The effect of NaCl on the growth of a *Halomonas* species: accumulation and utilization of compatible solutes

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The effect of NaCl on growth and compatible solute utilization was investigated in a *Halomonas* species. Growth of *Halomonas* was observed in medium of low osmolarity (high water activity) when only 0.1 mM Na⁺ was present. However, lowering the water activity, by addition of KCl or sucrose, inhibited growth in this low-Na⁺ medium, but growth could be restored by the addition of NaCl. The bacterium could grow on glucose as the sole carbon source in up to 3.25 M NaCl and was shown also to metabolize glycine betaine. However NaCl concentrations greater than 2 M inhibited growth when glycine betaine was the sole carbon source. Glycine betaine was transported into the cells by a process stimulated by NaCl irrespective of whether the carbon source was glucose or glycine betaine. Cytoplasmic levels of glycine betaine were monitored throughout the growth cycle in 2 M NaCl medium with glycine betaine as sole carbon source. As the culture aged, glycine betaine was increasingly replaced by the tetrahydropyrimidine ectoine as the major cytoplasmic solute. The increased sensitivity to high NaCl concentrations when grown on glycine betaine may be due to the glycine betaine catabolic pathway enzymes being inhibited by the increasing external solute concentration.

Keywords: *Halomonas*, sodium, compatible solutes, glycine betaine

INTRODUCTION

Large increases in external salinity can exert profound effects on the physiology of the bacterial cell. These increases often change the composition of the cell membrane (Adams & Russell, 1992; Kates, 1986) and give rise to the production and/or accumulation of high cytoplasmic concentrations of organic compounds (compatible solutes) to maintain positive turgor pressure (Brown *et al.*, 1986). Many halotolerant bacteria also have a requirement for low levels of NaCl, which reflects the utilization of the Na⁺ ion to produce a gradient across the cytoplasmic membrane (Unemoto *et al.*, 1990). Such Na⁺ gradients are essential in solute transport processes (MacLeod, 1986).

Increasing levels of salt lower environmental water activity. The presence of high concentrations of NaCl causes many organisms difficulties in regulating and maintaining cell turgor. To overcome low external water activity two strategies are employed. Some halophiles maintain the ionic strength of their cytoplasm at a level

equal to the external concentration; these organisms possess modified metabolic enzymes capable of functioning in elevated ion concentrations (Ginzburg *et al.*, 1971). KCl is normally accumulated and this mechanism has been found in all halophilic archaeobacteria studied to date. Some groups of anaerobic eubacteria (e.g. *Halo-bacteroides* and *Haloanaerobium*) also accumulate KCl in response to salt stress (Galinski, 1993). Nevertheless, a second strategy is used by the majority of eubacteria, which actively exclude ionic solutes but lower the water activity of the cytoplasm by accumulating organic compatible solutes which do not disrupt normal cell function (Brown *et al.*, 1986). Organic compatible solutes are also employed in halophilic methanogenic archaeobacteria in addition to the accumulation of KCl (Galinski, 1993). Both strategies enable the cell to maintain turgor and proceed with metabolic functions.

The strategy of compatible solute accumulation adopted by most eubacteria has been extensively studied. To regulate cell turgor bacteria accumulate one or several compatible solutes, such as trehalose (Giaeffer *et al.*, 1988), glycine betaine (Imhoff & Rodriguez-Valera, 1984) and ectoine (Galinski *et al.*, 1985). The latter is a cyclic amino acid derivative and is synthesized *de novo* by the majority of

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heterotrophic halotolerant bacteria (Trüper *et al.*, 1991). Until recently it was believed that glycine betaine is only synthesized *de novo* by phototrophic salt-tolerant bacteria (Galinski & Trüper, 1982). However Severin *et al.* (1992) showed that the actinomycete *Actinopolyspora halophila* could also synthesize glycine betaine. Nevertheless, if glycine betaine, or its precursor choline, is present in the environment then heterotrophic halotolerant bacteria utilize transport systems to accumulate glycine betaine (Cairney *et al.*, 1985; Whatmore *et al.*, 1990; Pourkomailian & Booth, 1992; D'Souza *et al.*, 1993; Ko *et al.*, 1994). Under such conditions *de novo* synthesis of ectoine appears to be suppressed (Regev *et al.*, 1990; Severin *et al.*, 1992; Cummings *et al.*, 1993). In this paper we investigate the utilization of ectoine and glycine betaine in an unusual species of heterotrophic halotolerant bacteria, *Halomonas* SPC1, which falls within the *Halomonas/Deleya* complex described by Franzmann & Tindall (1990).

