentrations when cells were grown at 30 °C or 40 °C. Its relative proportion within the spectrum of solutes is further clarified in Table 2. It is remarkable that Y could not be found at low temperatures and low salt concentrations. With both rising temperature and salinity its relative proportion increased to a maximum of 36% of total solutes in glucose-grown cells (at 20% NaCl and 40 °C).

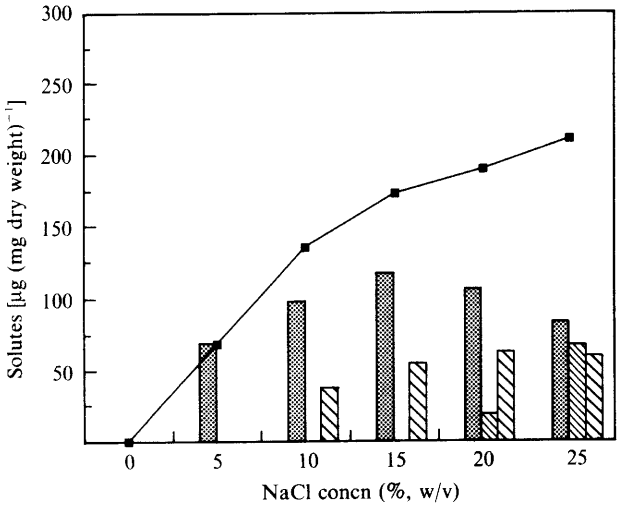

Fig. 4. Compatible solutes in *H. elongata* grown on yeast extract at 40 °C and at various salt concentrations. (▨) Glycine betaine, (▩) Y, (▧) ectoine, (■—■) total solutes.

Osmoadaptation in other members of the Halomonadaceae

From NMR spectroscopy and growth experiments we conclude that the osmoregulatory responses of *D. halophila* and *F. halmephilum* are similar to those of *H. elongata*. Studies by HPLC and NMR (data not shown) showed that glycine betaine was only accumulated when the cells were grown on a yeast extract medium. On the other hand, ectoine and Y played a dominant role and were influenced by external parameters in the same way as shown for *H. elongata*.

Discussion

Until now there had been no evidence that halophilic heterotrophic micro-organisms were able to balance the osmotic pressure of the medium by an accumulation of intracellular compounds. The apparent deficit of intracellular osmotica led Vreeland (1987) to propose a hypo-osmotic cytoplasm or the presence of an undetected compound.

Natural-abundance ^{13}C-NMR spectroscopy proved to be a useful technique for the identification of the solute spectrum of the *Halomonadaceae*. Based upon the results obtained from NMR spectroscopy and chromatographic investigations we are now able to present data on new compatible solutes which show the occurrence in these organisms of the newly described amino acid ectoine (Galinski *et al.*, 1985) and prove that an osmotic balance is achieved by these solutes.

The uncertainty about the presence of compatible solutes was mainly due to the fact that ectoine had escaped detection, as it does not react with the common reagents for amino acids, like dinitrofluorobenzene, dansyl chloride or ninhydrin. At present ectoine is only detectable by HPLC or NMR spectroscopy. Ectoine meets all requirements for compatible solutes: it is a small, highly soluble organic molecule, neutral at physiological pH values and does not interfere with normal cellular functions (Galinski, 1985).

The ^{13}C-NMR spectra show that ectoine is the dominant compatible solute in the *Halomonadaceae*, when grown on a glucose/mineral salt medium. Glycine betaine is only detected in cells cultivated on yeast extract. It is also obvious from a comparison with the internal reference that glycine betaine, ectoine and the new solute Y are present at concentrations at least ten times higher than the amino acids, which stresses their role as osmoregulatory solutes in the *Halomonadaceae*. These findings are also supported by a comparison of the data given by Vreeland *et al.* (1983) for the dominant amino acid glutamate [0·036 µmol (mg dry weight)$^{-1}$] and the cell-associated ions [0·084 µmol (mg dry weight)$^{-1}$] at 20% NaCl with the values presented in Table 1. We determined a maximum total solute concentration of 20–27% of the cellular dry weight. The slightly lower values at elevated salt concentrations and temperatures are probably due to an increased amino acid pool and higher internal ion concentrations as have been reported before (Vreeland *et al.*, 1983; Sadler *et al.*, 1980).

