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The role of trehalose as a substitute for nitrogen-containing compatible solutes (*Ectothiorhodospira halochloris*)

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Abstract. The halophilic phototrophic bacterium *Ectothiorhodospira halochloris* is able to synthesize both nitrogen-containing (betaine, ectoine) and nitrogen-free (trehalose) compatible solutes. In the absence of external ammonium and under nitrogen-limited growth conditions ectoine was metabolized and trehalose partly replaced betaine. The cytoplasmic trehalose concentration did not exceeded 0.5 mol/kg water (approx. 30% of total compatible solutes). A decreasing content of betaine in cells growing under nitrogen limitation is a result of decreased biosynthesis. Apparently, the betaine pool cannot be used as a nitrogen source, not even in a situation of total nitrogen depletion.

Key words: Anaerobic phototrophic bacteria – Halophilic eubacteria – Osmoadaptation – Betaine Ectoine – Trehalose – Compatible solutes – *Ectothiorhodospira halochloris*

Organisms which thrive in highly saline natural environments have to cope with the physiological stress of lack of water. Low water activity of the surrounding medium will cause dehydration of the organisms' cytoplasm and subsequently impair metabolic functions, unless they employ a mechanism to retain cytoplasmic water. Two different strategies have been evolved:

– The "salt-in-cytoplasm" type maintains a cytoplasmic salt concentration close to that of the environment. Consequently this mechanism of adaptation, which was discovered in the family *Halobacteriaceae*, relies on a number of physiological changes, mainly with respect to their metabolic machinery (Reistad 1970; Visentin et al. 1972; Lanyi 1974). The presence of salt dependent cytoplasmic enzymes can be taken as a marker for this type of haloadaptation, which restricts the range of suitable natural habitats.

Most of the halophilic and halotolerant eubacteria, but also algae and fungi, are able to adapt over a wide range of salinities. They possess cytoplasmic enzymes of the "normal" type, which show optimum activity at low salt concentrations and would not function in a cytoplasm of high ionic strength. These organisms are, therefore, dependent on their capability to accumulate or synthesize compatible solutes (Brown 1976), which are responsible for osmotic balance and compatible with the organisms' metabolism. The spectrum of compatible solutes encountered in halophilic microorganisms – including halophilic algae and fungi – has been reviewed elsewhere (Trüper and Galinski 1986). In general compatible solutes fall into four typical classes of compounds, namely polyols, sugars, amino acids and betaines.

The role of betaine as a compatible solute in halophilic eubacteria was first described in the halo-alkaliphilic sulfur bacterium *Ectothiorhodospira halochloris* (Galinski and Trüper 1982) and has since then been reported in a wide variety of halophilic and halotolerant prokaryotes (Mohammad et al. 1983; Mackay et al. 1984; Reed et al. 1984). Besides this major osmoticum, which might reach a cytoplasmic concentration of approx. 2.5 mol/kg water, extremely halophilic *Ectothiorhodospira* species synthesize two additional compatible solutes, the disaccharide trehalose and the novel, so far unknown, cyclic amino acid ectoine (Galinski et al. 1985). In view of the high nitrogen demand for the compatible solute production and the possibly restricted nitrogen supply in an alkaline environment we decided to monitor changes in the relative proportion of compatible solutes under various nitrogen-limited growth conditions. *Ectothiorhodospira halochloris* served as a model organism to assess the necessity of nitrogen compounds like glycine betaine and to back up the general concept proposed by others (Reed et al. 1984; Mackay et al. 1984) that the type of osmolyte used reflects, to a certain extent, the degree of halophily.

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Materials and methods

Culture methods

Ectothiorhodospira halochloris DSM 1059 (Imhoff and Trüper 1977) was grown anaerobically in 1 l screw cap bottles and 10 l carboys at a light intensity of approx. 3,000 lux and 10,000 lux respectively. The temperature was controlled at 40° C and growth was monitored as optical denstiy (OD_{650}) or by protein determination according to Schmidt et al. (1963) using the Lowry method (Lowry et al. 1951).