METHODS

Organism and growth medium. The bacterium was isolated as a contaminant of a culture of the halotolerant green alga *Dunaliella parva* CCAP 19/9 and it has been deposited in the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany, as DSM 6507 (DUNCON). It was grown on a defined medium (MBM), which contained 50 mM MgSO₄, 50 mM Tris/HCl (pH 7.6), 10 mM KCl, 19 mM NH₄Cl, 0.1 mM Na₂SO₄, 0.025 mM Fe-EDTA, 0.33 mM K₂HPO₄ · 3H₂O, 1 ml trace element mix l⁻¹ (Hajibagheri *et al.*, 1986) and 20 mM carbon source. The pH was adjusted to 7.6 using 1 M NaOH or HCl. NaCl was added as the osmotic solute unless otherwise stated. The growth requirement for NaCl was investigated using MBM medium in which all sodium salts were replaced with analytical grade potassium salt equivalents. Inocula were obtained by twice washing (3000 g, 5 min) 5 ml aliquots of mid-exponential-phase cultures (grown in 0.4 M NaCl MBM) in the sodium-free medium. The inoculum was resuspended in its original volume and inoculated into 100 ml MBM free of sodium salts. The concentration of sodium in this medium was determined by flame photometry (Corning model 410), calibrated using a standard solution of sodium (Corning).

¹³C-NMR spectroscopy. NMR analysis of compatible solutes was performed on mid-exponential-phase cultures which were harvested and washed twice by centrifugation (10000 g, 15 min, 4 °C) in MBM iso-osmotic with growth medium without added carbon source. After the final wash the pellet was resuspended in 2 ml potassium phosphate buffer iso-osmotic with the growth medium. ¹³C-NMR spectra were obtained as previously described (Cummings *et al.*, 1993).

Synthesis of [¹⁴C]glycine betaine. Synthesis of [¹⁴C]glycine betaine from [¹⁴C]choline was performed enzymically using choline oxidase (Sigma) according to the method of Landfald & Ström (1986).

Measurement of [¹⁴C]betaine uptake. Cells for uptake assays, grown in 0.4 M NaCl MBM medium, were harvested after overnight growth and diluted with fresh medium to an OD₆₆₀ of 0.3. Cultures were then incubated until the OD₆₆₀ reached 0.4; the cells were washed twice in carbon-free MBM (10000 g, 15 min), concentrated 20-fold in wash medium and kept on ice. Cells were used for assays within the next few hours. Glycine betaine uptake assays were performed in 2.8 ml MBM medium with no carbon source or NaCl added. The protocol was a modified version of that employed by Thompson & MacLeod

(1973). A 175 µl aliquot of cell suspension was added to the reaction vessel and incubated aerobically (30 °C, 5 min). Solid NaCl was added to achieve the required salinity. [¹⁴C]Glycine betaine (0.2 µCi; 7.4 kBq) was added, 300 µl samples were withdrawn at 30 s intervals, vacuum filtered through 0.45 µm nitrocellulose filters, and subsequently washed in 3 ml MBM (no carbon present and isotonic with reaction medium). Filters were then placed in 5 ml scintillation fluid and counted using a scintillation counter (Beckman LS1801). Glycine betaine was added such that the ratio of [¹⁴C]glycine betaine to cold glycine betaine was approximately the same regardless of substrate concentration. Susceptibility of uptake to osmotic shock was determined by the method of Neu & Heppel (1965).