We have calculated the cytoplasmic solute concentrations from our chromatographic data using the values given by Vreeland *et al.* (1983), who determined a cytoplasmic volume of 0·28 ml (g dry weight)$^{-1}$ for *H. elongata* grown at 30 °C and 8% NaCl. For cells grown under similar conditions (30 °C, 10% NaCl) we obtained a value of 0·63 µmol ectoine (mg dry weight)$^{-1}$. This would be equivalent to a cytoplasmic concentration of 2·25 M-ectoine. Added up with about 400 µmol of cations and amino acids (Vreeland *et al.*, 1983), and assuming equivalent amounts of inorganic ions, this would amount to a total solute concentration of 3·05 M, which is close to achieving osmotic equilibrium.

On the basis of a cytoplasmic volume of only 0·12 µl (mg dry weight)$^{-1}$ for cells grown at salinities as high as 20% NaCl (Vreeland *et al.*, 1983), our experimental ectoine data would suppose an intracellular concentration of 11 M. This ectoine concentration exceeds not only the osmotic equilibrium but also the maximum solubility of ectoine. We therefore believe that the reported values of cytoplasmic volume of cells grown at 20% NaCl may be too low.

It should be considered that cytoplasmic volumes vary during the cell cycle and due to external parameters (Kubitschek, 1987; Stock *et al.*, 1977) and that small

Table 1. *Cellular content [μmol (mg dry weight)$^{-1}$] of glycine betaine, solute Y and ectoine in H. elongata at various salt concentrations and temperatures in two growth media*

With the exception of the single determinations (marked with an asterisk), values represent the mean of at least two measurements. The standard deviation was less than 6% and 8% of the mean for glucose-grown cells and for cells from complex medium, respectively. Abbreviations: b, glycine betaine; Y, new solute; e, ectoine.

NaCl concn (%, w/v)	Glucose/mineral medium						Yeast extract medium					
	20 °C			40 °C			20 °C			40 °C		
	b	Y	e	b	Y	e	b	Y	e	b	Y	e
0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0·24	0	0	0·26	0·62	0	0·17	0·58	0	0
10	0	0	0·98	0	0·07	0·63	0·88	0	0·4	0·82	0	0·27
15	0	0	1·63	0	0·29	0·74	0·47	0	0·84	0·98	0	0·39*
20	0	0·03	1·83	0	0·44	0·86	0·55	0	1·16	0·9	0·12	0·46*
25	–	–	–	–	–	–	0·78*	0	0·58*	0·7	0·42	0·42*

–, No growth observed.

Table 2. *Temperature and salt dependence of solute Y in H. elongata grown on a glucose/mineral salt medium and its contribution to the cellular solute pool*

Values represent the mean of at least two measurements; the standard deviation was less than 6% of the mean. Figures in parentheses represent the contribution made by the concentration of solute Y to the cellular salt pool (%, w/w, of detectable solutes).

NaCl concn (%, w/v)	Concn of Y [μg (mg dry weight)$^{-1}$]		
	20 °C	30 °C	40 °C
10	0 (0)	3·0 (2·8)	10·3 (9·8)
15	0 (0)	4·6 (9·4)	42·6 (28·8)
20	6·1 (2·3)	42·1 (17·7)	69·3 (36·2)