Medium 160 (modified after Imhoff and Trüper 1977) contained the following components in 1 kg of water: 160 g NaCl (2740 mmol), 20 g Na_2SO_4 (141 mmol), 14 g $NaHCO_3$ (167 mmol), 6 g Na_2CO_3 (57 mmol), 2 g sodium acetate (24 mmol), 0.8 g NH_4Cl (15 mmol), 0.5 g KH_2PO_4 (3.7 mmol), 0.1 g $MgCl_2 \times 6\ H_2O$ (0.5 mmol), 0.05 g $CaCl_2 \times 2\ H_2O$ (0.3 mmol), 0.5 g $Na_2S \times 7-9\ H_2O$ (approx. 2 mmol). The medium was supplemented with 1 ml/l of vitamin solution "VA" (Imhoff and Trüper 1977) and immediately filtered through a sterile filter. The sterile medium was then supplemented with 1 ml/l of sterile trace element solution "SLA" (Imhoff and Trüper 1977). It proved unnecessary to adjust the pH as the present ratio of $NaHCO_3/Na_2CO_3$ served as a carbonate buffer in the region of pH 8.5 The standard medium described above contained 20% (w/w) inorganic salts ($NaCl/SO_4/CO_3$). The osmotic strength was determined using a Knauer vapour pressure osmometer ($p = 5.86$ osm; $\psi = -15.25$ MPa; $a_w = 0.899$).

For the determination of the ammonium requirement of *E. halochloris* the prepared medium was supplemented with sterile NH_4Cl solution prior to inoculation. A starting culture medium containing no more than 4 mM NH_4Cl and 1 g/l (12 mM) of sodium acetate proved to be appropriate to obtain a stationary cell culture ($OD_{650} = 1.0$) deprived of all extracytoplasmic ammonium. To avoid a possible mobilization of atmospheric nitrogen the culture was kept under an inert atmosphere of argon throughout the experiment. Stationary phase cells were fed twice with 0.5 g/l sodium acetate to induce further growth and concomitant mobilization of internal nitrogen sources. The assimilation of acetate and changes in the cytoplasmic concentration of trehalose and betaine were monitored. The second cycle of acetate assimilation was followed by the addition of NH_4Cl (final concentration 14 mM) to release nitrogen stress.

The medium for the cultivation of N_2-fixing cells was essentially the same as above except that NH_4Cl was omitted completely. The semicontinuous device for the cultivation of nitrogen fixing cells is described in Fig. 1. The culture vessel was designed to allow sufficient illumination within a dense cell suspension and rapid diffusion of molecular nitrogen (high surface to volume ratio). It consists of a central glas container divided by a semi-permeable membrane. Two of the medium reservoirs supply culture medium for the upper and lower half, respectively, of the bioreactor. Whereas bacterial growth takes place in the upper volume of approx. 700 ml (15 cm in diameter, 4 cm in depth), which stays in contact with a nitrogen atmosphere, the lower volume serves to enable exchange of the culture medium in order to remove any growth inhibiting substances and to gain a high cell density ($OD_{650} = 6.4$). Similarly, a mild dilution stress can be applied if the lower volume is replaced by a dilution medium of low salinity (3rd reservoir). The whole device was kept under a nitrogen pressure of approx. 400 mm water column (3.92 kPa). All rubber connections were made of Viton (Dupont), oxygen free nitrogen was obtained by use of a copper catalyst (Type CHS, P. Ochs, Bovenden).

Determination of medium components

Acetate was determined enzymatically in the fed batch experiments employing the combined reactions of acetyl-CoA synthetase, citrate synthase and malate dehydrogenase (Test kit Nr. 148261; Boehringer, Mannheim). In N_2-fixing cultures acetate assimilation was monitored on a Packard-Becker (Model 430) gas