Enzyme assays of the catabolic enzymes of glycine betaine metabolism. Assays of the catabolic enzymes of glycine betaine metabolism were performed on cell-free extracts from mid-exponential-phase cultures which were harvested and washed twice in MBM (10000 g, 15 min, 4 °C). The cells were sonicated and cell debris removed (14000 g, 20 min, 4 °C). The cell-free extract was kept on ice and used within 4 h. Glycine betaine transmethylase was assayed by the enzyme-catalysed donation of [¹⁴C]methyl group from glycine betaine to homocysteine to form [¹⁴C]methionine (Smith *et al.*, 1988). Dimethylglycine dehydrogenase and sarcosine dehydrogenase activities were assayed by monitoring the substrate-dependent reduction of dichlorophenolindophenol spectrophotometrically at 600 nm, according to the methods of Smith *et al.* (1988).

Cell volume and protein determination. Cell volume was determined by the method of Stock *et al.* (1977), but [¹⁴C]dextran (M_r 70000) was used as the impermeable solute rather than [¹⁴C]sucrose. Protein determinations were estimated by the Lowry method.

Statistics. Where error bars are shown they represent plus and minus one standard error calculated from three replicates. If no error bars are present the data are from one experiment whose trends were confirmed in a second independent experiment.

RESULTS

Growth experiments

Halomonas SPC1 showed optimum growth in 0.4 M NaCl with a generation time (*g*) of 3 h. Strain SPC1 also grew well in 2 M NaCl (*g* = 5.6 h) and grew slowly in salinities up to 3.25 M NaCl (*g* = 34 h). In basal medium in which all sodium salts had been replaced by potassium salts there was a period of slow growth for about 30 h before a steady rate was attained. Once established the fastest growth rate in Na⁺-free medium (*g* = 15 h) was significantly slower than in 0.4 M NaCl, but faster than growth at 3.25 M NaCl. Measurement of the Na⁺ in the medium using flame photometry showed that 0.1 mM was present. If the water activity was lowered in the basal medium by the addition of 1.5 M KCl or sucrose which gave an equivalent NaCl concentration of 1.44 M for KCl and 1.47 M for sucrose (Weast & Astle, 1982), *Halomonas* SPC1 was unable to grow. However, adding NaCl (50 mM) restored growth, but the growth rates observed (*g* = 10.4 h for 1.5 M KCl and *g* = 8.4 h for 1.5 M sucrose) were much less than cultures grown in 1.5 M NaCl (*g* = 4 h).

The inhibition of growth caused by 3.25 M NaCl medium for glucose-grown strain SPC1 cells could be overcome

Fig. 1. Stimulation of growth of *Halomonas* SPC1 at 3.25 M NaCl induced by various compatible solutes (20 mM). Glucose was present as the carbon source (20 mM). ●, No addition; ■, proline; ▲, trehalose; ▼, choline; ◆, glycine betaine. Other solutes tested which had no effect on growth were glycine, sarcosine and dimethylglycine.

Fig. 2. Growth of *Halomonas* SPC1 with glycine betaine as sole source of carbon (20 mM) at different salinities. ■, 0.4 M NaCl; ▲, 2.0 M NaCl; ▼, 2.5 M NaCl.

partially by the addition of several known compatible solutes (Fig. 1). The extent of growth stimulation varied. Most effective were glycine betaine and choline; the latter is usually a precursor of glycine betaine. Also, trehalose and proline gave significant stimulation of growth. Ectoine was not tested in these experiments since sufficient quantities are not yet commercially available. The role of compatible solutes is usually in lowering the water activity of the cytoplasm by accumulation of high concentrations of the compounds. However, it was found also that glycine betaine could be utilized as the sole carbon source by the bacterium; under these conditions the highest salinity at which growth was observed was 2 M NaCl (Fig. 2). Therefore, at salinities up to 2.0 M NaCl, glycine betaine may play a dual role.

Fig. 3. Effect of NaCl concentration on the uptake of glycine betaine by *Halomonas* SPC1 cells grown on glucose (●) or glycine betaine (■) as sole source of carbon (20 mM). The cells were in mid-exponential phase in 0.4 M NaCl medium at the time of harvesting.