deviations in the determination cause large errors. Most other data on cytoplasmic volumes of halophilic bacteria are related to cellular protein. Values available per mg protein are: 2·7 μl for an unidentified halophile in 4·25 M-NaCl (Matheson *et al.*, 1976), 1·2–1·4 μl for *Ectothiorhodospira halophila* at 15% NaCl (Imhoff & Riedel, 1989), 3·3 μl for *Vibrio alginolyticus* (Tokuda & Unemoto, 1982) and 1·92 μl for *Bacillus subtilis* at 10% NaCl (Müller, 1986). For *H. elongata* we have determined a protein content of 20–30% of the cellular dry weight. Ferrer *et al.* (1987) have reported similar values for *D. halophila*. A recalculation of the data published by Vreeland *et al.* (1983) on the basis of protein content would therefore result in a value of 0·6 μl cell water (mg protein)$^{-1}$ in cells grown at 20% NaCl. This value is by far the lowest reported. Furthermore, the cytoplasmic volumes available for other halophilic bacteria are not so strongly affected by external salt concentrations as described for *H. elongata*. We therefore believe that the cytoplasmic volume of *H. elongata* has probably been underestimated for cells grown at high salinities, whereas a value of 0·28 μl (mg dry weight)$^{-1}$ for 8% NaCl appears to be reasonable.

Although the values given above are approximate estimations we believe that the data presented suffice to propose that the cells are able to counteract the external osmotic pressure by accumulating organic solutes. The concentration of the compatible solutes is in the molar region and clearly exceeds the ion and amino acids concentrations. There is no need to propose a hypo-osmotic interior for the *Halomonadaceae* (Vreeland, 1987) since previously undetected osmotically active solutes are present in molar concentrations.

Growth of the organisms on a yeast extract medium led to the accumulation of glycine betaine. *De novo* synthesis of betaine is as yet only known in phototrophic bacteria (Galinski & Trüper, 1982; Mackay *et al.*, 1984). The ability to accumulate glycine betaine from the medium, however, has been shown (Larsen *et al.*, 1987; Le Rudulier & Bernard, 1986) for a number of organisms and the synthesis from methylated precursors such as choline has also been demonstrated (Landfald & Strom, 1986; Styrvold *et al.*, 1986; Pocard *et al.*, 1989).

It is therefore assumed that yeast extract contains betaine or suitable precursors which are taken up by the cells. Thus reports of glycine betaine in halophiles (Imhoff & Rodriguez-Valera, 1984) are explained by the use of yeast extract as a carbon source. The authors concluded that betaine is of general importance for osmotic adaptation of most or all eubacteria. They noted

a correlation between the concentration of betaine and the medium salt concentration over a range of 3 to 10% NaCl, but pointed out that the data did not correlate in most strains between 10 and 20% salts. It becomes clear from our results (Fig. 4) that this phenomenon is explained by the increasing concentration of ectoine and Y at elevated salt concentrations. We believe that betaine can serve as an osmoticum in eubacteria, but it is, however, not essential for osmoregulation in the *Halomonadaceae*, as glucose-grown cells are capable of *de novo* synthesis of ectoine and other unidentified compatible solutes.

Previous authors have noted that the use of complex media extended the salt tolerance of different organisms such as *Vibrio costicola*, *Micrococcus* (now *Paracoccus*, see Ventosa, 1988) *halodenitrificans* (Forsyth & Kushner, 1970) and *H. elongata* (Vreeland & Martin, 1980). Our results with three strains of the *Halomonadaceae* suggest that this may be due to the accumulation of glycine betaine from yeast extract. Uptake of glycine betaine by the cells is probably advantageous, as it may be more economical either to transport this solute or synthesize it from precursors rather than to perform a *de novo* synthesis. It has not been shown yet that glycine betaine applied to the medium can suppress ectoine synthesis. Our results support the idea that ectoine synthesis is shifted to higher salt concentrations in the presence of betaine. Possibly the concentration of betaine or suitable precursors in a complex medium is too low to totally replace ectoine. Alternatively, the betaine transport system may also be affected at high salinities.

Table 2 shows that the concentration of the ectoine derivative Y increases with rising salt concentration and temperature. At the moment it is speculative to suggest that this component is responsible for the observed temperature effects, i.e. a shift of maximum growth rates to higher salinities at elevated temperatures. Although it is intriguing to speculate that the new substance, Y, represents a more suitable enzyme protector, the variation of the relative proportion of compatible solutes may also be explained by a secondary effect on the specific biosynthetic pathways. These questions are at present under investigation in our laboratory.

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