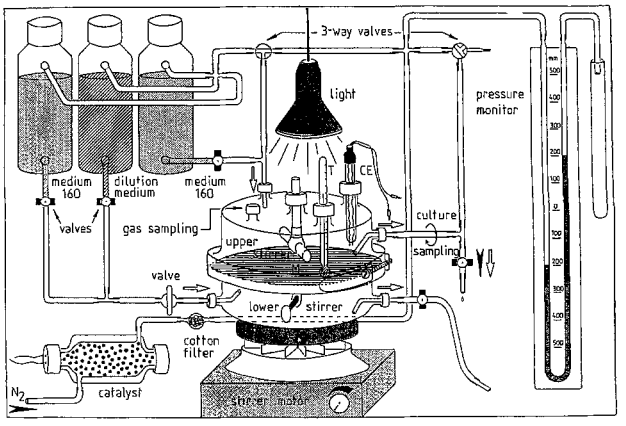

Fig. 1. Culture vessel for the semi-continuous growth of nitrogen-fixing *Ectothiorhodospira halochloris* cells. A semi-permeable membrane (*M*) divides the bioreactor into two separate compartments, the upper phase containing the organism in close contact with a nitrogen atmosphere and the bottom unit enabling a semi-continuous medium exchange. *Abbreviations: T* thermometer; *CE* conductivity electrode; Arrows illustrate flow of nitrogen (▶) and medium (⇨)

chromatograph using the following method: equal volumes of sample, internal standard (5 mM acetone) and formic acid (98–100%) were combined in a stoppered vessel and kept at 0° C for 5 min. A volume of approximately 1 µl was then injected and quantified following the internal standard method. Column: glass, 1 m × $^1/_4''$ × 2 mm (i.d.), packed with Porapak Q, 80–100 mesh (Packard-Becker, Delft); Carrier gas: nitrogen (99.996% purity) at a flow rate of 15 ml/min; Temperature: injector and flame ionization detector at 170° C, oven at 150° C.

Ammonium was determined according to Berthelot using the test kit from Boehringer Mannheim or using a modified version according to Krom (1980): 0.3 ml of sample containing 1–30 µg NH_4Cl were mixed with 5 ml of solution 1 and solution 2 and incubated for 30 min at 37° C. Absorption was recorded photometrically at 650 nm and quantified according to a standard. Solution 1: 50 mg of sodium prusside in 1 l of 0.1 M sodium salicylate; Solution 2: NaOH + 5 ml sodium hypochlorite (15% w/v) in 1 l of water.

Gas chromatographic determination of gases (H_2, N_2, CH_4, CO_2) was achieved with a Packard-Becker (Model 430) chromatograph using a thermal conductivity detector in the twin column mode. A sample of 500 µl was injected for each analysis. Reference column: glass, 0.91 m × $^1/_4''$ × 2 mm (i.d.) packed with SE-30 or Porapak Q (Packard-Becker, Delft); Analytical column: stainless steel, 1.83 m × $^1/_8''$ × 2 mm (i.d.) packed with Carbosieve S II, 120–140 mesh (Supelchem, Sulzbach); Carrier gas: helium (99.996% purity) and, for H_2 determinations, nitrogen (99.996% purity) at a flow rate of 20 ml/min and 5 ml/min ($^1/_8''$ column), respectively; Temperature: injector and detector were kept at 180° C. The oven temperature programme started at 40° C and, after 5 min, was followed by a linear increase (20° C/min) to a final temperature of 180° C at which it was kept for a minimum of 5 min.

Methods of extraction

Low molecular weight compounds (including compatible solutes and inorganic salts) were extracted following the perchloric acid method by Sutherland and Wilkinson (1971). The volume of the protein-free solution (obtained from 1 g cell wet weight) was reduced by freeze drying and subsequent dissolution in 5 ml demineralised water. Further purification was achieved by vigorous shaking with an equal volume of chloroform. For the quantitative determination

of betaine the aqueous top layer was then desalted on a Biorad AG 11A8 ion retardation column.

Poly-β-hydroxybutyrate was extracted following the sodium hypochlorite/chloroform method of Law and Slepecky (1961). The chloroform fraction containing the polyester was then further purified by the addition of two volumes of diethylether to precipitate poly-β-hydroxybutyrate.

A universal fast extraction method (modified Bligh & Dyer technique) was developed for nuclear magnetic resonance (NMR) of compatible solutes and storage products like poly-β-hydroxybutyrate and glycogen. Starting from 1 g cell material (wet weight) the following extraction procedure was applied: add 18.5 ml extraction mixture (methanol, chloroform, water: 10:5:3.4 by vol.); homogenize for 2 min in a teflon homogenizer (Braun-Melsungen); add 5 ml chloroform; shake vigorously and centrifuge (5,000 × g); add 5 ml water to supernant; centrifuge (5 min) to promote phase separation; recover a) aqueous top layer for determination of compatible solutes, b) chloroform layer containing poly-β-hydroxybutyrate and c) sediment for the determination of glycogen.