Glycine betaine transport

To clarify the role of glycine betaine, its transport into strain SPC1 cells was investigated. Glycine betaine was transported into the cytoplasm of cells which were grown on glucose (Fig. 3); the uptake was dependent on the presence of NaCl and was unaffected by osmotic shock. Glucose-grown bacteria showed optimal uptake with 300 mM NaCl. However, increasing the concentration of salt to 500 mM had no deleterious effect on glycine betaine uptake (Fig. 3). Glycine betaine transport was also assayed in cultures grown on glycine betaine as the sole carbon source. In these bacteria uptake of glycine betaine was shown to be dependent on NaCl and optimal in the presence of 400 mM NaCl, followed by a slight decline in transport activity when the salt concentration was increased to 500 mM (Fig. 3).

When the substrate concentration was varied the glycine betaine transport system followed typical Michaelis-Menten kinetics (data not shown). When *Halomonas* SPC1 was utilizing glucose as the carbon source, the K_t of the system was 72.9 ± 1.4 nM, with a $V_{\max}$ of 19.5 ± 0.4 nmol min⁻¹ (mg protein)⁻¹. Substituting LiCl for NaCl did not support glycine betaine uptake. When the bacterium was grown on glycine betaine, the K_t was 26 ± 0.9 nM and the $V_{\max}$ 20.9 ± 4.2 nmol min⁻¹ (mg protein)⁻¹. In this case LiCl was effective in allowing glycine betaine transport (data not shown). Addition of the uncoupler carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) or the respiratory inhibitor KCN had no effect on either glycine betaine uptake or on respiration rate. After EDTA treatment to permeabilize the cells (Peleg *et al.*, 1980), uptake of glycine betaine in either glucose- or glycine-betaine-grown cells was completely inhibited by the addition of 10 μ M CCCP or 0.5 mM KCN (final concentrations). Sodium vanadate (final concentration 1 mM) had no effect on glycine betaine uptake,

Fig. 4. NMR spectra of *Halomonas* SPC1 cells grown at 2.0 M NaCl with glycine betaine as the sole carbon source showing the increase in cytoplasmic concentration of ectoine (E) at the expense of glycine betaine (B) as the cells pass from mid-exponential phase (a) to stationary phase (b). Peaks for the internal standard lactate (L) are indicated. The B $\times$ 3 label refers to the three methyl groups present in glycine betaine. The intensity of the signals between 160 and 190 p.p.m. are variable from sample to sample because of slow relaxation rates and were therefore not used for the quantitative measurements shown in Table 1.

irrespective of EDTA treatment or the carbon source on which the cells were grown (data not shown).

¹³C-NMR studies

¹³C-NMR was used to determine those compounds which were accumulated to high cytoplasmic concentrations when the bacterium was grown in medium containing 2 M NaCl and glycine betaine as the sole carbon source. The spectra showed that glycine betaine and ectoine were the major cytosolic constituents during both the exponential and stationary phases of growth (Fig. 4). However, it was also clear that the ratio of glycine betaine to ectoine varied depending on the growth phase. Therefore NMR spectra were analysed to determine the compatible solute composition of the cytoplasm during the growth cycle. At early- to mid-exponential phase the

Table 1. Estimation of the intracellular concentration of glycine betaine and ectoine using ¹³C-NMR

The cells were grown in 2 M NaCl with 20 mM glycine betaine as the sole source of carbon. Cell suspension (500 μ l) was added to 50 μ l D₂O plus 11 μ l 0.17 M lactate. The concentration in the sample volume was calculated using peak area from spectra like those in Fig. 4 with reference to the internal lactate standard. This was then converted to intracellular concentration using a cell volume of 2 μ l (mg protein)⁻¹ (Miguel & Gilmour, 1994).