The compatible solute fraction was further purified following the perchloric acid method (above). The resulting protein-free extract was subjected to high performance liquid chromatography (HPLC). The chloroform layer containing lipids and poly-β-hydroxybutyrate was analysed without further purification by natural abundance ^{13}C NMR spectroscopy. The sediment was dried under a stream of nitrogen. Glycogen was then recovered by boiling in 5 ml of 30% (w/v) KOH, filtering through a Whatman GF/F glass fibre filter and precipitating in the cold with 4 ml ethanol. The water soluble fraction of the precipitant was analysed using nuclear magnetic resonance (NMR).

Chemical determinations of betaine, trehalose and poly-β-hydroxybutyrate

Betaine was determined according to the modified Dragendorff method of Stumpf (1984). Trehalose was quantified following the method of Loewus (1952), which employs the anthrone-H_2SO_4 reaction. As both these chemical methods rely on relatively unspecific universal reactions for both sugars and quaternary ammonium compounds the absence of any interferring substances was checked by thin layer chromatography. Poly-β-hydroxybutyrate was determined photometrically following the crotonic acid method of Law and Slepecky (1961).

Nuclear magnetic resonance (NMR) spectroscopy

Natural abundance ^{13}C NMR spectra were recorded in the pulsed Fourier transform mode on a Bruker WP-80-FT spectrometer operating at 20.115 MHz and 80 MHz for the decoupling channel. 1 ml of aqueous protein-free extracts (containing at least 20 mg of substance) was supplemented with 0.2 – 0.3 ml D_2O to provide an internal lock signal. An aliquot of CD_3OD (approx. 50 µl) served as internal standard. Chloroform extracts were recorded in a mixture of 1 ml $CDCl_3$ and 0.5 ml CD_3OD.

High performance liquid chromatography (HPLC) of compatible solutes

Betaine, ectoine and trehalose were determined quantitatively from aqueous desalted extracts (Biorad AG 11A8). An aliquot was diluted with 2.33 volumes of acetonitrile and analysed on an LDC/Milton-Roy chromatography system in isocratic mode. The separation method was developed as a modification of the technique described by Vialle et al. (1981).

Column: Bio-sil Amino 5-S (Biorad, Munich), 250 × 8 × 4 mm (i.d.); Solvent: 70% (v/v) acetonitrile at a flow rate of 1 ml/min (2,000 psi or 14 MPa); Detector: refractive index monitor (model 1109) from LDC/Milton-Roy.

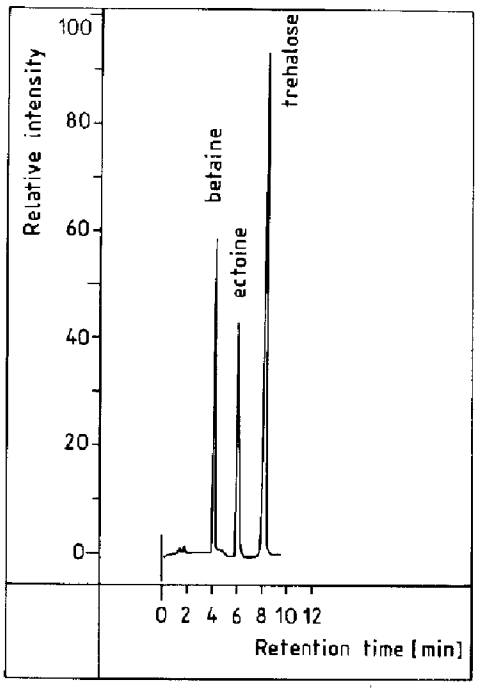

Fig. 2. High performance liquid chromatography separation of the compatible solutes of *Ectothiorhodospira halochloris* on a Nucleosil 5-NH_2 column (70% acetonitrile) using a refractive index monitor for peak detection

Determination of cytoplasmic concentrations of solutes

The cytoplasmic volume was determined following a modification of the method of Takacs et al. (1964). ^{14}C dextrane, ^{14}C sucrose and ^3H_2O were used to assess the various accessible volumes of a firm cell pellet (25,000 × g). Over a salinity range of between 160 and 240 g NaCl/kg water the cytoplasmic volume remained constant at 0.21 ml/g pellet wet weight independent of the growth phase. This low value is not unusual for a phototrophic organism packed with photosynthetic membrane stacks. It was used to calculate cytoplasmic concentrations.

Chemicals

Ectoine standards were prepared from *Ectothiorhodospira halochloris* following the method described by Galinski et al. (1985). All other chemicals were obtained commercially.

Results

The cytoplasmic concentration of compatible solutes (normal growth medium)

Figure 2 presents a typical HPLC chromatogram of solute standards which served to quantify the cellular concentration of betaine, ectoine and trehalose. Using the HPLC method described and on the basis of a cytoplasmic volume of 0.21 ml/g wet weight the intracellular concen-

tration of betaine in *Ectothiorhodospira halochloris* was shown to vary between 1.8 mol/kg cytoplasmic water an 2.5 mol/kg cytoplasmic water depending on the salinity of the growth medium (16 g NaCl and 24 g NaCl per 100 g water, respectively). The colorimetric determination of betaine according to Stumpf (1984) showed a slightly lower betaine level of 1.65 mol/kg cytoplasmic water for cells grown in standard growth medium. The intracellular concentration of ectoine and trehalose was generally much lower and amounted to approx. 0.1 and less than 0.05 mol/kg cytoplasmic water, respectively, in the early stationary growth phase. The relative proportion of ectoine and trehalose to betaine was, however, markedly increased under salt stress conditions. Ectoine and trehalose reached concentrations of approx. 0.4 and 0.1 mol/kg cytoplasmic water, respectively. Although betaine is without doubt the dominant compatible solute in halophilic species of the bacterial genus *Ectothiorhodospira* (Galinksi 1986), the observed variations have led us to suppose that the minor components also play an important role in osmoadaptation (Galinski et al. 1985). The high cytoplasmic concentration of betaine (1.8 M is equivalent to 14% of the cells' dry weight) also appears to impose an additional burden on the cells' nitrogen metabolism, especially in an alkaline environment where ammonia is lost into the atmosphere. One might therefore expect that an organism capable of both trehalose and betaine synthesis might increase the relative proportion of nitrogen-free compatible solutes when the availability of ammonium becomes growth limiting.

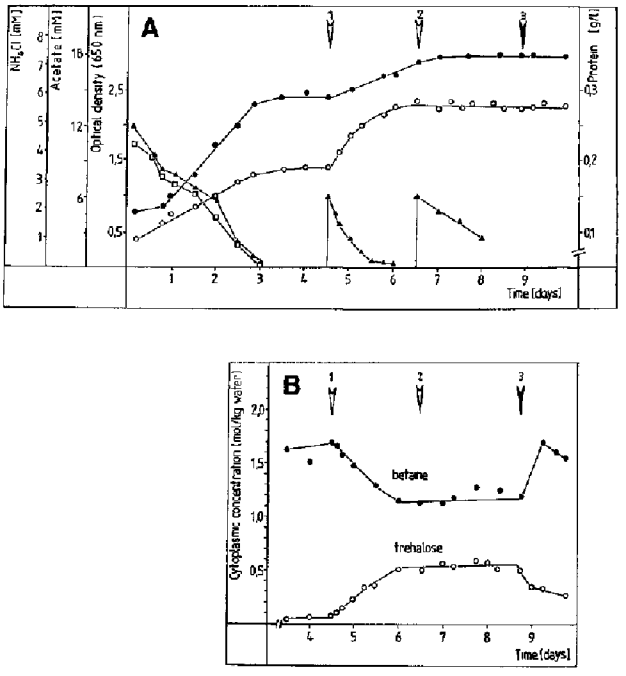

Fig. 3A, B. Growth characteristic of *Ectothiorhodospira halochloris* during depletion of external ammonium (**A**) and concomitant changes of its cytoplasmic betaine and trehalose concentration (**B**). Cells were repeatedly fed with sodium acetate (▽) in the absence of external ammonium, which was finally added (▼) to release nitrogen stress. Symbols: □ ammonium chloride; ▲ sodium acetate; ○ optical density (**A**) and cytoplasmic trehalose concentration (**B**); ● protein (**A**) and cytoplasmic betaine concentration (**B**)