Growth phase	OD ₆₆₀	Concentration in intracellular volume (mM)		Ratio
		Glycine betaine	Ectoine	
Early exponential	0.450	592	193	3.1:1
Late exponential	0.900	494	394	1.3:1
Stationary	1.338	375	561	0.7:1
Late stationary	1.440	314	494	0.6:1

Table 2. Dimethylglycine and sarcosine dehydrogenase activities assayed from cultures grown under various salinities with either glycine betaine or glucose as carbon source

Carbon source*	Salinity (M)	Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]	
		Sarcosine dehydrogenase	Dimethylglycine dehydrogenase
Glycine betaine	0.4	346.6	24.1
Glycine betaine	2.0	32.4	4.8
Glucose	0.4	NA	NA
Glucose + glycine betaine	3.25	NA	NA

NA, No activity detected.

* Carbon sources were added to give a final concentration of 20 mM.

ratio of glycine betaine to ectoine in the cytoplasm was approximately 3:1 (Table 1, Fig. 4a), but by late-exponential phase the proportion had fallen to 1.3:1. The onset of stationary phase saw the level of glycine betaine fall below that of ectoine, with the ratio being estimated at 0.7:1 (Table 1, Fig. 4b). No further change in the ratio was observed in late-stationary phase. Miguel & Gilmour (1994) determined the cell volume of the closely related species *H. elongata* grown at 1.37 M NaCl and found it to be $2.06 \pm 0.09 \mu$ l (mg protein)⁻¹. Using a cell volume of 2 μ l (mg protein)⁻¹, the intracellular concentration of the two compatible solutes was calculated in *Halomonas* SPC1 (Table 1). Although large errors are involved, it appeared that the overall concentration of the two compounds was similar in each phase of growth. However, it should be noted that the total intracellular

concentration of ectoine plus glycine betaine does not osmotically equal the external salinity, again indicating that the absolute values are likely to be inaccurate (Table 1).

Catabolism of glycine betaine

The growth and NMR data implied that catabolism of glycine betaine occurred in the presence of up to 2 M NaCl. The pathway was found to be by oxidation to glycine via *N,N*-dimethylglycine and sarcosine. The dimethylglycine and sarcosine dehydrogenase enzymes were assayed under a range of growth conditions (Table 2) and were found to decrease significantly in activity as the salinity of the medium was increased from 0.4 to 2 M NaCl. No activity was detected in salinities greater than 2 M or in cultures grown on glucose to which glycine betaine was not added. Also, at 3.25 M NaCl with both glucose and glycine betaine present, there was no activity of the glycine betaine catabolic enzymes (Table 2).

DISCUSSION

The ability of *Halomonas* SPC1 to tolerate high levels of salinity is a normal feature of bacteria in this genus (Franzmann & Tindall, 1990). Many moderate halophiles have a requirement for NaCl in the growth media, indeed Vreeland (1987) indicated *Halomonas* species required up to 75 mM NaCl. In this study Na⁺ salts were excluded from the medium and replaced with potassium equivalents. The Na⁺ concentration of such media was determined to be 0.1 mM, but growth was still observed indicating that the Na⁺ requirement is less than 0.1 mM in a low-osmolarity medium. Lowering the water activity by the addition of 1.5 M KCl or sucrose inhibited growth in sodium-limited medium. However, addition of NaCl (50 mM) restored growth, but at a slower rate than in 1.5 M NaCl. Therefore, the Na⁺ requirement of the bacterium appears to be determined by the water activity of the surrounding medium. This effect is unlikely to be due to the requirement of the bacteria to accumulate Na⁺ on exposure to elevated salinities as earlier work has indicated that although inorganic ions are required for growth under such conditions, *Halomonas* SPC1 utilizes K⁺ in this role (Cummings *et al.*, 1993).