Growth and solute variation of a nitrogen-depleted culture

Figure 3A describes the growth of a batch culture of *E. halochloris* which, after complete assimilation of external carbon and ammonium, was repeatedly fed with a sole carbon source. Starved and nitrogen-depleted cultures display a marked increase in optical density upon addition of sodium acetate (marker no. 1). The increase in optical density per unit acetate was approximately twice as high in the nitrogen-free culture during the first feeding period (4.5 – 6 days) as compared to the culture before it became nitrogen-free (note that only half the amount of acetate yielded a similar increase in optical density). This strongly suggested the simultaneous accumulation of light-scattering storage material. A comparison of the gain of cellular protein per gram acetate (standard yield = 0.15 g protein/g sodium acetate), however, proved that the cells were actually growing. The protein content increased by 0.05 g upon addition of 0.5 g sodium acetate, equivalent to 66% of the increase expected from a "normal" cell culture. Further feeding (marker no. 2) did not increase the protein content although acetate was assimilated by the cells.

Figure 3B depicts the concomitant changes in the cytoplasmic solute concentration of nitrogen starved cells. It is shown that the cytoplasmic concentration of betaine drops from 1.65 mol to 1.2 mol/kg water during growth in the absence of external ammonium. Concomitantly, the cytoplasmic trehalose concentration is raised from 0.05 to 0.5 mol/kg water and thus compensates the osmotic deficit. Further supply of carbon source (marker no. 2) does not seem to initiate any further changes in the relative proportion of solutes. Upon addition of ammonium (marker no. 3) the original situation is apparently reconstituted. The accumulation of trehalose (30 mg/g wet weight) is accompanied by the synthesis of poly-β-hydroxybutyrate as a storage material which reaches a maximum value of 70 mg/g wet weight (470 mg/g protein) at the end of the first feeding period (data not shown). Although it is intriguing to suppose that betaine and trehalose are interconvertible, our results favour the assumption that betaine synthesis ceases under nitrogen stress, i.e. trehalose is synthesized in growing cells as an osmotic substitute when betaine synthesis is inhibited (see Discussion). As no further growth was induced by a second feeding period it remains open whether trehalose can replace betaine completely during nitrogen limited growth.

The physiological response of a nitrogen-fixing culture

In order to study the cells' reponse when external nitrogen is present but growth limiting, we performed similar experiments with a nitrogen-fixing culture of *Ectothiorhodospira halochloris*. This should evoke a situation where trehalose synthesis is preferred to betaine synthesis. The evolution of hydrogen served as a marker for nitro-

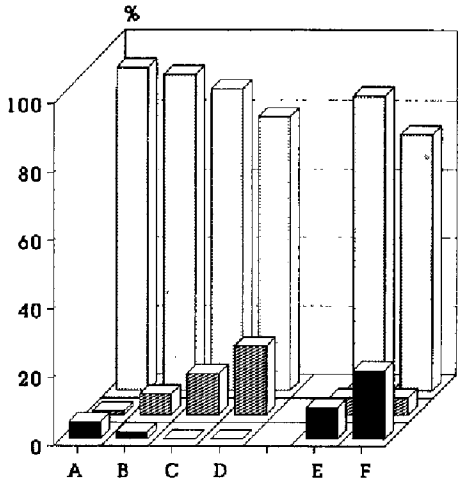

Fig. 4. Variation of the relative proportion of the compatible solute spectrum of *Ectothiorhodospira halochloris* in the course of the nitrogen-fixation experiment. 100% equals a cytoplasmic concentration of 1.8 mol/kg water. *A* Cells from the early exponential growth phase ($OD_{650} = 0.8$) containing all three solutes. *B*–*D* Increase of trehalose content with each doubling of cell mass ($OD_{650} = 1.6$, 3.2 and 6.4). At a maximum trehalose concentration of 20% of total solutes (*D*) no further growth was obtained. *E* Trehalose containing cells from *D* after nitrogen stress was released by the addition of 1 g/l NH_4Cl. *F* Salt-stressed cell material (160 → 200 g/l NaCl upshock) in the presence of external ammonium. ■ ectoine, ▨ trehalose, □ betaine

genase activity. Newly adapted cells of *E. halochloris* reached a doubling time of 24 h ($\mu = 0.029$), which was only half as fast as growth on ammonium as a nitrogen source. It is therefore justified to assume that the availability of nitrogen was the growth-limiting factor in these experiments. Beginning with an optical density of 0.2 we succeeded in obtaining 5 doublings of cell mass to a final optical density of 6.4. While the overall cytoplasmic osmolality stayed constant during the entire experiment, we observed relative changes in the compatible solute spectrum (Fig. 4). It was shown that betaine synthesis did not cease under nitrogen-fixing conditions and that the composition of solutes in the early exponential growth phase (Fig. 4A) did not differ much from that of an ammonium grown culture. Trehalose was very low and ectoine took a slightly lower level (5%) than under normal conditions. The picture, however, changed gradually in the course of the nitrogen-fixation experiment. Ectoine disappeared rapidly and the trehalose content steadily increased to a maximum of 20% of total solutes in actively growing cells (Fig. 4B–D). This remarkable shift towards nitrogen-free compatible solutes was parallelled by a steady decline of the growth rate (e.g. $\mu = 0.014$ at OD 1.6). A medium exchange, which was performed at an optical density of 2.7, served a dual purpose: firstly to dialyse any growth-inhibiting substances and secondly to supply fresh medium and promote further growth. Despite this manipulation the tendency towards lower growth rates was not relieved (e.g. $\mu = 0.010$ at OD 3.2). In the carbon-limited stationary phase the high trehalose content slowly declined, indicating that a conversion of trehalose to betaine is possible in the absence of an exogenous carbon sources. When nitrogen stress was relieved by the addition of ammonium chloride, growth was rapidly resumed and ectoine regained its place as the second strongest solute (Fig. 4E). Additional salt stress then invoked an even higher ectoine level of as much as 20% of total compatible solutes (Fig. 4F).

Cell samples of the highest trehalose content were subjected to NMR analysis, which proved the absence of any other so far undetected compounds (not shown). The observed chemical shift values (resonances at 93.5, 72.9, 72.4, 71.4, 70.0, 60.9 ppm) also confirmed the anomeric structure of α,α-trehalose. A comparison of the integrated spectral data further enabled us to approximate a molar proportion of 4:1 (betaine: trehalose) which agrees well with out HPLC determination. NMR analysis of the chloroform fraction (not shown) gave evidence that high amounts of poly-β-hydroxy-butyrate were also produced at the end of the nitrogen-fixation experiment (signals at 169.3, 67.4, 40.3, 19.1 ppm). The second potential storage material, glycogen, could not be detected under these experimental conditions.

Discussion

In order to test the organism's physiological response under nitrogen-limited growth conditions, we inforced nitrogen stress firstly by the omission of nitrogen and secondly by supplying molecular nitrogen as the only N-source. If our preconception of an efficient adaptation mechanism were correct, the need to mobilize nitrogen should not only abolish betaine production in favour of trehalose synthesis as an alternative osmoticum, but also induce the conversion of betaine into nitrogen-free organic solutes. We were, however, unable to provide evidence for the above-mentioned principle. Our results show that the apparent drop of the betaine concentration upon depletion of external ammonium was in fact the result of a dilution effect of a growing cell culture (see below). Although it is presently not known whether internal nitrogen reserves or biogenic external nitrogen sources were mobilized, growth, as monitored by the increase in protein content, continued for $^1/_3$ of a generation in the absence of external ammonium. The initial cytoplasmic betaine concentration would therefore be diluted gradually from 1.65 to 1.24 mol/kg water. Within the margins of experimental error this value coincides well with our observations of a lower betaine level of 1.2 mol/kg water (Fig. 3). We therefore propose that, under the applied conditions of nitrogen limitation, protein synthesis has priority over betaine synthesis, the betaine pool itself is not metabolized and trehalose is synthesized *de novo* to compensate the osmotic deficit. This view is supported by the following pieces of evidence:

1) On the basis of a standard yield of 0.15 g protein/g sodium acetate one can calculate that the observed protein gain upon nitrogen depletion (0.05 g/l) will require 66% (0.333 g/l) of the sodium acetate supplied. After complete consumption of all nitrogen reserves, the sodium acetate surplus of 166 mg (33%) can then partly serve as a source for the synthesis of trehalose and/or

poly-β-hydroxybutyrate (PHB). Taking a typical mass conversion rate of 60% for both biosynthetic pathways (2 sodium acetate → 1 PHB-repeating unit; 8 sodium acetate → 1 trehalose) one would expect *de novo* synthesis of approx. 100 mg/g wet weight in total for both compounds. This theoretical calculation agrees well with the observed production of poly-β-hydroxybutyrate (70 mg/g) and trehalose (30 mg/g).