In the absence of external compatible solutes, *Halomonas* SPC1 accumulates the tetrahydropyrimidine ectoine by *de novo* synthesis (Cummings *et al.*, 1993). Under these conditions the maximum salinity at which growth of strain SPC1 occurs is 3.25 M NaCl and growth is exceedingly slow. However, the addition of known compatible solutes to 3.25 M NaCl medium allowed growth to proceed at much faster rates (Fig. 1). The largest stimulation of growth was induced by the addition of glycine betaine. Therefore, the transport of glycine betaine was investigated in both glucose- and glycine-betaine-grown bacteria. The transport system was shown to be constitutively expressed and was strongly stimulated by the presence of NaCl (Fig. 3). The kinetic data indicated a threefold difference in affinity for glycine betaine between glucose- and glycine-betaine-grown bacteria. In

both cases, however, uptake appeared to be driven by an ion gradient across the membrane, which from the stimulatory effect of NaCl, and previous work on moderate halophiles (Unemoto *et al.*, 1990), is probably Na⁺. Glycine betaine transport has also recently been studied in the phototrophic haloalkaliphilic bacterium *Ectothiorhodospira halochloris* (Peters *et al.*, 1992). The $V_{\max}$ of this transport system [29 ± 1 nmol min⁻¹ (mg protein)⁻¹] was similar to that of *Halomonas* SPC1 [20.9 ± 4.2 nmol min⁻¹ (mg protein)⁻¹], although the affinity was more than 1000-fold lower ($K_t = 60 \pm 2$ μ M versus 26 ± 0.9 nM).

In common with several species of moderately halophilic eubacteria (Severin *et al.*, 1992), *Halomonas* SPC1 utilized glycine betaine as the sole source of carbon and energy. Growth on glycine betaine was possible in salinities up to 2 M NaCl but salinities in excess of this level were completely inhibitory (Fig. 2). Therefore, ¹³C-NMR spectroscopy was used to follow the fate of the glycine betaine when it was the sole source of carbon at 2.0 M NaCl to see if it was utilized as a compatible solute as well as a carbon source, or whether it was simply metabolized to produce another osmoprotective compound. The spectra obtained showed that glycine betaine and ectoine were the major cytosolic constituents (Fig. 4) implying that betaine was utilized as both carbon source and compatible solute. The presence of ectoine indicated that *de novo* synthesis of ectoine using carbon derived from glycine betaine catabolism also occurred. This phenomenon was further explored by monitoring the compatible solute composition of the bacterium through the growth cycle and estimating the ratio of glycine betaine to ectoine (Table 1). *Halomonas* SPC1 appears to accumulate glycine betaine rapidly in the early phase of growth, enabling the cell to take advantage of the capacity of glycine betaine to act as a compatible solute. However, as growth continues the bacterium metabolizes glycine betaine and maintains the osmotic potential of the cytoplasm by *de novo* synthesis of ectoine. The overall concentration as expected remained constant at around 650 mM; only the proportion of the two compounds changed, although these concentrations are very approximate (Table 1). The data clearly imply that glycine betaine is progressively catabolized to form ectoine by *Halomonas* SPC1. However, Severin *et al.* (1992) found that *Micrococcus halobius* grown on glycine betaine in the presence of 10% salts (~ 1.7 M NaCl) did not synthesize ectoine, although ectoine synthesis was observed in a complex medium.

It was thought highly likely that the glycine betaine catabolic pathway was by oxidation to glycine via *N,N*-dimethylglycine and sarcosine (Smith *et al.*, 1988). This was confirmed when it was found that the dimethylglycine and sarcosine dehydrogenase enzymes of this pathway could be assayed under a range of growth conditions (Table 2). Cytoplasmic activities of both were shown to be subject to a significant decrease in activity as the salinity of the medium was increased from 0.4 to 2 M NaCl. This would imply that the sensitivity of the catabolic enzymes to increasing external solute concentrations is responsible

for the apparent cessation of glycine betaine catabolism at salinities in excess of 2 M NaCl. A similar decrease in glycine betaine catabolic activity was seen in *Rhizobium meliloti* when the solute concentration of the medium was increased (Smith *et al.*, 1988). How the increased Na⁺ or Cl⁻ concentration affects the activity of the glycine betaine catabolic pathway enzymes is unclear. It is unlikely to be a direct effect of Na⁺ entering the cells but may reflect the cells' response to increased osmotic pressure across the membrane (Walter *et al.*, 1987).

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