2) Although the nitrogen pool trapped in compatible solutes seems to be only minor when compared with the protein requirement for nitrogen (5 mg/l vs. 24 mg/l, as calculated from an average N-content of 16%) a degradative nitrogen recycling from betaine would be beneficial for an organism capable of producing nitrogen-free solutes. When growth ceased at the end of the first feeding period (due to a total depletion of nitrogen) betaine was — unexpectedly — not mobilized in favour of protein synthesis.

3) As part of their salt stress response *E. halochloris* cells are able to take up external betaine into the cytoplasm and use it as an osmoticum, but are unable to grow on betaine as a carbon source (Tschichholz and Trüper 1990).

We therefore conclude that a degradative pathway for the mobilization of betaine is not established, even under the most stringent nitrogen limitations. The physiological basis of this one-way-only biosynthetic pathway is presently not understood, but it surely emphasizes the importance of betaine not only as an inert solute but also as a cytoplasmic protectant.

The sudden rise of the betaine pool when nitrogen stress is relieved (Figs. 3, 4) appears to be a consequence of the conversion of either trehalose and/or poly-β-hydroxybutyrate to betaine and therefore reflects the organism's capability of a rapid biosynthesis from internal carbon sources. As has been shown by Herzog et al. (1990) the trehalose degrading enzyme, trehalase, is, however, responsible for a rather slow adaptational mechanism which may occur in response to changes of the internal milieu (possibly the betaine level). This is reflected in its slow degradation compared with the rapid betaine synthesis (Fig. 3B). One must therefore assume that other internal carbon sources like poly-β-hydroxybutyrate provide the building blocks for rapid betaine synthesis upon relief of nitrogen stress.

In a physiological situation of nitrogen-limited growth (nitrogen fixing cells) ectoine disappeared gradually and faster than expected from a dilution effect (Fig. 4). This suggested a physiological degradation. Betaine synthesis, on the other hand, was not shut off completely in course of the experiment but proceeded at a slower rate than protein synthesis, while trehalose partly replaced betaine (Fig. 4B—D). We were however unable to achieve a relative proportion higher than 20% (in nitrogen-fixing cells) and 30% of total solutes (in ammonium-depleted cells), which seems to be the upper limit for replacement of betaine under the conditions employed. This upper limit of approximately 500 mM trehalose seems to agree very well with the unusually high K_m-value (0.5 M) of a recently described constitutive trehalase from this organism (Herzog et al. 1990) which probably controls the cytoplasmic trehalose concentration. It is in this connection of interest to note that, with increasing trehalose accumulation, a concomitant decline of growth rate was observed (Fig. 4). A possible growth inhibition by metabolic waste products in both the gas an liquid phase can be excluded as several exchanges of the growth medium and the nitrogen atmosphere failed to cause any improvement. Similarly, nitrogen limitation in a dense population is also unlikely to be the major cause for the cell's decreasing growth rate as inoculation of trehalose-containing cells into fresh medium did not result in enhanced growth rate. We therefore have some reason to believe that an adverse influence of trehalose accumulation upon the metabolic machinery limits the degree of betaine replacement. It is, therefore, probably justified to conclude from our results that compatible solutes differ with regard to their compatibility. Sugars (like glucose and fructose) and disaccharides (sucrose, trehalose), which have so far been investigated for their possible implication in osmoadaptation, seem to be less compatible with enzyme action at high concentrations and have never been found to accumulate to concentrations higher than 0.5 mol/kg water. As proposed by Reed et al. (1984) and Mackay et al. (1984) the occurrence of sugars as primary solutes may, therefore, be confined to fresh water and marine species of limited halotolerance and hence low cytoplasmic solute concentration.

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